EFFECT OF HYPERGLYCAEMIA ON VEGF-A SPLICE VARIANTS, JUNCTIONAL INTEGRITY AND VASCULAR LEAKAGE IN HUMAN FETO-PLACENTAL VESSELS FROM NORMAL AND TYPE 1 DIABETIC PREGNANCIES

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Abstract

Hyperglycaemia is a main feature of diabetes, and this pathology is a main complication of pregnancy. Diabetic patients often require tighter glycaemic control in pregnancy, as glucose metabolism changes, and insulin dosages need to be adjusted. Throughout gestation, the placenta is therefore subjected to periods of hyperglycaemic insult. Thus, we wished to study how brief periods of hyperglycaemia affected the feto-placental vasculature, through study of important permeability molecules, pro-permeability VEGFa, anti-permeability VEGFb, and junctional stability molecule VE-cadherin. A 15mM concentration of glucose was chosen because it is a level seen postprandially in diabetic pregnancies. We explored this by explant and perfusion methods.

We tested two chorionic villous explants methodologies to validate them for 4h and 24h duration studies, and found that a free-floating methodology which sought to replicate the *in utero* flow was not appropriate, as this resulted in high basal levels of endothelial VEGF and low junctional VE-cadherin, a feature that we hypothesised to be a wound healing response. Therefore, we chose the stationary explants methodology, with no simulation of flow, for our subsequent experiments.

The stationary method, where chorionic villi were incubated with hyperglycaemia (15mM glucose) vs. euglycaemia (5mM glucose) for the two different durations (4h and 24h) revealed that 15mM glucose was affecting junctional stability and the pro-permeability molecule VEGF-A after a 24h hyperglycaemic insult, but that VEGF was not affected by 4h. Given that 4h is a physiologically important timepoint (representing a postprandial glucose peak seen in diabetic pregnancies), we continued with this timepoint in further experiments.

Whilst we found no effect of 15mM glucose insult after a 4h incubation on total VEGF expression, we found that the recently discovered anti-permeability VEGFb splice variant was

decreased in hyperglycaemia compared to euglycaemic explants. Furthermore, the there was a significant negative correlation between total VEGF and VEGFb levels, indicating that the ratio of the two molecules was changing in diabetic explants compared to normal explants. The diabetic explants showed no further down-regulation of VEGFb on 15mM glucose insult, indicating a tolerance of the diabetic placental vessels to hyperglycaemia.

We then investigated whether total VEGF and/or VEGFb were important predictors of vascular dysfunction as measured by an increased in leakage to 76Mr dextran-TRITC tracer in a well established perfusion model. The vascular bed was perfused with 15mM glucose administered to the maternal circuit. After a 3h hyperglycaemic perfusion, we observed high total VEGF, low VEGFb, and a loss of VE-cadherin from the endothelial junctions. These changes corresponded to a mild increase in leakage to 76Mr dextran tracer measured by counting vessels showing 'hotspots' of tracer at perivascular regions (18% of vessels leakage in the hyperglycaemic perfusions vs. 10% leakage in the euglycaemic perfusions). The percentage of vessels exhibiting tracer leakage showed a significant negative correlation with VEGFb (Spearman r value -0.8857) but not total VEGF, indicating that the former may be an important predictor of vascular dysfunction.

It would be clinically important to be able to predict placental vascular dysfunction in diabetic pregnancies. Further experiments are needed to see whether the VEGFa/VEGFb ratio can predict vascular leakage under hyperglycaemia and the other main feature of diabetic pregnancies, hyperinsulinaemic insult, and whether the hyperglycaemic insult resulting in the diabetic phenotype is reversible upon euglycaemic conditions being restored. Our studies so far have shown that the diabetic phenotype can be partly replicated, in terms of vascular leakage, with a single 15mM hyperglycaemic insult. Chronic insult may well prove to result in the fully leaky vessels observed in diabetic placentae. VEGFb might be an important predictor of this leakage, and may be clinically used for assessment of risks in diabetic pregnancies.

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Abbreviations

α- and β-catenin	Alpha- and beta-catenin
Å	Angstrom (0.1 nanometre)
AGEs	Advanced glycation end products
Ang-1 and -2	Angiopoietin-1 and -2
ARP 2/3	Actin-related protein 2/3 complex
ASF/SF2	Alternative splicing factor/splicing factor 2
ATP	Adenosine triphosphate
BMI	Body mass index
bp	Base pairs
BSA	Bovine serum albumin
°C	Degrees Celsius
Ca^{2+}	Calcium
CO_2	Carbon dioxide
CVS	Chorionic villous sampling
D15	Diabetic explants incubated with 15mM glucose
D5	Diabetic explants incubated with 5mM glucose
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DPX	Di-n-butyle phthalate in xylene
DSS	Distal splicing site
EC	Endothelial cell
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
ET-1	Endothelin-1
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GDM	Gestational Diabetes Mellitus
GLUT	Glucose transporter
h	hour(s)
HbA_{1c}	Glycosylated haemoglobin
H&E	Haematoxylin and eosin
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HO	Haeme oxygenase
HUVEC	Human umbilical vein endothelial cells
ICR	Intracranial pressure
IDDM	Insulin independent diabetes mellitus
IGF	Insulin-like growth factor
ISE	Intronic splicing enhancer
ISS	Intronic splicing silencer
IRS-1	Insulin receptor substrate-1
IU	International unit
IUGR	Intrauterine growth restriction
JAM	Junctional adhesion molecule
kDa	kilo Dalton
KDR	Kinase domain region (also VEGFR-2)

LGA	Large for gestational age
M0	Medium 199 supplemented with 200IU/ml penicillin, 200μg/ml s treptomycin, 2g/ml fungizone
M199	Medium 199
M5	M0 supplemented with 5% fetal bovine serum
MAB	Monoclonal antibody
MAM	Meprin, A-5, Mu domain
MAPK	Mitogen-activated protein kinase
min	minute(s)
ml	millilitres
mM	millimolar
mmHg	millimetres of mercury
mOsm	milliosmole
mRNA	messenger ribonucleic acid
μg	microgram
μl	microlitre
μm	micrometre
N15	Normal explants incubated with 15mM glucose
N5	Normal explants incubated with 5mM glucose
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NFκB	Nuclear factor κB
nm	nanometre
NO	Nitric oxide
NOS	NO synthase
Nrp	Neuropilin
OCT	Optimum cutting temperature compound
p	probability value
p.c.	post-conception
P120	p120-catenin
PBS	Phosphate buffered saline
PECAM-1	Platelet Endothelial Cell Adhesion Molecule -1
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-3-kisane
PLCγ	Phospholipase Cγ
PIGF	Placental growth factor
PKB	Protein kinase B
PKC	Protein kinase C
PP1	Protein phosphatase 1
PSS	Proximal splicing site
RAGE	Receptor to advanced glycation end products
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
rpm	Revolutions per minute
RS domain	Arginine-serine reapeats domain
snRNP	Small nuclear RNP
SR proteins	Serine/Arginine-rich proteins
SRp	SR protein
SRPK1-2	SR protein kinases 1 and 2
TEER	Transendothelial electrical resistance

TGF-β	Transforming growth factor beta
TNF-α and β	Tumour necrosis factor alpha and/or beta
TRITC	Tetra methyl rhodamine isothiocyanate
Tyr	Tyrosine
VE-cadherin	Vascular Endothelial cadherin
VEGFa	Pro-permeability Vascular Endothelial Growth Factor splice variant a
VEGFb	Anti-permeability Vascular Endothelial Growth Factor splice variant b
VEGFR-1	VEGF receptor 1 (also Flt-1)
VEGFR-2	VEGF receptor 2 (also KDR)
vs.	versus
ZO	Zonula occludens

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Chapter 1
General introduction

1.1 The placenta

The function of the human placenta is vital for implantation, growth and healthy development of the embryo and fetus. The human placenta is a haemochorial organ, where the maternal blood comes in direct contact with the (chorionic) trophoblast of the placenta. The maternal circulation and the feto-placental circulation do not mix whilst allowing vital nutrient and gas exchange. It connects the developing fetus to the uterine cavity (Figure 1.1), and acts as an important discriminatory barrier between the mother and the fetus. Any abnormality in the structure and/or function of the placenta can result in fetal morbidity and mortality. Maternal diabetes, which is a major complication of pregnancy, does not spare the placenta and results in morbidity with long-term consequences.

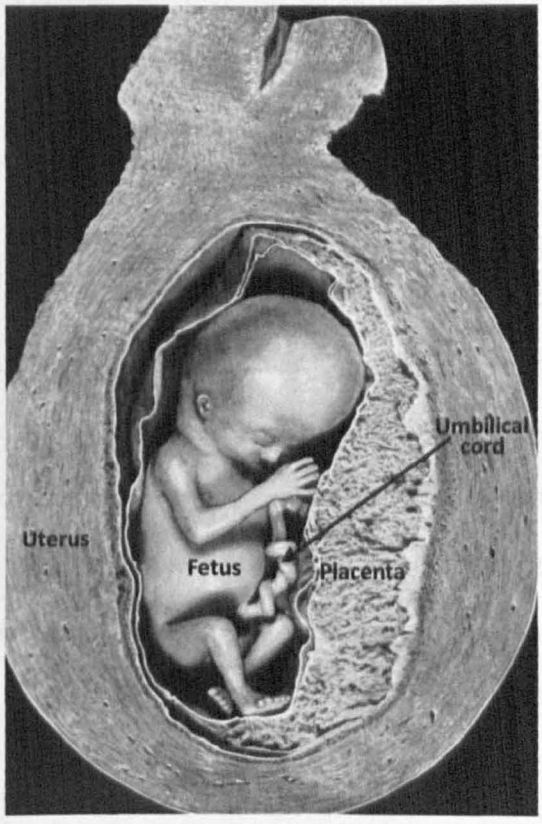


Figure 1.1: The pregnant human uterus, showing a third month gestation fetus, placenta, and uterine wall. The placenta is embedded in the decidualised endometrium, and connected to the fetus via the umbilical cord. Taken and adapted from Boyd and Hamilton (1970) with permission from John Wiley and Sons.

1.1.1 Placental function

The main functions of the human placenta are to carry out the exchange of gases and nutrients from the mother to the fetus (and vice versa), to produce hormones, predominantly progesterone, for the maintenance of the pregnancy (Munro *et al.*, 1983), and to protect against eventual xenobiotic substances in the maternal blood. In order to carry out the first function, exchanging gases such as oxygen, carbon dioxide and nutrients such as amino acids, free fatty acids, carbohydrates and vitamins, the placenta has an intricate and complex vascular system (Burton and Jauniaux, 1995). The maternal blood comes from the maternal spiral arteries and enters the trophoblastic lacunae (also called the intervillous space or 'blood lakes'), and the exchange of gases takes place between these and the terminal villi derived from the trophoblast layer of the embryo. The maternal blood then drains from the blood lakes via uterine veins (Figure 1.2). The fetal blood is transported from the umbilical cord's two arteries, through the placental circulation to the exchange sites in the terminal villi, and back to the fetus through the single vein in the umbilical cord, and to the fetus (Figure 1.2) (Heifetz, 1996; Boyd and Hamilton, 1970).

The placenta is highly permeable to gases, so the exchange of these is by simple diffusion. The diffusion of oxygen and carbon dioxide is so quick that the rate-limiting step in this exchange is the blood flow to and from the site of exchange. Interestingly, fetal haemoglobin has a higher affinity for oxygen and a lower affinity for carbon dioxide than the maternal adult haemoglobin (Blackburn, 2003), therefore favouring the transfer of oxygen to the fetus and of carbon dioxide to the mother.

As for the transfer of nutrients across the placental barrier, the main nutrients transported are carbohydrates, amino acids, lipids and vitamins and minerals, and each of these has its own system for transport.

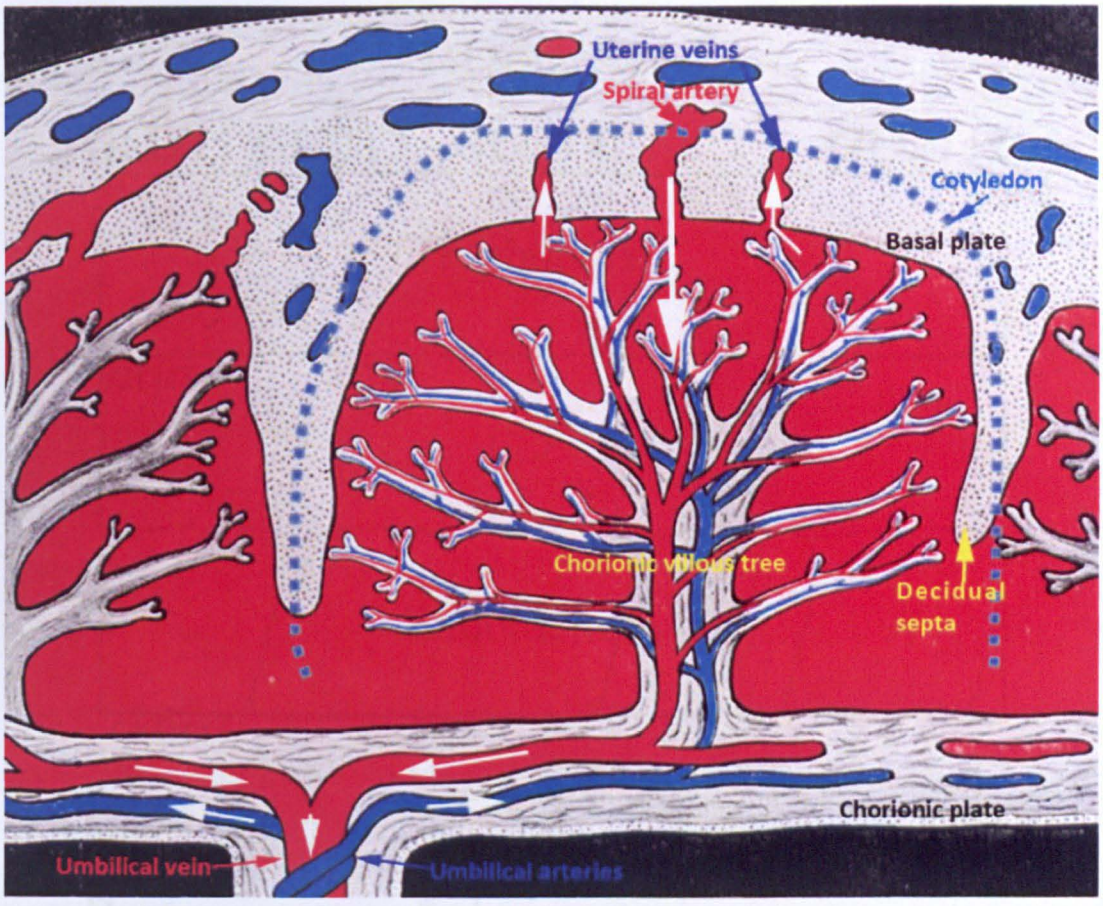


Figure 1.2: Diagram of a cross-section of the placenta, showing a chorionic villous tree with vessels directly connected to the umbilical vein (red) and the umbilical arteries (blue). The maternal blood flows (white arrows) into the blood lakes through spiral arteries, and drains from the intervillous space from the uterine veins. Fetal blood flows (white arrows) from the fetus through the umbilical arteries, through the placental circulation and back to the fetus from the umbilical vein. The blue dotted lines represent a cotyledon. The space (in red) surrounding the chorionic villous tree is the maternal blood from the maternal spiral arteries. The basal plate, chorionic plate, and decidual septa have also been annotated. Taken and adapted from Boyd and Hamilton (1970) with permission from John Wiley and Sons.

Glucose is the main source of carbohydrate for the fetus, as the fetus can carry out very little gluconeogenesis. Transfer of glucose from the maternal circulation is carried out by protein-mediated facilitated diffusion through the syncytiotrophoblast, with a number of glucose transporters (GLUTs) present in this layer of the placenta in pregnancy, and through the endothelial paracellular cleft to the fetal circulation. However, the location of GLUT receptors in the placenta changes throughout pregnancy (Figure 1.3).

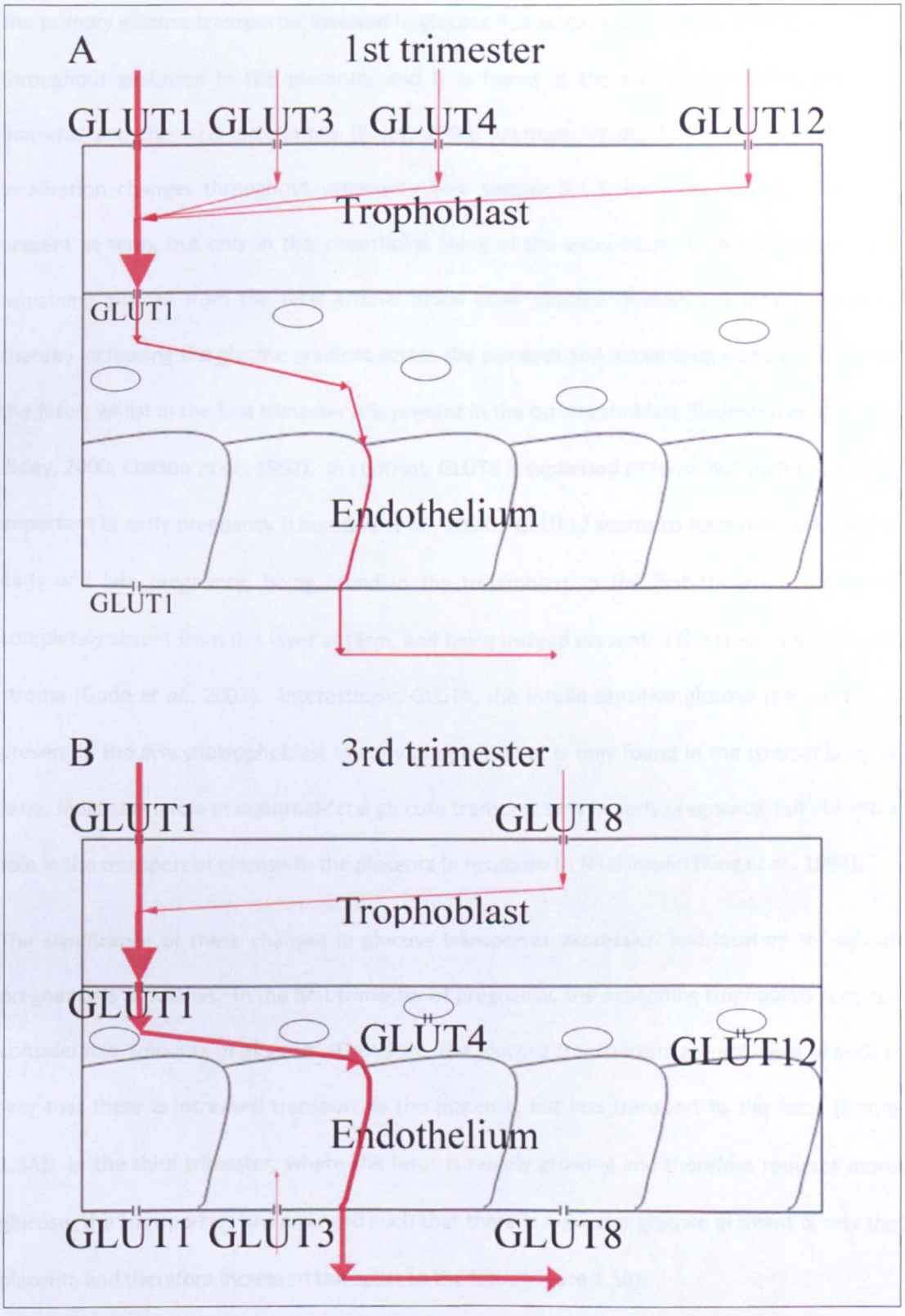


Figure 1.3: Location of GLUT transporters in early and late pregnancy and effect on glucose flux. In early pregnancy (A), the glucose transporters' location is such that there is increased placental uptake of glucose, therefore less transport to the fetus, whilst in late pregnancy (B), glucose transporters are arranged so that there is increased transport to the fetus. Red arrows: glucose path, the relative width of which corresponds to the relative amount of glucose present in that area, with bolder lines representing higher glucose levels.

The primary glucose transporter involved in glucose flux across the placenta, GLUT1, is present throughout gestation in the placenta, and it is found in the membranes facing both the maternal and the fetal circulations (Illsley, 2000; Baumann *et al.*, 2002), although its exact localisation changes throughout pregnancy (see section 5.1.3. for more detail). GLUT3 is present at term, but only in the endothelial lining of the vasculature, where it removes the remaining glucose from the fetal arterial blood after passage through the fetal circulation, thereby increasing the glucose gradient across the placenta and maximising glucose transfer to the fetus, whilst in the first trimester it is present in the cytotrophoblast (Baumann *et al.*, 2002; Illsley, 2000; Clarson *et al.*, 1997). In contrast, GLUT8 is expressed at term, but seems to be less important in early pregnancy (Limesand *et al.*, 2004). GLUT12 seems to have different roles in early and late pregnancy, being found in the trophoblast in the first trimester, but being completely absent from this layer at term, and being instead present in the smooth muscle and stroma (Gude *et al.*, 2003). Interestingly, GLUT4, the insulin-sensitive glucose transporter, is present in the syncytiotrophoblast in early pregnancy but is only found in the stromal layer at term, indicating a role in maternal-fetal glucose transport only in early pregnancy, but at term a role in the transport of glucose to the placenta in response to fetal insulin (Xing *et al.*, 1998).

The significance of these changes in glucose transporter expression and location thoughout pregnancy is as follows. In the first trimester of pregnancy, the expanding trophoblast requires considerable amounts of glucose. Therefore, the glucose transporters are arranged in such a way that there is increased transport to the placenta, but less transport to the fetus (Figure 1.3A). In the third trimester, where the fetus is rapidly growing and therefore requires more glucose, the transporters are arranged such that there is a greater glucose gradient across the placenta and therefore increased transport to the fetus (Figure 1.3B).

Glucose is initially uptaken by the syncytiotrophoblast, and then, once in the syncytiotrophoblast's cytoplasm, it can be transferred to the fetal circulation via the endothelial lining of the capillaries. The rate-limiting step for glucose transport to the fetus seems to be the

former, as in the syncytiotrophoblast it is also metabolised into glucose-6-phosphate or glycogen for placental nutrition, and therefore not all glucose which is transported to the placenta is transferred to the fetus (narrowing red arrows in Figure 1.3A-B) (Baumann *et al.*, 2002).

The transport of amino acids is essential for the fetus for protein synthesis and metabolism. This is evidenced by the presence of essential amino acids in fetal plasma. Some amino acids can be synthesised by the fetus itself. The transport of amino acids occurs via the microvilli and basal membrane of the syncytiotrophoblast. The fetal-maternal ratio of amino acids in blood is generally between 1 and 4, indicating that there is active transport of amino acids from mother to fetus (Yudilevich and Sweiry, 1985).

Lipids can be transported from the maternal circulation; however, the trophoblast can synthesise oleic, palmitic and palmitoleic acids, and small amounts of stearic, lauric and myristiric acids (Coleman and Haynes, 1987). Lipids are normally bound to proteins within the maternal plasma, such as free fatty acids to serum albumin, so there are lipoprotein lipases on the maternal surface of the placenta which are able to release the lipids from the proteins. They can then either cross the syncytiotrophoblast membrane by simple diffusion, or cross by binding to membrane bound and cytosolic fatty acid binding proteins (Haggarty, 2002). Once in the cytoplasm of the syncytiotrophoblast, they can either be metabolised or transported out of the trophoblast by cytoplasmic binding proteins. In the adult, the liver is responsible for the elimination or biotransformation of bile acids and many other lipid-soluble exogenous compounds; however, the fetal liver is immature, so the placenta takes over this task (Marin *et al.*, 2003).

The transfer of water depends on osmotic and hydrostatic pressure, and can move across the placenta passively; however, there may be a water channel in the trophoblast to facilitate such a transfer (Stulc, 1997). Calcium, potassium, magnesium and phosphate are all transported actively across the placenta, whilst sodium and chloride are thought to transfer passively, and

this is evidenced by the fact that the former group's levels are higher in the fetal circulation than in the maternal circulation, whilst the levels of the latter group are similar in both circulations (Shennan and Boyd, 1987; Stulc, 1997). Vitamins and minerals also are transferred to the fetal circulation.

The placenta is not only involved in the transfer of nutrients, but also in the production of hormones, which go on to have endocrine, paracrine and autocrine effects. Progesterone is produced by the placenta and released into both maternal and fetal circulations; it shares this production with the corpus luteum until the 9^{th} week of pregnancy, after which it becomes solely responsible for the production of this hormone. Progesterone inhibits uterine contraction and suppresses the menstrual cycle. At 9 weeks, the placenta also becomes the main producer of oestrogens, which act as growth hormones for the mother's reproductive organs, such as breasts, uterus, cervix and vagina, whilst the fetus is protected from oestrogen's effects by its conjugation with sulphate and glucoronide (Page, 1993). Human chorionic gonadotrophin is produced by the trophoblast and released to the maternal circulation in very early pregnancy to maintain the corpus luteum; it also acts in an autocrine fashion to stimulate formation of the syncytiotrophoblast and differentiation of villous trophoblast (Malassine and Cronier, 2002). The syncytiotrophoblast produces human placental lactogen, releasing it in both the maternal and fetal circulations; in the fetus, it regulates embryonic development and intermediary metabolism, it stimulates the production of hormones, and may be involved in angiogenesis (Handwerger and Freemark, 2000; Corbacho *et al.*, 2002). Placental growth hormone is secreted by the placenta to the maternal circulation and may have a role in maternal adjustment to pregnancy and placental development (Lacroix *et al.*, 2002). The placenta also produces a number of vasoactive autacoids and eicosanoids (Gude *et al.*, 1998) including endothelins (Grabau *et al.*, 1997), adrenomedullin (Al-Ghafra *et al.*, 2003), nitric oxide (Gude *et al.*, 1994), and many others, which may be involved in blood flow control in the placenta. The placenta also produces large amounts of acetylcholine (King *et al.*, 1991),

released by organic cation transporter subtypes 1 and 3 into the extracellular space (Wessler *et al.*, 2001). Acetylcholine is thought to act on through nicotinic acetylcholine receptors to have a role in the regulation of nutrient transport, blood flow and fluid levels in placental vessels, and also seems to have a role in vascularisation (Sastry, 1997).

Finally, the placenta has a protective function. Although some bacteria, protozoa, viruses and other xenobiotic molecules can pass through the placental barrier, the placenta has a number of mechanisms to defend the fetus from potentially toxic substances. Some of the features of these mechanisms include export pumps in the maternal-facing membrane of the syncytiotrophoblast, multidrug resistance-associated protein family members, a placenta-specific ATP-binding cassette protein, cytochrome P450 enzymes to metabolise drugs and xenobiotics, and phase I and II xenobiotic metabolising enzymes amongst others (Marin *et al.*, 2003; Pasanen, 1999). Although many molecules are not passed through the placenta, immunoglobulin G class is transported through the placenta by pinocytosis, and these antibodies provide an important passive immunity when the baby is born (as reviewed by Schneider and Miller (2010)). The placenta is referred to as providing resistance in series, with both the syncytium and endothelial junctions presenting a physical barrier for transport (Leach and Firth, 1992).

1.1.2 Placental structure

The term placenta derives from the Greek *plakóenta*, meaning flat, round pancake. This is an accurate description of its appearance. On average the placenta has a diameter of 22cm, a thickness of 2.5cm in the centre, and weighs 470g. The umbilical cord generally inserts in the centre of the placenta, but this can vary between individuals, and an abnormal positioning does not necessarily indicate pathology (Benirschke and Kaufmann, 2000).

The fetal side of the placenta, where the umbilical cord attaches, is called the chorionic plate (annotated in Figure 1.2). The chorionic plate is covered by the amnion, and chorionic vessels branch normally in a star-like pattern from the umbilical cord insertion. These vessels are continuous with the microvascular component of the chorionic villous trees.

There are three layers in the chorionic villous tree, each with different cell types. The first layer from the fetal circulation is composed of fetal vascular cells which surround the blood vessels, including endothelial cells, pericytes, and vascular smooth muscle cells. Then, there are mesenchymal cells in the villous core, which include Hofbauer cells and fibroblasts. Lastly, in direct contact with the maternal circulation and completely covering the surface of villous trees is the trophoblast, which can be of two types: undifferentiated cytotrophoblasts and fully differentiated syncytiotrophoblast. The syncytiotrophoblast bathes in the maternal blood and is a functional unit in the transport of oxygen and nutrients to the fetus, whilst cytotrophoblasts act as stem cells for the syncytiotrophoblast, and are responsible for the invasion of uterine spiral arteries.

The maternal surface (or basal plate, as annotated in Figure 1.2) is a heterogeneous mixture of fetal (trophoblast) and maternal (decidual) tissue as the trophoblast invaded the decidua in early pregnancy with subsequent remodelling of uterine spiral arteries (Boyd and Hamilton, 1970). The placenta at term is composed of 10-40 cotyledons (lobes of the placenta, blue dotted line in Figure 1.2), which are incompletely separated from each other by decidual septa. Between the chorionic and basal plates are the chorionic villous trees. In between the chorionic villous trees are the intervillous blood lakes, where the maternal blood comes in contact with the placental villi (Boyd and Hamilton, 1970).

1.1.2.1 The chorionic villous tree

The [umbilical] vessels join on the uterus
like the roots of plants and through them
the embryo receives its nourishment.
Aristotle. On the Generation of Animals *(ca. 340 B.C.)*

This quote from Aristotle compares the placental vasculature to the structure of a tree, where the largest roots and branches from the trunk of the tree (equivalent to stem villi) are large conduit vessels, which then branch into smaller roots and branches, and it is only at the smallest vessels at the ends of the branches and roots (equivalent to terminal villi) which participate in the uptake of nutrients. This is a great visual representation of the chorionic villous tree, which contains three types of chorionic villi in the mature placenta: stem, intermediate and terminal villi.

Stem villi represent the 'trunk' of the chorionic villous trees (Figure 1.4), and are thus located near the chorionic plate. They vary in size between 80μm in diameter for the smallest stem villi to 3mm for the largest, and they make up between 20 to 25% of the total volume of the villi (Benirschke and Kaufmann, 2000). They are covered by a thick layer of trophoblast (which will degenerate at term and be mostly replaced with perivillous fibrinoid; this degeneration is more marked in larger stem villi than smaller ones). The vasculature of stem villi consists of arteries and veins (or arterioles and venules in the smaller stem villi) surrounded by the adventitia which continues without a sharp demarcation into the stroma, which contains fibroblasts, myofibroblasts (the central core of which is called the perivascular sheath) and Hofbauer cells; larger stem villi also contain peripheral arterioles and venules (Demir *et al.*, 1997, Benirschke and Kaufmann, 2000). The vascular walls of stem villi also contain occasional mast cells. Stem villi form from immature intermediate villi (Demir *et al.*, 1997; Castellucci *et al.*, 2000). These large villi provide mechanical support of the villous trees structures, but they have little involvement in the materno-fetal exchange (Benirschke and Kaufmann, 2000). The myofibroblasts present in the perivascular sheath of stem villi may allow the regulation of blood

flow by regulating the longitudinal contraction of the anchoring villi (Farley *et al.*, 2004), therefore decreasing intervillous blood volume and increasing utero-placental flow impedance (Chernyavsky *et al.*, 2010).

The mature intermediate villi (hereafter referred to as intermediate villi) are the intermediate branches of the tree, which link the stem villi to the terminal villi (Figure 1.4). They vary in size between 60-150μm in diameter (about the same diameter of terminal villi), and they make up about 25% of total villous volume. Intermediate villi are defined by their stroma having less than 50% vascular lumen. Another important function of intermediate villi is vasoregulation and intravillous blood distribution regulation, which is allowed by the presence of terminal arterioles. Intermediate villi also produce hormones (Castellucci *et al.*, 2000).

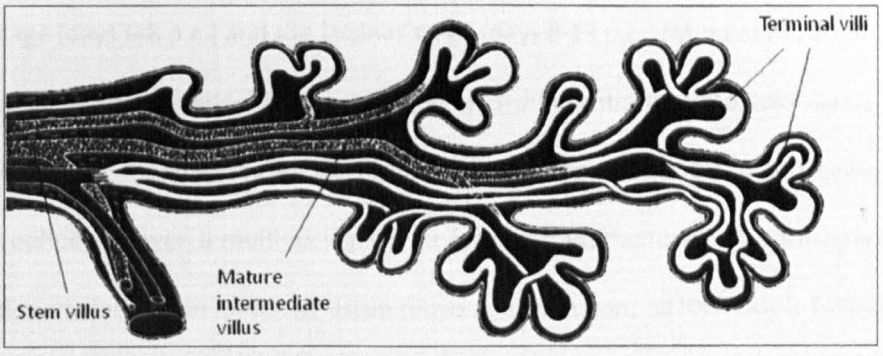


Figure 1.4: Structure of a chorionic villous tree. Stem, intermediate and terminal villi shown. Taken from Benirschke and Kaufmann (2000) with kind permission from Springer Science and Business Media.

The terminal villi are the smallest and most distant from the stem villi. Terminal villi sprout from the surface of intermediate villi, and are characterised by highly dilated sinusoids and a high degree of capillarisation. They have much thinner trophoblast than stem villi and so are able to participate in the feto-maternal exchange function of the placenta in the first and second trimesters. They connect to the mature intermediate villi through narrow necks, and resemble grapes (the terminal villi) on a vine (the intermediate villi) (Figure 1.4). It is these terminal villi which participate most in the gas and nutrient exchange between the two circulations. The average diameter of terminal villi is between 30-80μm, and they form about 40% of villous volume, but due to their small diameters, they constitute 50% to the total

villous surface (Benirschke and Kaufmann, 2000). They are characterised by thin trophoblast and a stroma with connective tissue with few fibres and cells and rare macrophages. The vessels found in terminal villi are capillaries, some of which are sinusoidally dilated. The latter are in close contact with the trophoblast, forming the "epithelial plate", which is the most suitable place for the exchange of nutrients, gases and water, as the diffusional distance between maternal and fetal blood is reduced to around 3.7μm here (Feneley and Burton, 1991).

1.1.3 Vascular development in the developing placenta

The development of the human placenta begins around 6 days post conception (p.c.), as soon as the embryo has implanted. This first placental development period is divided into the pre-lacunar stage (days 6-8 p.c.) and the lacunar stage (days 8-13 p.c.) (Mihu *et al.*, 2009). The pre-lacunar stage is characterised by the trophoblast's differentiation into two layers, an inner single cell layer of mononucleate cytotrophoblast cells around the blastocyst cavity, and the syncytiotrophoblast layer, a multinucleate layer formed from the fusion of cytotrophoblast cells, which is directly facing the maternal tissue (Boyd and Hamilton, 1970). This is followed by the lacunar period, which is characterised by the proliferation of the syncytiotrophoblast and the appearance of lacunae within this layer; the lacunae are initially small, but they merge with each other and will become the intervillous blood lakes once the trophoblast has eroded the spiral arteries' walls (Benirschke and Kaufmann, 2000).

The syncytiotrophoblast continues to penetrate deeper and deeper into the endometrial stroma, until it reaches the endothelial lining of the sinusoids. This is then eroded away, and therefore the lacunae become continuous with the spiral arteries (Kaufmann and Kingdom, 2000). The extravillous trophoblast that invades the spiral arteries is phenotypically distinct from the villous cytotrophoblast, as they have been shown to express different angiogenic

factors in tissue culture experiments (Lash *et al.*, 2010). The lacunae fill with blood, and will subsequently be known as 'blood lakes'. The utero-placental circulation is thus established.

Prior to implantation, decidualisation occurs. The endometrial cells surrounding the embryo become loaded with glycogen and lipids, and polyhedral in shape. The intercellular space becomes filled with extravasate and the tissue is therefore oedematous. The endometrium in these circumstances is called the decidua, and is composed of three parts. The decidua basalis is the part closest to the embryonic pole, and forms a compact layer of cells which is called the decidual plate. This plate is the part of the maternal tissue which is connected to the chorion frondosum (the part of the chorion at the embryonic pole, which is bushy in appearance, and in which the villous structures will form). The decidua capsularis is instead located over the abembryonic pole, and as the embryo grows this part becomes stretched and then degenerates. Finally, the decidua parietalis is on the opposite side of the uterus and as the fetus grows fuses with the chorion laeve (the abembryonic part of the chorion, which is devoid of villi from the third month, and is smooth in appearance).

The chorionic villi begin to be formed on the 13th day p.c. as the cells of the cytotrophoblast invade the local endometrium, forming cellular columns covered with syncytia (Boyd and Hamilton, 1970). These are referred to as primary villi, and initially they completely surround the embryo around the circumference of the chorion (Pijnenborg *et al.*, 1981). The secondary villi begin to be formed at 15 days p.c, when mesodermal cells invade the core of the primary villi, from the chorion in the direction of the decidua. Only one layer of cytotrophoblast cells are present between the surface covering of the syncytiotrophoblast and the inner mesodermic core. The secondary villi are formed before the start of villous vasculogenesis (Demir *et al.*, 2006). Finally, the tertiary or definitive placental villi are formed by the end of the third week p.c. when the mesodermal cells begin to differentiate into blood cells and blood vessels (Boyd and Hamilton, 1970). This process of vasculogenesis and development from secondary to tertiary villi is influenced by the trophoblast (Kingdom *et al.*, 2000). That the trophoblast is an

essential factor in the development of the chorionic villous tree was demonstrated in knockout experiments, where the absence of trophoblastic transcription factor glial cell missing-1, a protein which regulates syncytiotrophoblast differentiation in mice, resulted not only in failure of syncytiotrophoblast formation but also in the decreased formation of blood vessels in the feto-maternal unit (Anson-Cartwright *et al.*, 2000). In the following months, the tertiary villi then sprout to create a villous tree, comprised of stem villi, intermediate villi and terminal villi.

As the embryo grows and becomes a fetus (at 8-10 weeks gestation), more nutrient and gas exchange is required to maintain growth of the fetus at the appropriate rate. In order to achieve this, the composition of the terminal villi, and to a certain extent the intermediary and stem villi, changes. The cytotrophoblastic layer in the villi and some of the connecting tissue starts to disappear, leaving only the syncytium and the endothelial layer of the blood vessels to separate the two circulations, therefore allowing a more intimate connection, and a greater exchange of molecules, as there are only two layers instead of four separating the maternal and fetal circulations. Furthermore, the syncytium becomes very thin, facilitating the exchange. This latter process begins in the fourth month, and at birth no cytotrophoblast is found in the terminal villi, although some is found in the stem villi (Benirschke and Kaufmann, 2000).

During the fourth/fifth month, another morphological change occurs in the placenta: 10-40 cotyledons are formed, which persist until birth. They are formed by a core of maternal tissue, covered in syncytial cells to disallow direct contact between the two circulations, called the decidual septa, projecting into the intervillous spaces. It is very important that the two circulations remain separated at all times, as even a minor bleed can lead to haemolytic disease due to Rh-incompatibility (if the embryo is Rh-positive and the mother Rh-negative, the mother will produce antibodies against the fetus' blood cells, which penetrate the placental barrier and cause a breakdown of the fetal red blood cells). The septa penetrate the intervillous space, however they do not reach the chorionic plate, allowing contact between the various

intervillous spaces in the different cotyledons to be maintained (Benirschke and Kaufmann, 2000).

The cotyledons receive blood from spiral arteries which perforate the decidual plate and enter the intervillous space. The extravillous trophoblast invaded the muscle wall (interstitial extravillous cytotrophoblast) and the endothelium (endovascular extravillous cytotrophoblast) of the spiral arteries, amd might be replacing these cell layers with trophoblast (Brosens *et al.*, 1967; Kaufmann *et al.*, 2003). However, recent views dispute this endothelial replacement (Bulmer *et al.*, 2012). The result of this invasion is the loss of maternal muscular and vascular control, vasomotor control and vessel elasticity, in order to create a high flow but low resistance environment which maximises gentle but high blood flow into the intervillous space with exposure of the chorionic villi to minimal shear. If this remodelling is in part or fully absent, the narrow lumen of the spiral artery and intact muscular wall may restrict and perturb blood flow to the placenta, and the physical influence of the high shear stress on the chorionic villi can induce the villi to secrete vasoactive substances into the intervillous space, possibly leading to maternal hypertension and pre-eclampsia later in the pregnancy (Brosens *et al.*, 1972; Pijnenborg *et al.*, 2006). The intervillous spaces hold about 150ml of blood, which is replenished 3-4 times per minute. The surface area of the villi is around $13m^2$ (Luckhardt *et al.*, 1996), although it is mostly the terminal villi which contribute to the placental exchange (Benirschke and Kaufmann, 2000).

By the end of the pregnancy, terminal villi contain dilated and highly coiled capillaries, and the endothelium of these capillaries is only separated from the maternal circulation by a thin layer of syncytiotrophoblast, minimising the distance between maternal and fetal circulation and thus maximising exchange capabilities without compromising the barrier function of the placenta (Demir *et al.*, 1989; Kingdom *et al.*, 2000).

The placenta throughout the pregnancy increases in parallel to the developing fetus, and it covers approximately 15-30% of the internal surface of the uterus. The increase in thickness of

the placenta during the pregnancy is not due to the further penetration of the maternal tissue; rather, it is due to the further arborisation of the villous trees (Benirschke and Kaufmann, 2000).

1.1.4 Placental vasculogenesis and angiogenesis and its control

Vasculogenesis in the placenta occurs in three steps (Figure 1.5). The first step starts in the third week, and comprises the formation of haemangiogenic cords from angioblastic progenitor cells, which form aggregates arranged in strings. These cells can then differentiate into two cell types: cells in cords located peripherally will become angioblasts, which will go on to become the lining of the blood vessels (the endothelium), whilst the cells in centrally located cords will become haematopoietic cells, which will go on to form blood cells (Demir *et al.*, 2004). They contain desmosomes and primitive tight junctions but have few organelles and no cellular extensions at this stage (Kaufmann *et al.*, 2004). This first step is controlled by the villous cytotrophoblast and Hofbauer cells, as they begin to produce the angiogenic molecules vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (Seval *et al.*, 2007). These two growth factors, via a paracrine mechanism, initiate the differentiation, migration and proliferation of pluripotent mesenchymal cells, which in turn differentiate to haemangiogenic progenitor cells.

The second step of vasculogenesis involves the formation of a lumen by the enlargement of centrally located intercellular clefts, and the presence of cytoplasmic protrusions and outgrowths, as well as pericytes surrounding these primitive vessels (Demir *et al.*, 1989). This step is regulated by the cytotrophoblast, Hofbauer cells and fibroblasts cords, which produce VEGF, PlGF and fibroblast growth factor (FGF) and activate the cords formed in the first step, which then respond by differentiation into endothelial precursor cells (Kaufmann *et al.*, 2004).

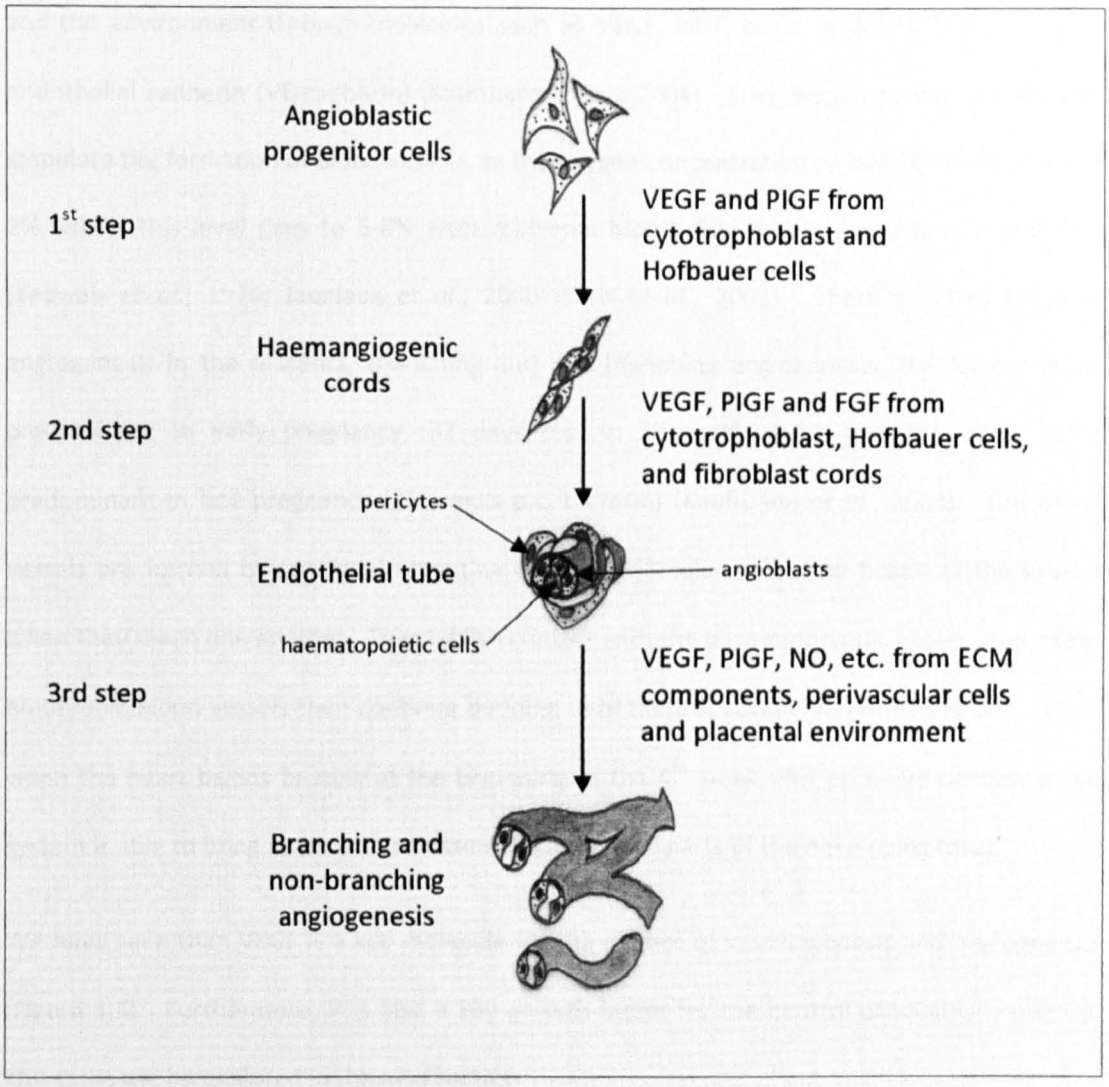


Figure 1.5: Vasculogenesis and angiogenesis in the human placenta. Angioblastic progenitor cells are arranged in haemangiogenic cords in the first step, which then form endothelial tubes in the second step, and vessels grow by branching and non-branching angiogenesis in the third step. On the right, the mediators for these steps are annotated. FGF: fibroblast growth factor, NO: nitric oxide, ECM: extracellular matrix. Adapted and redrawn from Charnock-Jones *et al.* (2004).

The endothelial tubes remain isolated until they join up with the fetal circulation, which at this point has not begun yet. Vasculogenesis starts at the same time in both the placenta and the extraembryonic allantois, which then connects to the placenta and embryo allowing the feto-placental circulation to start. At the same time as the developing vasculature, haematopoietic stem cells start to develop from primitive vessel wall into lumen (Downs *et al.*, 1998).

The third stage is formally referred to as angiogenesis, and consists of the remodelling of the vessels. This stage is mediated by extracellular matrix (ECM) components, perivascular cells,

and the environment through molecules such as VEGF, PlGF, nitric oxide (NO), and vascular endothelial cadherin (VE-cadherin) (Kaufmann *et al.*, 2004). Low oxygen concentrations also stimulate the formation of blood vessels, as the oxygen concentration before 10 weeks p.c. is 1-2%, whilst this level rises to 6-8% after maternal blood flow to the placenta is established (Yedwab *et al.*, 1976; Jauniaux *et al.*, 2000; Lash *et al.*, 2002). There are two types of angiogenesis in the placenta, branching and non-branching angiogenesis, the former being predominant in early pregnancy (32 days p.c. to 25 weeks p.c.), and the latter being predominant in late pregnancy (25 weeks p.c. to term) (Kaufmann *et al.*, 2004). The blood vessels are formed by continual sprouting of the endothelial cells, and fusion of the vessels when they reach one another. To establish contact with the intra-embryonic vessels, the extra-embryonic blood vessels then continue budding until there is contact between the two. Thus, when the heart begins beating at the beginning of the 4th week, this primitive cardiovascular system is able to bring nutrients and essential gases to all parts of the developing fetus.

We have seen than VEGF is a key molecule for the control of vasculogenesis and angiogenesis (Figure 1.5). Furthermore, it is also a key growth factor for the control of vascular integrity. This topic will be explored in the next section.

1.2 Control of endothelial integrity

1.2.1 VEGF-A

Vascular endothelial growth factor (VEGF) is one of the most important growth factors involved in angiogenesis and permeabiility. It is 40,000 more times effective than histamine, in a molar ratio, in inducing an increase in permeability (Senger *et al.*, 1993). Therefore, any molecules which either inhibit or potentiate the action of VEGF are important players, especially in the placentation research area, as the placenta requires tight regulation of angiogenesis and permeability.

1.2.1.1 Molecular biology

VEGF is a family of growth factors which includes 7 members: VEGF-A, -B, -C, -D, -E and -F, and PlGF. VEGF-E and -F were discovered from non-human sources (VEGF-E from a virus (Lyttle *et al.*, 1994), VEGF-F from viper venom (Suto *et al.*, 2005)), but they are not endogenous to the placenta, and thus shall not be discussed further. Three members for which limited information in the placental context is available, and which do not seem to be the major members for VEGF action, are VEGF-B,-C, and -D. VEGF-B can only bind VEGFR-1 (for more detail about VEGF receptors, see section 1.2.1.2); however, VEGF-B can form heterodimers with VEGF-A, and may influence VEGF receptor signalling in this way (Nash *et al.*, 2006). VEGF-B is present in two isoforms in humans, VEGF-B_{167} and $_{186}$. VEGF-C and -D can bind VEGFR-2, but not VEGFR-1, and although VEGF-C is expressed in the placenta, it is mainly a lymphangiogenic agent (Karkkainen *et al.*, 2004), and thus it is not the subject of this thesis. VEGF-D is not present in the placenta. The member of the VEGF family we are interested in for this thesis is VEGF-A.

The VEGF-A gene has 8 exons divided by 7 introns, and it gives rise to six isoforms arising through alternative splicing: VEGF-$A_{121, 145, 165, 183, 189}$ and $_{206}$ (Figure 1.6), the most abundant of which are VEGF-A_{121} and $_{165}$, while other isoforms are expressed only in certain tissues (interestingly, VEGF-A_{145} and $_{206}$ are found only in embryonic and placental tissue) (Pages and Pouyssegur, 2005). The heparin binding isoforms bind heparin in the homodimeric 45kDa protein form (Ferrara and Henzel, 1989) (exons 6-7 in Figure 1.6), and each isoform is differentially regulated (Pages and Pouyssegur, 2005).

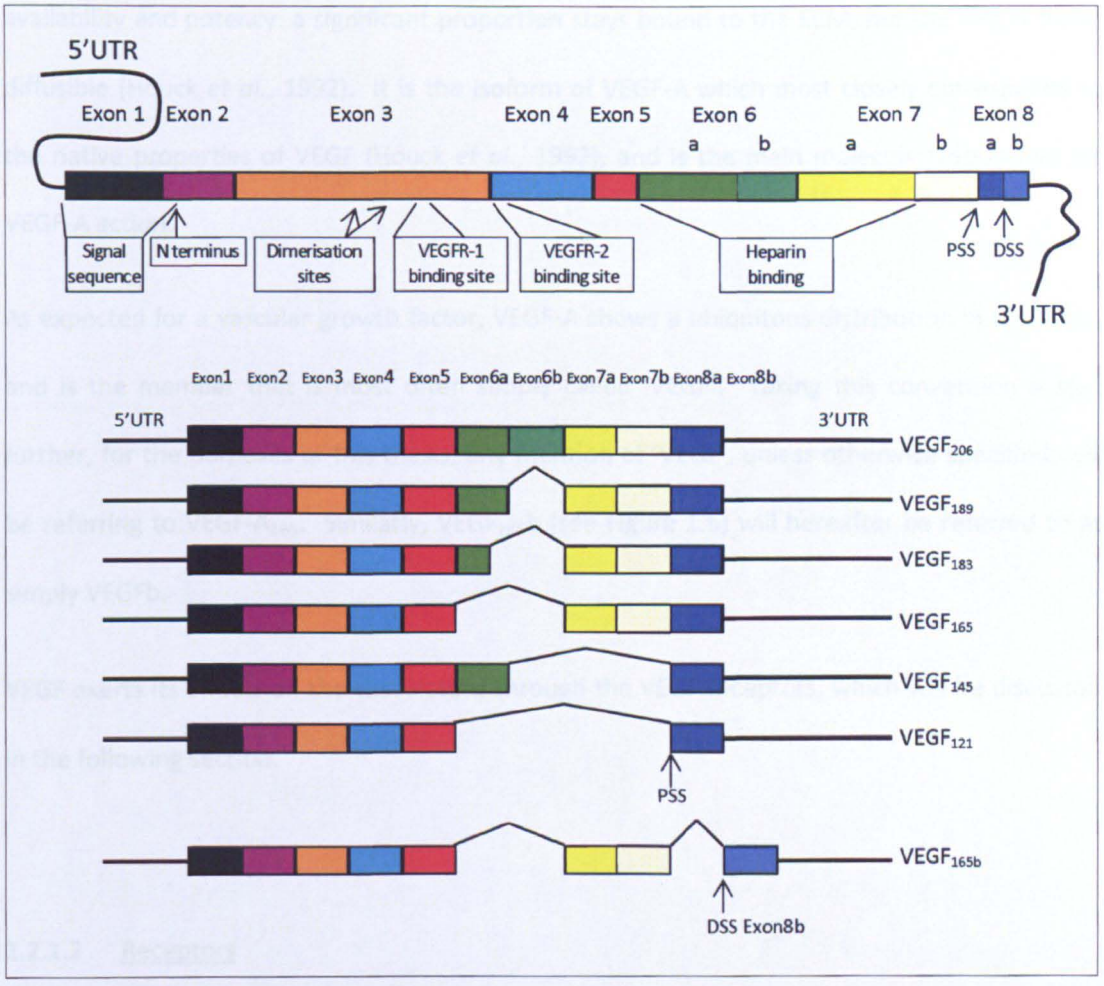


Figure 1.6: VEGF-A gene and pre-mRNA splicing pattern and sequence. The six 'conventional' isoforms of VEGF and $VEGF_{165}b$ are shown. PSS: proximal splicing site, DSS: distal splicing site, UTR: untranslated region. Adapted and redrawn from Woolard *et al.* (2004).

VEGF-A isoforms have different characteristics which allow them to have different properties in the extracellular space. VEGF-A_{121} is an acidic polypeptide (lacking exons 6-7, Figure 1.6), and therefore does not bind heparin or heparan sulphate (a component ECM). It is therefore freely diffusible in the extracellular space, which makes it a very potent but short acting VEGF isoform. VEGF-$A_{183, 189}$ and $_{206}$, in contrast, are highly basic (as they include exons 6-7, Figure 1.6) and therefore bind to the ECM with high affinity, and are almost completely sequestered in the ECM. When the heparin-binding domain is cleaved by plasmin, 110 amino acid active fragments are released, and they are free to bind to the VEGF receptors, and in this way, they are more biologically available than VEGF-A_{121}. VEGF-A_{165} has intermediate properties (as it lacks exon 6 but includes exon 7, Figure 1.6), which give it optimal characteristics for both biological

availability and potency: a significant proportion stays bound to the ECM, but the rest is freely diffusible (Houck *et al.*, 1992). It is the isoform of VEGF-A which most closely corresponds to the native properties of VEGF (Houck *et al.*, 1992), and is the main molecule responsible for VEGF-A action.

As expected for a vascular growth factor, VEGF-A shows a ubiquitous distribution in the body, and is the member that is most often simply called 'VEGF'. Taking this convention a step further, for the purposes of this thesis, any mention of 'VEGF', unless otherwise specified, will be referring to VEGF-A_{165}. Similarly, $VEGF_{165}b$ (see Figure 1.6) will hereafter be referred to as simply VEGFb.

VEGF exerts its effects on the vasculature through the VEGF receptors, which will be discussed in the following section.

1.2.1.2 Receptors

The VEGF receptor family consists of three members: VEGFR-1 (also called fms-related tyrosine kinase-1, or Flt-1), VEGFR-2 (also called kinase domain region, or KDR), and VEGFR-3 (Flt-4) (Figure 1.7). All three receptors are members of the tyrosine kinase family of transmembrane receptors. VEGFR-3 only binds with VEGF-C and -D, and as such will not be discussed further. VEGFR-1 is a negative regulator for VEGF, whilst the primary receptor for VEGF signalling is VEGFR-2 (Mukherjee *et al.*, 2006). The Neuropilins (Nrp), receptors of the semaphorin-collapsin family (Chen *et al.*, 1997), also bind VEGF, VEGF-B, and PlGF, even though they are not tyrosine kinase receptors.

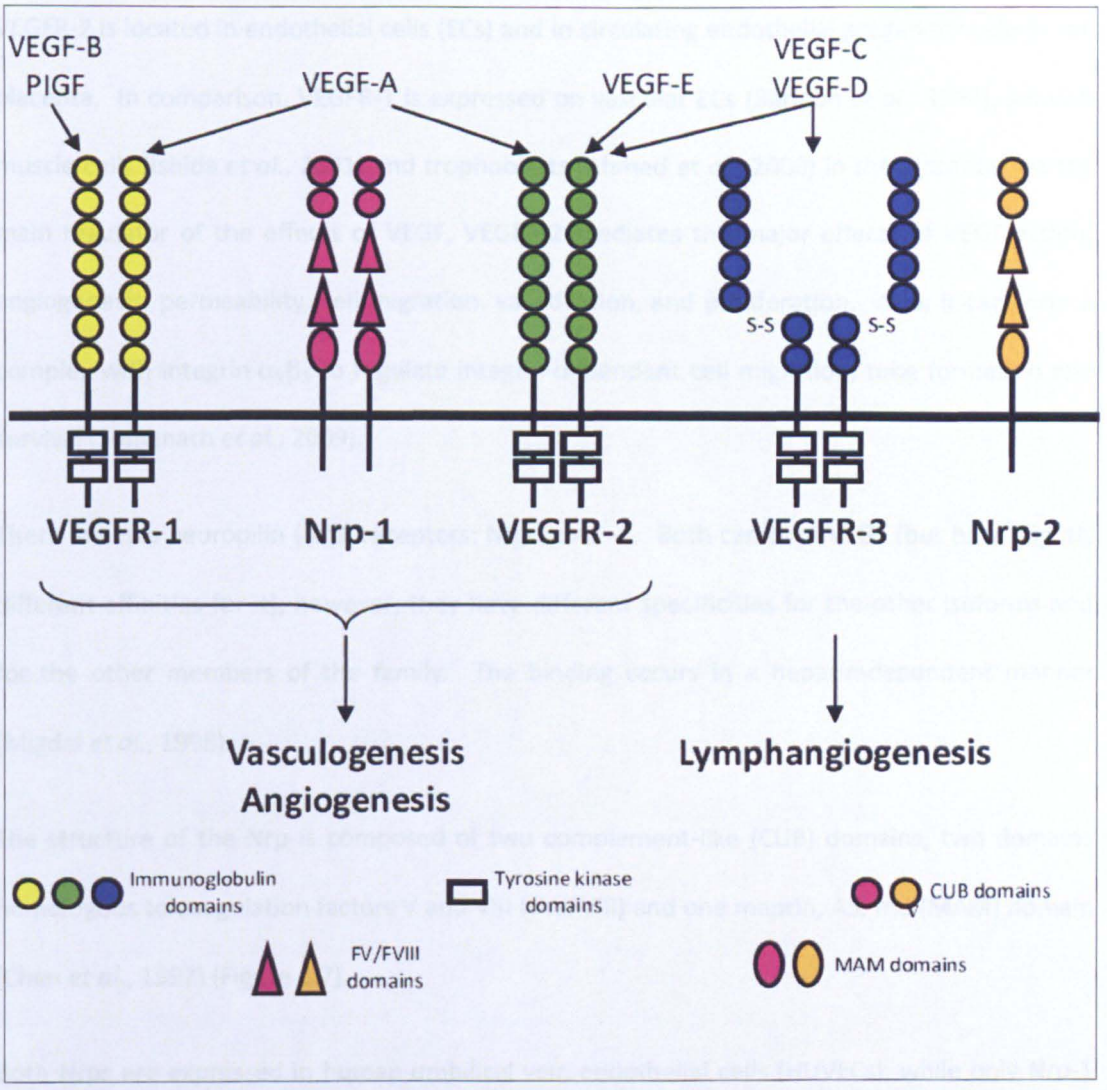


Figure 1.7: VEGF receptors and their ligands, showing which VEGF family member binds to each VEGF receptor (VEGFR). In addition, the structural details of these and the neuropilin receptors (Nrp) are shown. Red, blue and yellow horizontal ovals: immunoglobulin domains, green reclangles: tyrosine kinase domains, horizontal purple and green ovals: complement-like (CUB) domains, purple and green diamonds: homologous to factors V and VIII (FV/VIII) domains, vertical purple and green ovals: meprin, A5, mu (MAM) domains. Adapted and redrawn from Hicklin and Ellis (2005).

The structure of both VEGFR-1 and VEGFR-2 is composed of seven extracellular immunoglobulin domains, each of which have a specific function, a single transmembrane domain, and a consensus tyrosine kinase sequence which is interrupted by a kinase-insert domain (Shibuya *et al.*, 1990) (Figure 1.7). However, slight differences in the immunoglobulin domains result in the VEGF binding with VEGFR-2 to cause strong tyrosine autophosphorylation leading to VEGF signalling activation, whilst VEGF binding to VEGFR-1 causes only weak tyrosine autophosphorylation leading to negative regulation of VEGF signalling.

VEGFR-2 is located in endothelial cells (ECs) and in circulating endothelial progenitor cells in the placenta. In comparison, VEGFR-1 is expressed on vascular ECs (Barleon *et al.*, 1994), smooth muscle cells (Ishida *et al.*, 2001) and trophoblasts (Ahmed *et al.*, 2000) in the placenta. As the main regulator of the effects of VEGF, VEGFR-2 mediates the major effects of VEGF action: angiogenesis, permeability, cell migration, vasodilation, and proliferation. Also, it can form a complex with integrin $\alpha_V\beta_3$ to regulate integrin-dependent cell migration, tube formation and survival (Somanath *et al.*, 2009).

There are two neuropilin (Nrp) receptors: Nrp-1 and -2. Both can bind VEGF (but have slightly different affinities for it); however, they have different specificities for the other isoforms and for the other members of the family. The binding occurs in a heparin-dependent manner (Migdal *et al.*, 1998).

The structure of the Nrp is composed of two complement-like (CUB) domains, two domains homologous to coagulation factors V and VIII (FV/FVIII) and one meprin, A5, mu (MAM) domain (Chen *et al.*, 1997) (Figure 1.7).

Both Nrps are expressed in human umbilical vein endothelial cells (HUVECs), while only Nrp-1 can be found in capillaries (Soker *et al.*, 1998). They are expressed in development in the endothelium of all blood vessels and mesenchymal cells surrounding the blood vessels, amongst others.

Nrps are co-receptors for many members of the semaphorin/collapsin family. It is thought that they affect VEGF's signalling by binding VEGF with high affinity, but acting as co-receptors and therefore not directly transducing a signal (Soker *et al.*, 1998). In support of this theory, VEGF has a different binding site on Nrp-1/2 than VEGFR-2, and therefore does not affect VEGF's binding affinity to VEGFR-2, but greatly potentiates its effects (probably through higher order clustering) (Whitaker *et al.*, 2001). However, Nrps do not seem to influence endothelial cell proliferation *in vitro* (Neufeld *et al.*, 2002).

The signalling pathways mediating the effects of VEGF are discussed in the following section.

1.2.1.3 Signalling

There are several signalling pathways that VEGF is thought to activate via VEGFR-2, to mediate its various effects. When VEGF binds to VEGFR-2, the receptor dimerises and auto-phosphorylates on several tyrosines, which then acts on the different pathways to affect cell behaviour.

VEGF is a survival factor in both vascular endothelium (Baffert *et al.*, 2006) and non-vascular tissues such as neuronal cells and neuronal stem cells (Kilic *et al.*, 2006; Schanzer *et al.*, 2004), lymphocytes (Farahani *et al.*, 2005) and haematopoietic stem cells (Gerber *et al.*, 2002). In order to accomplish this role, VEGF mediates the VEGFR-2-dependent phosphorylation of the phosphatidylinositol-3-kinase (PI3K) pathway which induces Bcl-2 and A1 expression, which then leads to EC survival (Fujio and Walsh, 1999; Gerber *et al.*, 1998). Tyr1175 on VEGFR-2 seems to be especially important for pro-survival signalling (Holmqvist *et al.*, 2004). This effect can be seen *in vitro*, as when ECs are starved of serum, there is PI3K-dependent inhibition of apoptosis by VEGF (Abid *et al.*, 2004). When basal VEGF is inhibited in the vasculature, endothelial cell apoptosis is preceded by local thrombosis and a decrease in vascular perfusion (Inai *et al.*, 2004). Hence, small amounts of VEGF are essential for endothelial cell survival.

In addition to PI3K, the related proteins phospholipase Cγ (PLCγ), rat sarcoma (Ras) GTPase-activating protein and the Src family are also phosphorylated, leading to mitogenic, chemotactic and prosurvival signals (Guo *et al.*, 1995). VEGF also mediates endothelial cell growth through activation of the Raf-Mek-Erk mitogen-activated protein kinase (MAPK) pathway; however, this VEGF-induced activation is protein kinase C (PKC)-dependent but Ras-independent, which is an unusual situation (Takahashi *et al.*, 1999).

The production of NO by endothelial nitric oxide synthase (eNOS) (see section 1.3.3.1 for more details on NO synthesis) can be induced independently by both VEGFR-1 (via PI3K) and by VEGFR-2 (via PLCγ1) (Ahmad *et al.*, 2006). This creates an increase in EC-derived NO, which then increases vasodilation (Hood *et al.*, 1998; Kroll and Waltenberger, 1999). In a related, NO-dependent pathway, VEGFR-2 activates guanylyl cyclase, which then produces cyclic guanosine monophosphate (cGMP), which leads to an increase in vesico-vacuolar organelles, fenestrations and transcellular gaps, thereby increasing permeability. This has been shown to occur *in vitro* (Dvorak and Feng, 2001). Calcium influx is another mechanism for VEGF-induced permeability, as it has been shown that VEGF causes an increase in microvessels' hydraulic conductivity (Bates and Curry, 1997).

The effects mediated by these signalling pathways are discussed next.

1.2.1.4 Effects and role

VEGF is the driving force behind neo-vascularisation: both during embryonic and placental development and during solid tumour vascularisation (Pages and Pouyssegur, 2005). VEGF is produced under hypoxic conditions, which then drives the growth of vessels towards the hypoxic areas. As well as vascularisation, the main member of the VEGF family is responsible for proliferation, sprouting, migration, survival, changes in permeability and tube formation of ECs, and induces angiogenesis in embryogenesis and corpus luteum formation amongst other processes (Ferrara and Davis-Smyth, 1997; Dvorak *et al.*, 1995; Hippenstiel *et al.*, 1998).

Isoforms of VEGF which bind heparin are essential stimulatory cues for the initiation of the formation of vascular branches. This further role is possible because VEGF is regulated by plasmin, which cleaves the heparin-binding domain of ECM-bound forms when more bioavailable VEGF is needed (Carmeliet *et al.*, 1999).

VEGF promotes vascular inflammation (Detmar *et al.*, 1998), and adhesion molecule expression might be stimulated by VEGF, and angiopoietin-1 (Ang-1) has been shown to suppress this, leading to inhibition of leucocyte adhesion *in vitro* (Kim *et al.*, 2001). VEGF is also able to regulate its inhibitor, Ang-1, as Ang-1's inhibitor angiopoietin-2 (Ang-2) mRNA expression and release is strongly upregulated by VEGF (Oh *et al.*, 1999).

VEGF studies have led to insights into its importance in the body. VEGF was the only known example of haploinsufficiency that led to lethality; $Vegf^{+/-}$ mutants died between days 11 and 12 (Ferrara *et al.*, 1996), due to severe vascular abnormalities. This means that VEGF must be carefully regulated both spatially and temporally (Ferrara and Davis-Smyth, 1997).

VEGF overexpression studies by Bhardwaj *et al.* (2003) in rabbit showed a strong angiogenic effect in carotid arteries, and several groups have also shown a similar effect on various animal tissues (as reviewed by Rissanen *et al.* (2004)). However, this often produced large, dilated and leaky vessels. Excessive VEGF has been shown to be dangerous, as recombinant VEGF in pregnant mice led to an increase in resorption frequency, placental haemorrhage, and a decrease in embryo weight. Therefore, bioactive VEGF levels appear to be critical in the regulation of the growth and function of vessels, both during placental and embryonic development (Charnock-Jones, 2002).

In studies by Lim *et al.* (2004, 2005) in Type 2 diabetic patients, plasma VEGF was found to correlate to glycated haemoglobin (HbA_{1c}), a marker for high blood glucose, both in patients with and without cardiovascular disease. Additionally, VEGF levels associated more strongly to HbA_{1c} levels than to which group the sample belonged in (whether the sample was from controls or from diabetic patients with or without cardiovascular disease). The increased VEGF levels showed a correlation with hyperglycaemia in the entire subject group and also in the patients alone (however, the correlation between Ang-2 and hyperglycaemia was only significant in the entire group). Ang-1 expression, in contrast to Ang-2, was affected only in a limited way by VEGF, and this effect might be specific to the cell type. Blood pressure, body

mass index (BMI), smoking and sex did not correlate significantly with VEGF. Therefore, the studies showed that plasma levels of VEGF (and those of Ang-2) correlate with endothelial damage/dysfunction indexes, but not inflammation indexes. They also indicated a link between glycaemic control, endothelial abnormalities, and angiogenesis.

These are the effects of VEGF on the vasculature, but how is VEGF itself regulated and affected by its environment? These aspects are discussed in the following section.

1.2.1.5 Regulation

Hypoxia is one of the most important VEGF regulators, as VEGF gradients attract and guide the new sprouting vessels to the cells which need oxygen most urgently. Hypoxia inducible factor (HIF)1α binds to hypoxia responsive elements on the VEGF gene, increasing transcription (as reviewed by Pages and Pouyssegur (2005)).

As discussed in section 1.2.1.3, VEGF is a survival factor *in vivo*. This is developmentally regulated, as inhibition of VEGF causes apoptotic changes in the vasculature of newborn but not adult mice (Gerber *et al.*, 1999). Coverage of vessels by pericytes might be one of the key changes that cause the loss of dependence on VEGF in human cancer cells (Benjamin *et al.*, 1999).

Heparan sulphate proteoglycans are important for VEGF regulation. They are negatively charged cell-surface associated heparin-like molecules that bind basic VEGF molecules, thereby limiting their diffusion. However, both VEGF (Figure 1.6) and VEGFR-2 have heparin-binding sites. Low concentrations of heparin (0.1-10μg/ml) potentiate the effects of VEGF as they allow crosslinking between the molecules and therefore increasing local VEGF concentration at the VEGF receptor (Gitay-Goren *et al.*, 1992; Tessler *et al.*, 1994; Wijelath *et al.*, 2010); however, higher concentrations inhibit the effects of VEGF, as heparin saturates the VEGF and VEGFR-2 binding sites (Gitay-Goren *et al.*, 1992; Robinson and Stringer, 2001; Tessler *et al.*, 1994). Pre-

treatment with heparinase also inhibits VEGF effects (Pages and Pouyssegur, 2005). However, the $VEGF_{121}$ isoform, which does not bind heparin, is unaffected by heparin or heparinase.

In the earlier mentioned studies by Lim *et al.* (2004, 2005) looking at human plasma levels of VEGF in Type 2 diabetic patients compared to healthy controls, the authors found increased levels of VEGF in diabetic patients, and a significant positive correlation between these and glycosylated haemoglobin (HbA_{1c}) levels. It is thought that raised blood glucose leads to an increase in substrate flux through the sorbitol pathway, and this causes a hyperglycaemic pseudo-hypoxic state, which then induces VEGF production, in a mechanism shown in rat studies (Tilton *et al.*, 1997). With this mechanism, hyperglycaemia leads to an induction of VEGF expression.

Since the effects of glucose on two splice variants of VEGF-A (VEGFa and VEGFb) in the placental vasculature are a main focus of this thesis, it is useful to provide a description of the general spicing mechanisms involved. This is provided next in section 1.2.1.6.

1.2.1.6 General splicing mechanisms

Splicing is the process which removes intronic sequences from pre-mRNA to form mRNA. Splicing occurs at splice sites on pre-mRNA; each intron to be removed has a 5' splice site at its upstream end, and a 3' splice site at its downstream end. It is here that the splicing machinery (spliceosome) binds and initiates splicing. The spliceosome is made up of a large ribonucleoprotein (RNP) complex (comprised of 5 small nuclear (sn) RNP), and catalyses both intron excision and the subsequent exon ligation. The splicing process is thus a two step process (Figure 1.8).

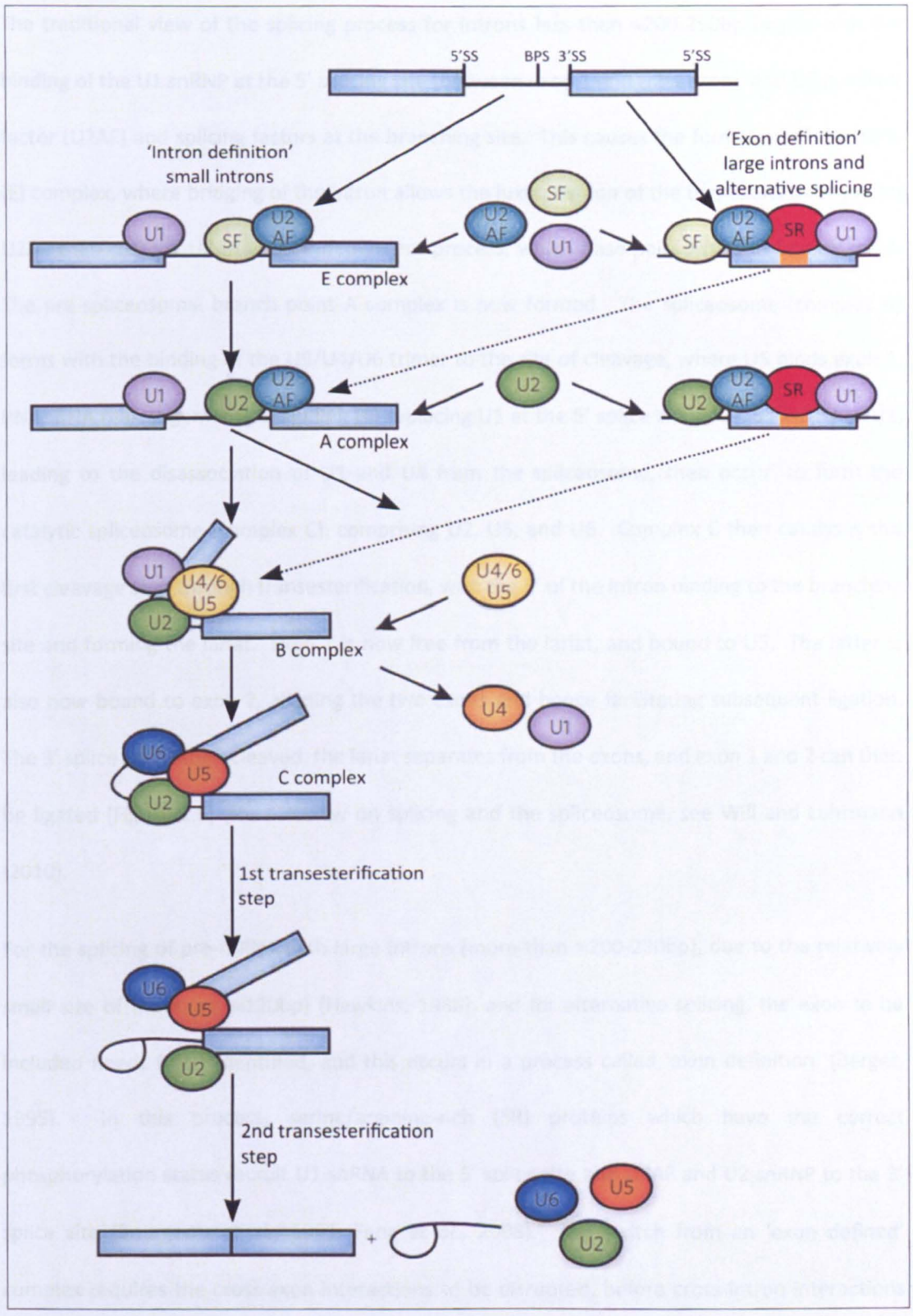


Figure 1.8: Two step splicing mechanism. After assembly of the spliceosome in the C complex, splicing occurs in two esterification steps. Both 'intron definition' (left), seen in splicing of short introns, and 'exon definition' (right), seen in both alternative splicing and splicing of long introns, complexes are shown. 5'SS: 5' splicing site, BPS: branching point site, 3'SS: 3' splice site, SR: SR proteins, dotted arrows: poorly understood processes, orange sequence: exonic splicing enchancer (ESE). Adapted by permission from Macmillan Publishers Ltd: [Nature Chemical Biology] (Schneider-Poetsch *et al.*, 2010), copyright (2010).

The traditional view of the splicing process for introns less than ≈200-250bp begins with the binding of the U1 snRNP at the 5' splicing site (between exon 1 and the intron), and U2 auxiliary factor (U2AF) and splicing factors at the branching site. This causes the formation of the early (E) complex, where bridging of the intron allows the juxtaposition of the two exons to be joined. U2AF then recruits U2 in an ATP-dependent process, which base pairs with the branch point. The pre-spliceosomal branch point A complex is now formed. The spliceosome (complex B) forms with the binding of the U5/U4/U6 trimer to the site of cleavage, where U5 binds exon 1. RNA: RNA rearrangements, including U6 replacing U1 at the 5' splice site and U6 binding to U2, leading to the disassociation of U1 and U4 from the spliceosome, then occur, to form the catalytic spliceosome (complex C), comprising U2, U5, and U6. Complex C then catalyses the first cleavage step through transesterification, with the 5' of the intron binding to the branching site and forming the lariat. Exon 1 is now free from the lariat, and bound to U5. The latter is also now bound to exon 2, aligning the two exons and hence facilitating subsequent ligation. The 3' splice site is then cleaved, the lariat separates from the exons, and exon 1 and 2 can then be ligated (Figure 1.8) (for a review on splicing and the spliceosome, see Will and Luhrmann (2010).

For the splicing of pre-mRNA with large introns (more than ≈200-250bp), due to the relatively small size of the exon (≈120bp) (Hawkins, 1988), and for alternative splicing, the exon to be included needs to be identified, and this occurs in a process called 'exon definition' (Berget, 1995). In this process, serine/arginine-rich (SR) proteins which have the correct phosphorylation status recruit U1 snRNA to the 5' splice site and U2AF and U2 snRNP to the 3' splice site (Bourgeois *et al.*, 1999; Feng *et al.*, 2008). The switch from an 'exon defined' complex requires the cross-exon interactions to be disrupted, before cross-intron interactions can be formed in 'intron definition' complex A or complex B (Smith and Valcarcel, 2000) (see Figure 1.8). The mechanisms behind the switch from 'exon definition' to 'intron definition' are currently poorly understood (shown in dotted arrows in Figure 1.8).

In order for RNA-binding proteins to localise to the right sequences to initiate splicing, the presence of exonic and intronic splicing enhancers (ESEs and ISEs, respectively) and silencers (ESS and ISS, respectively) is essential. These then recruit the SR proteins and heterogeneous RNPs necessary for alternative splicing to the site, which then further recruit the spliceosome. The binding of specific proteins to precise sequences on pre-mRNA found at splicing sites is the best understood process regulating alternative splicing, by increasing or decreasing binding of the spliceosome at a particular splice site (other less well understood processes regulating alternative splicing include elongation rates of the transcript and pre-mRNA secondary structure). Alternative splicing affects 30-50% of genes (Modrek and Lee, 2002; Roberts and Smith, 2002), greatly increasing the number of proteins arising from single genes, and therefore giving rise to greater diversity of proteins.

As discussed above, the VEGF-A gene is one of the genes that can be alternatively spliced. The alternative splicing of the VEGF-A gene results in proteins with different functional characteristics: VEGF splice variants containing exons 6 and 7 are capable of heparin binding (the extent of which is determined by having both exons or only one), neuropilin binding requires both exons 7 and 8a (Appleton *et al.*, 2007; Soker *et al.*, 1998), and the presence of exon 8a or 8b results in VEGFa (for VEGFa naming convention, see section 4.1.2) or VEGFb, respectively (see Figure 1.6). The splicing factors required for the exon 8a/8b selection are discussed below.

1.2.1.6.1 VEGF-A splicing

VEGF-A mRNA is produced through activity at two promoters of the VEGF gene: the classical expression of increased VEGF mRNA following different stimuli is produced through a promoter without a TATA-box, whilst the constitutive low levels of VEGF found ubiquitously are produced by mRNA formed through a second internal promoter.

It is the alternative splicing of this 8-exon VEGF-A pre-mRNA that results in differing protein products which are differentially expressed (Figure 1.6); they have strikingly different properties which confer different roles to these important proteins.

Splicing is a co-transcriptional process, which as stated above occurs when the sequences at the 5' and 3' of the intron (the splicing sites) of the pre-mRNA are recognised by the splicing machinery. The spliceosome is formed by splicing proteins, and the whole process is regulated by splicing regulatory factors. The distal region of the VEGF pre-mRNA (close to the splice region for exon 8) is predicted to contain a number of ESEs (Screaton *et al.*, 1995), to which different splicing factors can bind and thus favouring the production of either the pro-angiogenic or the anti-angiogenic splice variant.

To obtain the splicing product VEGFa requires alternative splicing factor/splicing factor 2 (ASF/SF2) and SRp40 splicing factors. Bioinformatics show that these cluster adjacently to the proximal splice site (Nowak *et al.*, 2008). ASF/SF2 has been shown in several studies (Ge and Manley, 1990; Ge *et al.*, 1991; Krainer *et al.*, 1990) to favour proximal site selection in alternative splicing, and this applies also to VEGF. In order for ASF/SF2 to localise exclusively to the nucleus and be released from its nuclear storage site, it needs to be phosphorylated by SR protein kinases 1 and 2 (SRPK1-2) (Kuroyanagi *et al.*, 1998; Wang *et al.*, 1998; Yun *et al.*, 2003), as inhibition of these then inhibits proximal splicing to produce VEGFa in favour of distal splicing to produce VEGFb in human epithelial cells and mouse models (Nowak *et al.*, 2010). SRPKs are thus important for VEGF splicing decisions.

In contrast, to obtain the splicing product VEGFb requires the SRp55 splicing factor. Bioinformatics show that this splicing factor clusters to 35 nucleotides immediately downstream of the distal splice site stop codon (Nowak *et al.*, 2008). Activation of MAPK p38 seems to correlate to increased phosphorylation and activity of SRp55, which then results in increased levels of VEGFb (Nowak *et al.*, 2008), whilst SRp55 is silenced by small hairpin RNAs and is relocated or degraded as a result of phosphorylation by kinase Clk/Sty (Lai *et al.*, 2003).

Treatment of human epithelial cells and podocytes with transforming growth factor β1 (TGFβ-1) was found to increase expression of VEGFb through the mechanism described above, whilst treatment with insulin-like growth factor-1 (IGF-1) and tumour necrosis factor α (TNF-α) showed an overall increase in VEGF-A but a decrease in VEGFb through activation of PKC which in turn activates SRPK1 and localises it to the nucleus, which ultimately results in the activation of ASF/SF2 and splicing in favour of VEGFa (Nowak 2008, 2010).

Although no studies have as yet been performed investigating the effect of glucose on VEGF splicing, a recent study shows the importance of the splicing factors in diabetic disease, as an SRp55 polymorphism significantly correlates with proliferative diabetic retinopathy (Carter *et al.*, 2011), a diabetic complication characterised by pathogenic angiogenesis. This diabetic complication is also associated with a switch from VEGFa to VEGFb (Perrin *et al.*, 2005), indicating that the polymorphism decreases SRp55's affinity to the VEGF mRNA.

1.2.1.7 VEGFb

A novel splice variant of VEGF-A, VEGFb was discovered in 2002 in renal cortex (Bates *et al.*, 2002). This splice variant was found to be identical to the various isoforms of VEGF ($VEGF_{165}$, $VEGF_{121}$ etc.) except for the last 6 amino acids at the C-terminus, with a sequence of Ser-Leu-Thr-Arg-Lys-Asp in VEGFb instead of Cys-Asp-Lys-Pro-Arg-Arg in VEGFa (Bates *et al.*, 2002). These amino acid differences were predicted to confer a different configuration to the entire molecule, altering its effects; in particular, the two charged arginine residues are replaced with neutral lysine-aspartic acid residues and the proline residue is replaced by an arginine residue (Cebe Suarez *et al.*, 2006).

After it was discovered, VEGFb was shown to be present in many tissues, both human and animal, including colon, islets, vitreous fluid, circulating plasma, urine, prostate, smooth muscle, and, crucially, the placenta. In the first three tissues, it is thought to account for more than 50%

of total VEGF protein, whilst in the other tissues it is thought to represent a significant portion of total VEGF protein, except in the placenta, where it is reported to make up only a small proportion (Bates *et al.*, 2006; Bevan *et al.*, 2008; Konopatskaya *et al.*, 2006; Perrin *et al.*, 2005; Varey *et al.*, 2008).

When it was discovered in the renal cortex, it was shown to be downregulated in tumour tissue compared to normal tissue. This led to the hypothesis that this splice variant of VEGFa is an anti-angiogenic molecule. VEGFb has subsequently been shown to be downregulated in a variety of tissues in pathologies where angiogenesis takes place, such as various cancers – colon (Varey *et al.*, 2008), prostate (Woolard *et al.*, 2004), and renal-cell carcinomas (Bates *et al.*, 2002), and in malignant melanoma (Pritchard-Jones *et al.*, 2007) – as well as diabetic retinopathy (Perrin *et al.*, 2005), pre-eclampsia (Bates *et al.*, 2006), and Denys-Drash syndrome (Schumacher *et al.*, 2007). It is thought that the ratio of angiogenic/anti-angiogenic splice variants might be important in initiating the angiogenesis seen in these pathologies: in normal colon tissue and vitreous fluid, for example, VEGFb comprises about 90% and 65% respectively of total VEGF, however in colon carcinomas and diabetic retinopathy these percentages drop to between 5 and 50% for the former (Varey *et al.*, 2008) and 13% for the latter (Perrin *et al.*, 2005).

VEGFb exerts its effect by being a weak agonist of the VEGFR-2 receptor, but instead of phosphorylating the same Tyrosine residues as VEGFa (Tyr^{1054}, ^{1075} and ^{1215}), it phosphorylates Tyr^{951} and might phosphorylate other tyrosine residues (Kawamura *et al.*, 2008). In human microvascular endothelial cells, but not in HUVECs, VEGFb activates MAPK and protein kinase B (PKB) phosphorylation (Woolard *et al.*, 2004); however, this signalling is attenuated when compared to VEGFa signalling through this pathway (Cebe Suarez *et al.*, 2006). Activation of the MAPK pathway by TGF-β has also been shown to upregulate expression of VEGFb, by downstream activation of CLK/sty kinases, which leads to the phosphorylation of SRp55 (see section 1.2.1.6.1), leading to the up-regulation of VEGFb expression (Nowak *et al.*, 2008).

VEGFb can instead be downregulated by IGF-1, TNFα (Nowak *et al.*, 2008), and by activation of PKC (Nowak *et al.*, 2010), which then phosphorylates alternative splicing factor/splicing factor 2 (ASF/SF2) (Sanford *et al.*, 2005), which can then translocate to the nucleus, leading to an increase in VEGFa (Amin et al, unpublished).

Studies in rodents have led to the theory that VEGFb is important in the reproductive system, where it is tightly regulated. Overexpression of VEGFb in various reproductive systems causes abnormalities: in the ovaries it causes defective follicle development, reduced litter size and inhibits the formation of the cumulus oocyte complex (Qiu *et al.*, 2009a; Qiu *et al.*, 2009b), whilst in the mammary, it inhibits alveolar development during lactation causing a reduction in milk production (Qiu *et al.*, 2008). Inhibition of the VEGFb splice variant replicates the effects seen with overexpression of VEGFa: in the ovary, it resulted in follicle progression (Artac *et al.*, 2009) whilst in males, it stimulated testicular vascular development and caused a perturbation in testicular cord formation (Cupp *et al.*, 2008). All these studies suggest that the balance between the two splice variants may be involved in fertility control.

Another tissue where VEGFb is thought to be important is the kidney, where it is expressed in the human glomeruli (Bevan *et al.*, 2008). Overexpression of the anti-angiogenic splice variant causes a gene-dose dependent decrease in murine glomerular permeability (Ferguson *et al.*, 2007), and has been shown to ameliorate the increased permeability to proteins and water due to diabetic nephropathy in streptozotocin-induced diabetic mice (Ferguson *et al.*, 2009). Recombinant VEGFb has also been shown to decrease water permeability in wild type mouse glomerulus (Ferguson *et al.*, 2009) and to inhibit the increased permeability induced by VEGFa in human kidneys (Bevan *et al.*, 2008). Therefore, it seems as though VEGFb also acts as an anti-permeability molecule, at least in the kidneys.

The paracellular cleft of the endothelium, which has a key role in the maintenance of vascular integrity by providing a physical barrier, is discussed in section 1.2.2.

1.2.2 The endothelial paracellular cleft and its role in endothelial permeability

The endothelium is the lining of the vasculature, and provides a barrier to plasma proteins, which would otherwise move freely in and out of vessels. There is great heterogeneity and diversity in the degree of this barrier function between different tissues, which is dependent on the type of endothelial arrangement: in the renal glomeruli and intestinal mucosa, fenestrated endothelium is comprised of large pores through which proteins can pass, contributing to the exchange functions of these tissues, whilst in the blood brain barrier, a tight continuous endothelium prevents the passage of proteins and harmful pathogens to the delicate brain (Kumar *et al.*, 1987; Garlanda and Dejana, 1997; Simionescu *et al.*, 2000).

In the placenta, the vessel wall comprises of a single continuous layer of endothelium and the secreted basement membrane (Firth and Leach, 1996). One of the characteristics of this barrier is the paracellular cleft, where two endothelial cells come closest to each other (Figure 1.9). This 18nm space between the two cells is filled with a layer of glycoprotein, which creates a net of fibrous molecules, therefore limiting permeability by acting as a 'molecular sieve' (Luft, 1966; Curry and Michel, 1980). Two features of the paracellular cleft in the placenta are adherens and tight junctions, which further restrict permeability.

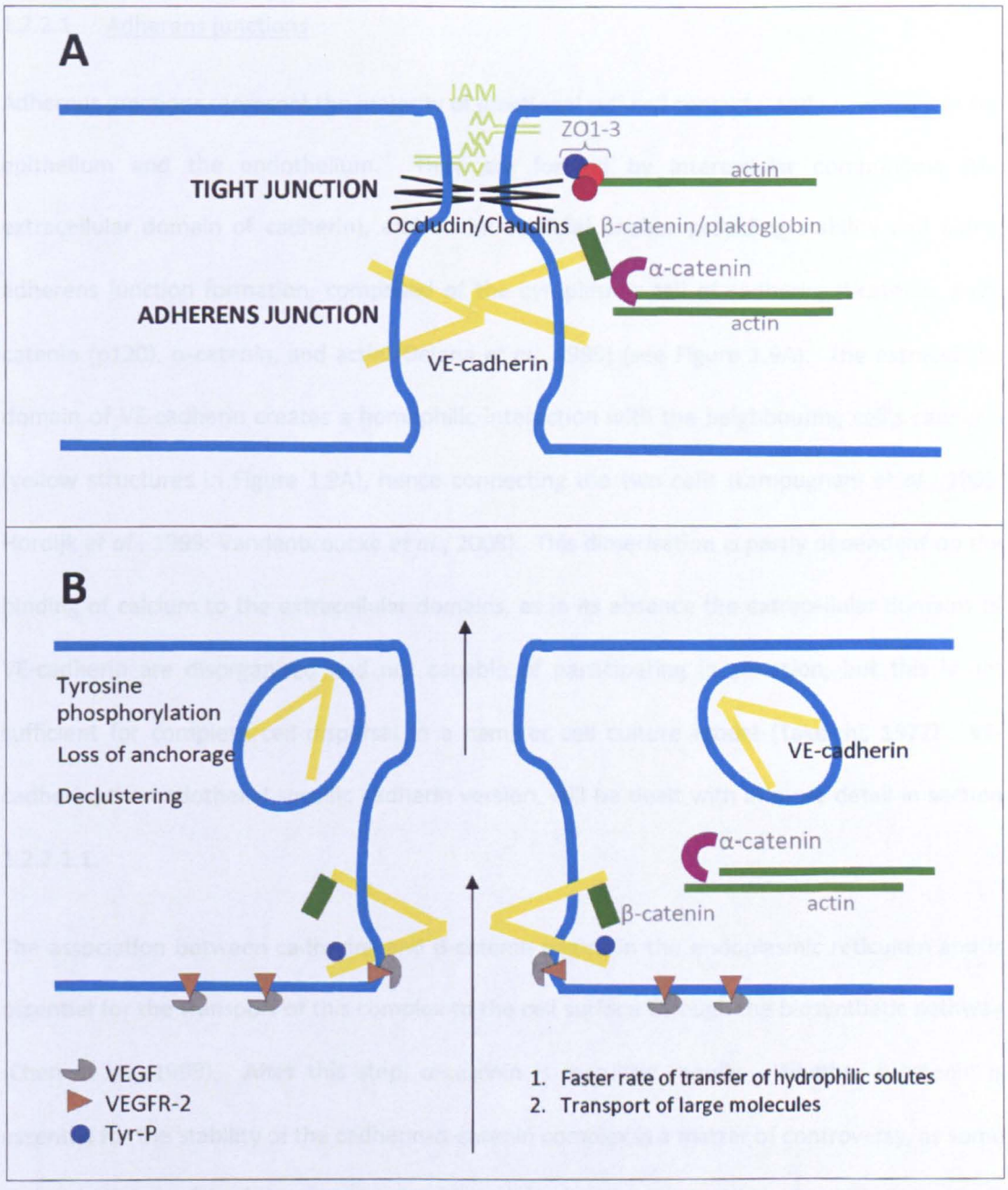


Figure 1.9: Stable (A) and open (B) endothelial junctions in the paracellular cleft. In A, the junction is stable, with VE-cadherin in its complex with β-catenin, plakoglobin, α-catenin and actin, and VE-cadherin homophilic binding with another VE-cadherin on an adjacent cell. In B, in response to VEGF binding to its receptor VEGFR-2 (for more detail, see section 1.2.1.2 on VEGF receptors), VE-cadherin has dissociated from both its intracellular complex with α-catenin and actin, allowing contraction of the cell by actin, and from its partner on the adjacent cell, allowing opening of the junction and paracellular permeability, and allowing VE-cadherin internalisation. Also of note is the tight junction in A, but its opening and disappearance from the paracellular cleft in B. Junctional adhesion molecule (JAM) is thought to have a role in monocyte infiltration in inflammation. Adapted and redrawn from Leach *et al.* (2009).

1.2.2.1 Adherens junctions

Adherens junctions represent the majority of junctional cell-cell contacts, and are present in the epithelium and the endothelium. They are formed by intercellular components (the extracellular domain of cadherin), and an intracellular anchor providing stability and aiding adherens junction formation, comprised of the cytoplasmic tail of cadherin, β-catenin, p120 catenin (p120), α-catenin, and actin (Dejana *et al.*, 1995) (see Figure 1.9A). The extracellular domain of VE-cadherin creates a homophilic interaction with the neighbouring cell's cadherin (yellow structures in Figure 1.9A), hence connecting the two cells (Lampugnani *et al.*, 1995; Hordijk *et al.*, 1999; Vandenbroucke *et al.*, 2008). This dimerisation is partly dependent on the binding of calcium to the extracellular domains, as in its absence the extracellular domains of VE-cadherin are disorganised and not capable of participating in adhesion, but this is not sufficient for complete cell dispersal in a hamster cell culture model (Takeichi, 1977). VE-cadherin, the endothelial-specific cadherin version, will be dealt with in more detail in section 1.2.2.1.1.

The association between cadherins and β-catenin begins in the endoplasmic reticulum and is essential for the transport of this complex to the cell surface through the biosynthetic pathway (Chen *et al.*, 1999). After this step, α-catenin is recruited rapidly. Whether β-catenin is essential for the stability of the cadherin-α-catenin complex is a matter of controversy, as some researchers have found that a cadherin-α-catenin fusion protein is able to function normally in the absence of DE-cadherin (the VE-cadherin homologue in *D. melanogaster*) and Armadillo (the β-catenin homologue in *D. melanogaster*) in the common fruit fly oogenesis (Pacquelet and Rorth, 2005), whilst others have found that this fusion protein cannot totally compensate for the lack of β-catenin during the dorsal closure in the development of the fruit fly (Gorfinkiel and Arias, 2007). Some researchers, as well as work performed in our lab (Wright *et al.*, 2002), on mammalian tissue, have shown that β-catenin plays a role in the dissociation of the cadherin-

catenin complex, resulting in cadherin endocytosis (Lilien and Balsamo, 2005; Delva and Kowalczyk, 2009) (yellow structure in blue oval, Figure 1.9B).

The function of p120 is less controversial, and is thought to be a positive regulator for the function of cadherin. When p120 is bound to the juxtamembrane domain of cadherins, it renders this domain unavailable for binding to the endocytic machinery and covers sites for cadherin ubiquination, thus inhibiting endocytosis (Davis *et al.*, 2003b; Ishiyama *et al.*, 2010). However, p120 has been shown to be not essential for the development of *D. melanogaster*, and a DE-cadherin mutant that cannot bind p120 can fully compensate for the lack of endogenous DE-cadherin (Pacquelet *et al.*, 2003). The fact that both p120 and β-catenin are redundant for the function of the oogenesis in the fruit fly suggests that, at least in this particular tissue in this organism, the morphogenesis actions of cadherins can be accomplished with cadherin-α-catenin alone.

The presence of α-catenin is necessary for the formation of adherens junctions, as reviewed by Kobielak (Kobielak and Fuchs, 2004). α-catenin acts between the cadherin-β-catenin complex and the actin cytoskeleton. Until recently, it was thought that the ability of α-catenin to bind both β-catenin and actin indicated that α-catenin existed in a complex with both of these molecules to keep the cadherin in place in the junctional space. However, recent evidence suggests that such α-catenin cannot bind β-catenin and actin simultaneously: α-catenin binds β-catenin in a monomeric configuration and actin in a dimer configuration, and furthermore, the binding site for β-catenin overlaps the dimerization domain, so whilst bound to β-catenin, α-catenin cannot form dimers (Pokutta and Weis, 2000). This evidence is supported by the fact that cadherin, β-catenin, α-catenin and actin have not been found in a complex together through a variety of approaches (Yamada *et al.*, 2005). A new model has emerged: binding to β-catenin, α-catenin's concentration near the junctional space increases, and on detachment from the former, α-catenin dimerises and competes with actin-related protein 2/3 complex (ARP2/3, a nucleator of branched actin) for binding to actin. This process would help to convert

actin networks from those found in lamellipodia to those seen associated with adherens junctions (Yamada *et al.*, 2005). How adherens junctions couple with actin and how these are able to withstand strong pulling forces is, however, not explained by this model, and remains to be investigated.

Interestingly, α-catenin, as well as binding to actin, β-catenin and plakoglobin (another molecule that binds to cadherins), binds to zonula occludens-1 (ZO-1), a key molecule for tight junctional formation and stability (Kobielak and Fuchs, 2004). Hence, it is thought that adherens junctions and tight junctions are somehow related, although the mechanisms behind this association are not well understood (and hence not shown in Figure 1.9A). This relationship could be essential for the coordinated opening of adherens and tight junctions (as if adherens junctions are open and tight junctions are not, permeability would not necessarily be increased, as another barrier would still be in place).

Studies on Madin Darby canine kidney cells have illustrated how adherens junctions are formed. First, lamellipodia (mediated by several molecules including ARP2/3) from adjacent cells probe a neighbouring cell. Cadherin-catenin complexes cluster at the site of cell-cell contact, α-catenin concentrations rise, and eventually the adherens junction matures (anchored by actin) as α-catenin replaces ARP2/3 in the actin strands, and the junction expands. Cadherin-catenin complexes aggregate and connect with actin, and subsequently actin promotes adherens junctional growth through its remodelling (McNeill *et al.*, 1993; Adams *et al.*, 1996; Vasioukhin and Fuchs, 2001).

Adherens junctional disassembly (Figure 1.9B) occurs when, for example, vessels undergo remodelling or wound healing. Cadherins are normally removed from the cell membrane by endocytosis, although some other mechanisms occasionally occur. The site where p120 binds is also the clathrin binding site (Ishiyama *et al.*, 2010). When cadherins are tyrosine phosphorylated at their intracellular domain, β-arrestin, a clathrin adaptor, is able to interact to

them, and hakai (an E3 ubiquitin ligase) is able to ubiquitylate the cadherin at the site of p120 binding, promoting endocytosis (Gavard and Gutkind, 2006). Indeed, cadherin turnover and endocytosis is increased in the absence of p120 (Ishiyama *et al.*, 2010). The mechanism of how the cadherin-catenin complex disassociates from the actin cytoskeleton is not known. The internalisation of VE-cadherin in the endothelium has of course the effect of increasing vascular permeability, as explained below.

1.2.2.1.1 VE-cadherin

VE-cadherin, also known as CD144 or cadherin-5, is the endothelial-specific cadherin, and, as other cadherins, it has three LDRE domains, three DXNDNXP sequences for the maintenance of cadherin conformation, and four cysteine residues near the transmembrane region (Figure 1.10). VE-cadherin has 5 extracellular domains which, when bound to calcium ions, contribute to the binding between VE-cadherin molecules from adjacent cells, in a zipper-like fashion (Figure 1.10). Ca^{2+} gives rigidity to these extracellular domains, which are otherwise disorganised and cannot bind to each other or provide adhesion (Lampugnani *et al.*, 1995; Hordijk *et al.*, 1999; Vandenbroucke *et al.*, 2008).

The junctional complex of VE-cadherin, β-catenin, plakoglobin and p120 is important for the strength of the junction, more so than its formation: a VE-cadherin mutant which lacks the domain responsible for β-catenin and plakoglobin binding still forms functional zipper-like structures, but the resulting junctions showed decreased strength (Navarro *et al.*, 1995). Other studies have shown that blocking VE-cadherin homophilic association with antibodies in the adult mouse led to vast increases in permeability, haemorrhages and overall vascular fragility (Corada *et al.*, 1999).

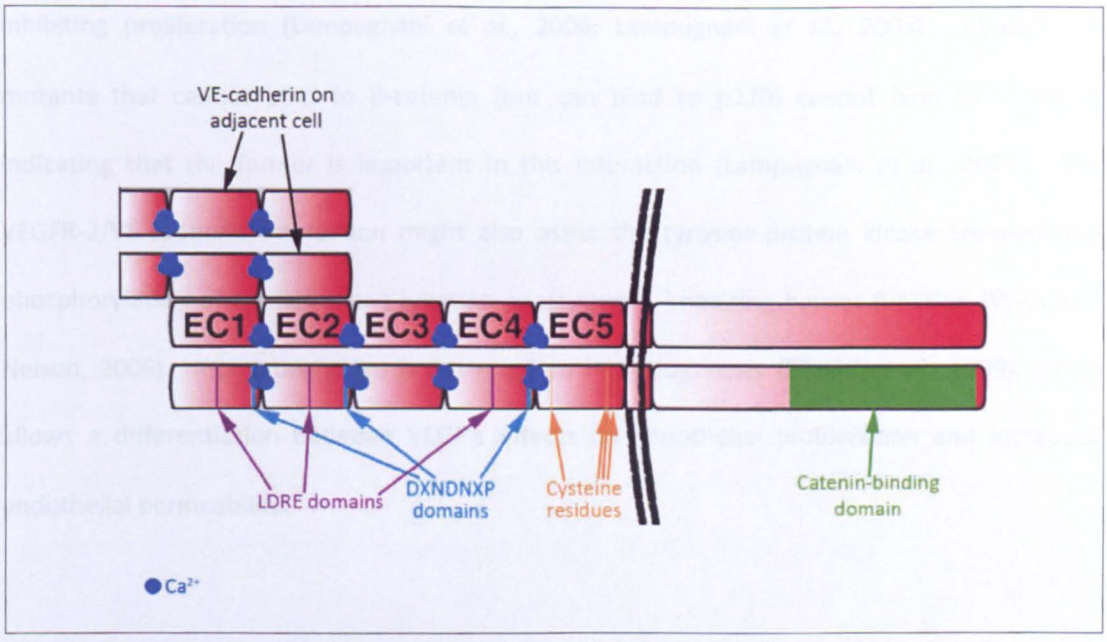


Figure 1.10: VE-cadherin structure showing important components and domains. A VE-cadherin dimer on one cell is shown binding to another VE-cadherin dimer on an adjacent cell through extracellular domains 1-2, whilst structural rigidity is being allowed by calcium binding to the extracellular domains. For simplicity, VE-cadherin binding to its intracellular complex with β-catenin, plakoglobin and p120 is not shown here. EC: extracellular domain, blue circles: Ca^{2+} ions, bold black lines: cell membrane. Adapted with permission, Journal of Cell Science, Dejana *et al.* (2008).

It has been shown that several agents which increase permeability (including tumour necrosis factor, histamine, and, importantly, VEGF) mediate tyrosine or serine phosphorylation of VE-cadherin's intracellular C-tail, and subsequently β-catenin, plakoglobin and p120 disassociate from the VE-cadherin complex (Andriopoulou *et al.*, 1999; Esser *et al.*, 1998), thereby increasing permeability. β-catenin, plakoglobin and p120 can also be phosphorylated by some of the same agents (notably VEGF) that phosphorylate VE-cadherin, and it is known that β-catenin tyrosine phosphorylation can reduce β-catenin/VE-cadherin affinity and increase cadherin turnover at the adherens junctions (Lampugnani *et al.*, 1997; Esser *et al.*, 1998), again increasing permeability (see Figure 1.9B).

Interestingly, VE-cadherin can interact with VEGF's receptor VEGFR-2 (see section 1.2.1.2). Activation of both the PLCγ and MAPK are reduced in VE-cadherin-VEGFR-2 associations (Miaczynska *et al.*, 2004). VE-cadherin also inhibits the internalisation of the receptor via density-enhanced phosphatase-1 (DEP-1) and this contact-inhibition has the overall effect of

inhibiting proliferation (Lampugnani *et al.*, 2006; Lampugnani *et al.*, 2003). VE-cadherin mutants that cannot bind to β-catenin (but can bind to p120) cannot bind to VEGFR-2, indicating that the former is important in this interaction (Lampugnani *et al.*, 2003). The VEGFR-2/VE-cadherin interaction might also assist the tyrosine-protein kinase Src-mediated phosphorylation of the adherens junction constituents, impairing barrier function (Weis and Nelson, 2006). Interestingly, Src is not required in angiogenesis (Eliceiri *et al.*, 1999), which allows a differentiation between VEGF's effects on endothelial proliferation and increased endothelial permeability.

1.2.2.1.2 Vascular adherens junctions and VE-cadherin in the control of vascular permeability

In the endothelium, permeability is mediated by transcellular and paracellular transport. The transcellular pathway is outside the scope of this thesis, as it occurs sparsely, if at all, in placental tissue, and shall not be discussed further.

Permeability involving paracellular junctions was first shown by Majno and Palade (1961) and with tracer studies by Williams and Wissig (1975) and involves adherens and tight junctions opening to allow passage. The openings of these junctions are tightly regulated, as to avoid exposing the subendothelial matrix (a highly thrombogenic layer) to blood, and to retain vascular integrity, but to allow passage of small hydrophilic solutes and to open up to allow cell trafficking to occur (Bazzoni and Dejana, 2004; Aird, 2007). When there is permanent vascular damage, requiring wound healing, endothelial cells retract, with accompanying haemorrhages, leucocyte adhesion, and small thrombi formation. In contrast, molecules able to increase permeability reversibly, such as VEGF and histamine, do not affect functional responses or endothelial cell viability, instead mediating the accumulation of fibrin and fibrinogen outside vessels, leucocyte transfer to inflamed tissue, or simply an increase in oxygen and nutrient access to the surrounding tissues (Dejana *et al.*, 2009).

1.2.2.2 Tight junctions

Tight junctions represent about 20% of cell-cell junctional complexes. As the spacing between cells at tight junctions, also called zonula occludens, is only 4nm, it is the most selective barrier of intercellular transport (Mehta and Malik, 2006).

Tight junctions are comprised of strands of claudin and occludin which are integral to the plasma membrane that run alongside the tight junction and that form the basis for the adhesion to the neighbouring cell (Furuse *et al.*, 1993; Furuse *et al.*, 1998a), and intracellular plaque proteins which anchor the tight junction to the cytoskeleton (Figure 1.9A).

The claudin family of membrane proteins are the key molecules in tight junctions. Their structure of claudins consists of two extracellular loops, the first loop being a selectivity filter for claudin pores and the second loop being involved in cell-cell adhesion, four transmembrane domains and cytoplasmic NH_2- and C-termini. Most claudins have a conserved sequence of GLWxxC in their first extracellular domain, which might assist in the homotypical binding of claudins on the adjacent cell, and a PDZ domain-binding motif at the C-terminus of the molecule, to associate with zonula occludens proteins 1-3 (ZO1-3) (Itoh *et al.*, 1999) (Figure 1.11). They interact with other claudin molecules in the adjacent cell to cause adhesion, and interact with other claudin molecules in the plasma membrane to create tight junctional strands (Tsukita *et al.*, 2001; Furuse *et al.*, 1999). The interaction between the two claudins' first extracellular loop creates a pore which is selective for charge and size: solutes under 4Å in size and with a charge opposite that of the loop can diffuse through the pore without the tight junction opening, whilst bigger solutes cannot.

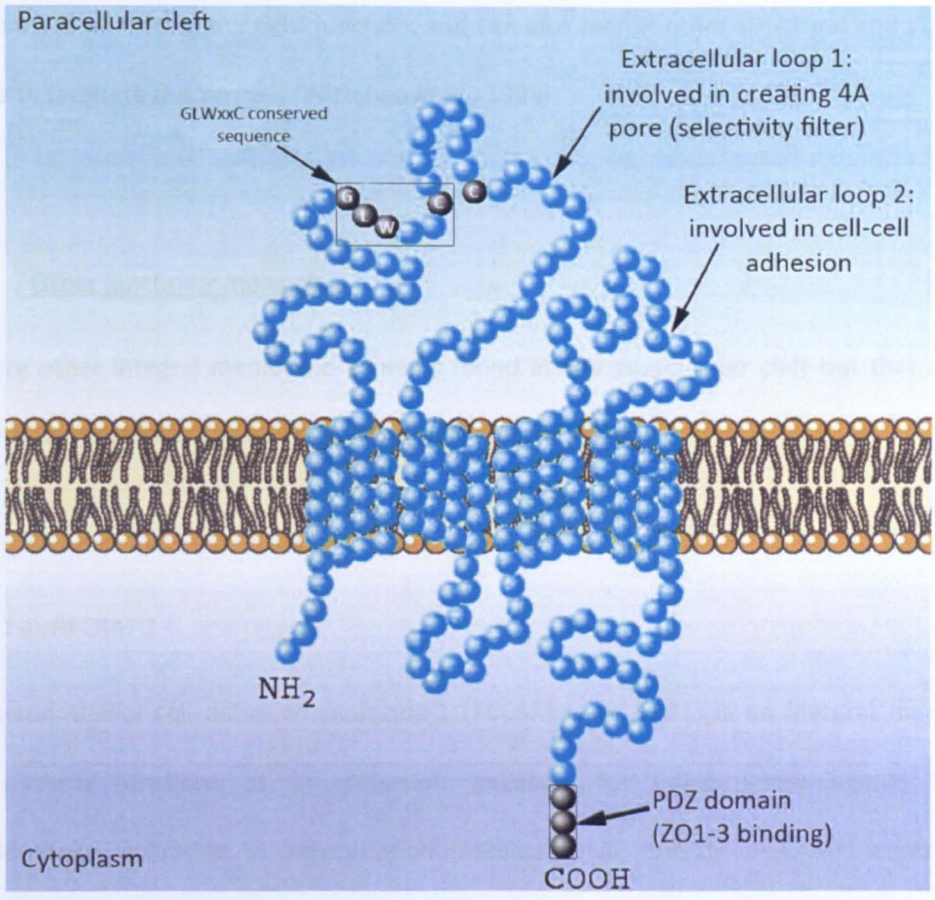


Figure 1.11: Claudin structure showing important components and domains. A claudin molecule is shown, annotated with conserved sequences (black circles) and important domains. In particular, the ZO1-3 binding site, the PDZ domain, is shown, and the conserved GLWxxC domain, which is thought to aid homotypic binding on the adjacent cell. Black circles: conserved residues, blue circles:non-conserved residues. Taken and adapted (permission not required) from Van Itallie *et al.* (2004).

Occludin has been shown by overexpression studies to play a role in tight junction formation and adhesion, although to a lesser extent than claudin: without the latter, cell-cell contacts form, but they are only observed as dots in immunofluorescence micrographs, short occluding strands are formed, and weak cell-cell adhesion occurs (Furuse *et al.*, 1998b; van Itallie and Anderson, 1997). However, occludin knockout mice and murine embryonic stem cell mutants negative for occludin could still form morphologically and physiologically normal tight junctions, and the knockout mice are viable (Saitou *et al.*, 1998; Saitou *et al.*, 2000).

ZO1-3 are cytoplasmic plaque proteins which contain three PDZ domains, the first of which can directly bind to the cytosolic C-terminal domain of claudins (Itoh *et al.*, 1999) (Figure 1.11); ZO-1 can also bind to occludin (Fanning *et al.*, 1998). ZO1-3 can bind to the actin cytoskeleton of the

cell, therefore anchoring the tight junction, and can also recruit other structural and signalling proteins to facilitate this process (Wittchen *et al.*, 1999).

1.2.2.3 Other junctional molecules

There are other integral membrane proteins found at the paracellular cleft but that are not located at the junctional space. Here, we will discuss PECAM-1.

1.2.2.3.1 PECAM-1

Platelet-endothelial cell adhesion molecule-1 (PECAM-1, or CD31) is an integral membrane protein which functions as an adhesion molecule for inflammatory agents to aid transendothelial migration in inflammation (Albelda *et al.*, 1991). PECAM-1 contains six immunoglobulin-like domains in its extracellular domain, and it is through homophilic binding of these with the corresponding CD31 on the leucocyte that PECAM-1 mediates leucocyte extravasation through the paracellular cleft of the endothelium (Sun *et al.*, 1996). In its intracellular domain, PECAM-1 can also associate with β-catenin, through which it may influence the barrier integrity of the endothelium (Ilan *et al.*, 2000).

Vascular integrity dysfunction is one of the characteristics of diabetes. In the next section, we will introduce and discuss this disease, which also affects the vasculature of the placenta, an aspect which is of course of particular interest to this thesis.

1.3 Diabetes mellitus

1.3.1 History of diabetes

The term 'diabetes mellitus' means 'to pass through sweet urine' in Greek. The term 'diabetes' was introduced into the medical field by Aretaeus of Cappadocia, the first person to extensively describe diabetes mellitus (hereafter referred to simply as diabetes). He wrote in his book *Therapeutics of chronic diseases* (taken from Henschen (1969)):

> 'Diabetes is a wonderful affliction, not very frequent among men, being a melting down of the flesh and limbs into urine. ... The course is the common one, namely, the kidneys and bladder; for the patients never stop making water, but the flow is incessant, as if from the opening of aqueducts. The nature of the disease, then, is chronic, and it takes a long period to form; but the patient is short-lived, if the constitution of the disease be completely established; for the melting is rapid, the death speedy. Moreover, life is disgusting and painful; thirst, unquenchable; excessive drinking, which, however, is disproportionate to the large quantity of urine, for more urine is passed; and one cannot stop them either from drinking or making water. Or if for a time they abstain from drinking, their mouth becomes parched and their body dry; the viscera seem as if scorched up; they are affected with nausea, restlessness, and a burning thirst; and at no distant term they expire. Thirst, as if scorched up with fire... But if it increase still more, the heat is small indeed, but pungent, and seated in the intestines; the abdomen shrivelled, veins protuberant, general emaciation, when the quantity of urine and the thirst have already increased; and when, at the same time, the sensation appears at the extremity of the member, the patients immediately make water. Hence, the disease appears to me to have got the name of *diabetes*, as if from the Greek word διαβήτης (*which signifies a siphon*), because the fluid does not remain in the body, but uses the man's body as a ladder (διαβάθρη), whereby to leave it. They stand out for a certain time, though not very long, for they pass urine with pain, and the emaciation is dreadful; nor does any great portion of the drink get into the system, and many parts of the flesh pass out along with the urine.'

The first instance of what Aretaeus called the 'wonderful affliction' was found in the Egyptian Ebers papyrus, dating from about 1550BC, which described various diseases including a disease characterised by polyuria which resembled diabetes mellitus. The Indian physicians Susruta and Charuka in the 5^{th}-6^{th} century made the link between polyuria and sugary urine, and differentiated between diabetes which affects the old and fat, and diabetes which affects the thin and young whose survival wasn't for long, giving the first account of what we now call Type 2 and Type 1 diabetes, respectively. The next significant advancement was the recognition of gangrene and impotence as two complications of diabetes by the Arabic Avicenna in the 10^{th} century. Other observations of the disease were made in the 17^{th}-19^{th} centuries, such as the

identification of the pancreas as vital for the disease, but the real breakthrough for treatment was made in 1921 with the discovery of insulin, which has been used ever since to treat diabetes.

Even though it has been known since antiquity, diabetes today still presents patient and physician with difficulties, providing only a treatment but not a cure. This highlights the challenging nature of tackling the diabetic milieu.

1.3.2 Types of diabetes

As all the diabetic work in this thesis has been performed on tissue from Type 1 diabetic patients, greater emphasis will be placed on this Type of diabetes.

1.3.2.1 Type 1 diabetes

Type 1 diabetes currently affects around 15% of diabetic patients in the UK (around 623,500 people) (Diabetes UK, 2010), and its incidence is increasing (EURODIAB ACE Study Group, 2000). Type 1 diabetes is sub-classified into two subgroups: in Type 1A there is the immune mediated destruction of the β-cells in the pancreas, whilst in type 1B there is a non-immune mediated severe insulin deficiency (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997).

Type 1A (hereafter referred to as simply Type 1) diabetes is characterised by the destruction of the pancreatic β cells by autoimmune mechanisms (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). This destruction subsequently leads to the insufficient production of insulin which is characteristic of this type of diabetes mellitus. Therefore, the symptoms of diabetes are dependent on the lack of insulin present, which explains its previous name of insulin dependent diabetes mellitus (IDDM).

The symptoms of the disease are often preceded by an autoimmune response, whereby autoantibodies against Type 1 diabetes-specific antigens (the three major antigens being insulin, glutamic acid decarboxylase, and insulin autoantigen 2) are present. The detection of two or more of these autoantibodies has a predictive value of over 90% for the development of Type 1 diabetes within the next 7 years (Verge *et al.*, 1996). These antigens act as markers for the T-lymphocytes, dendritic cells and macrophages which subsequently infiltrate the islets of Langerhans (Imagawa *et al.*, 1999). The β cells (which produce insulin) are uniquely targeted in this destructive infiltration, which does not affect α cells (producing glucagon) or δ cells (producing somatostatin) (Miao *et al.*, 2007).

Overt Type 1 diabetes progression from insulinitis is controlled by an alteration in the T helper cells Th1 to Th2 balance, with the subsequent attack and destruction of β-cells (Csorba *et al.*, 2010). By the time symptoms appear, most of the β-cells responsible for the production of insulin have been destroyed, leaving the body unable to produce insulin.

Both genetic and environmental factors contribute to the development of Type 1 diabetes. The genetic component involves several genes, the most important of which is the human leucocyte antigen (HLA) class II gene on chromosome 6p21 (Singal and Blajchman, 1973; Cudworth and Woodrow, 1974; Nerup *et al.*, 1974) and the insulin gene on chromosome 11p15 (Bennett and Todd, 1996; Bell *et al.*, 1984). HLA class II molecules are present on antigen-presenting cells such as dendritic cells, B lymphocytes, macrophages, and the thymus epithelium, and are responsible for presenting antigens to T cells. The defect around the insulin gene which predisposes to diabetes is in a variable number of tandem repeats (VNTR) found 596 base pairs upstream of the insulin gene. A short version of this VNTR predisposes to Type 1 diabetes in Caucasians, whilst longer versions offer protection from the disease (Bell *et al.*, 1984). It is thought that these polymorphisms act by regulating the transcription of the insulin gene *in cis*. The longer polymorphism is associated with a 20% lower insulin mRNA in the pancreas but a two- to three-fold increase in the thymus. It is thought that the higher thymic levels seen with

the protective variant facilitate the induction of immune tolerance (Vafiadis *et al.*, 1997; Pugliese *et al.*, 1997). Therefore, it is not surprising that Type 1 diabetes is associated most commonly with thyroid disease, along with other autoimmune conditions (Devendra and Eisenbarth, 2003).

There are two theories to explain the environmental factor of Type 1 diabetes, both arising to try to explain the rise in the incidence of the disease. The first theory hypothesises that there could be certain viruses or environmental triggers for the disease. Possible viruses implicated are congenital rubella syndrome (Ginsberg-Fellner *et al.*, 1984) and enteroviruses (reviewed by Tracy *et al.* (2010)). The second theory is the so called 'hygiene hypothesis', which states that our environment for infants and children is too clean, which inhibits the proper development of the immune system, leading to a predisposition to immune diseases such as diabetes and asthma (Bach, 2002; Wen *et al.*, 2008).

1.3.2.2 Type 2 diabetes

Type 2 diabetes was previously called non-insulin dependent diabetes, as it is a type which is characterised by insulin resistance, not total insulin deficiency (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). It affects around 85% of diabetic patients (Diabetes UK, 2010). This insulin resistance therefore leads to a relative insulin deficiency. Whilst β-cells in the pancreas are functional and insulin production seems normal or even elevated, insulin resistance and higher blood glucose levels result in the demand for insulin being greater than the supply. There is usually no autoimmune cause to this type. Most Type 2 patients are obese, which is a risk factor for it, as obesity itself is known to cause a limited insulin resistance (Turner *et al.*, 1979; Olefsky *et al.*, 1982; Reaven *et al.*, 1976).

In individuals with Type 2 diabetes, the development of hyperglycaemia is gradual, and the patient may at first not notice the classic symptoms of diabetes (Zimmet, 1992). Therefore, it

may be several years before the disease is diagnosed and treated, and as such they are at greater risk of developing macro- and microvascular complications.

Unlike Type 1 diabetes, Type 2 diabetes can potentially be completely ameliorated with lifestyle changes, as insulin resistance may improve with weight loss and exercise (Zanuso *et al.*, 2010). If this fails, treatment includes pharmacological intervention, most often with metformin, to control hyperglycaemia (Ripsin *et al.*, 2009). However, seldom does insulin resistance disappear completely.

Type 2 diabetes has a strong genetic association (Barnett *et al.*, 1981); it is therefore not surprising that it is more common in certain populations. However, unlike Type 1, the genetic association is very complex.

1.3.2.2.1 Developmental origins of Type 2 Diabetes

Both macrosomia and growth restriction have been associated with a predisposition to developing Type 2 diabetes. Both of these might have a genetic basis, but the fetus' environment is also crucial.

The evidence for macrosomia, an example of fuel-mediated teratogenesis predisposing the fetus to future type 2 diabetes, was initially shown in diabetic Pima Indians (McCance *et al.*, 1994), but later studies have subsequently found it in other populations as well (Dyck *et al.*, 2001; Wei *et al.*, 2003). It is thought that the main cause of this predisposition to Type 2 diabetes is the fetal exposure to hyperglycaemia and hyperinsulinaemia *in utero*.

That growth restriction, an example of nutrient-mediated teratogenesis, predisposes to Type 2 diabetes is explained by the 'thrifty phenotype hypothesis' (Hales and Barker, 1992), also known as Barker's hypothesis, after the author of the theory. In this hypothesis, the fetus makes adaptations *in utero* and in early post-natal life for its survival, based on the nutrient

deficient environment during gestation. If, in later life, this environment becomes more plentiful, the individual is prone to becoming obese and experience relative insulin deficiency.

1.3.2.3 Gestational diabetes

Gestational diabetes (GDM) is the development of diabetes during pregnancy. Gestational diabetes affects up to 5% of all pregnancies (Diabetes UK, 2010), but the frequency varies depending on which populations are studied. GDM represents around 80-90% of diabetic pregnancies (Osborn, 2005).

Gestational diabetes develops in the second and third trimester of pregnancy (Diabetes UK, 2010), and as such it does not affect the early development of the placenta, as do Type 1 and Type 2 diabetes mellitus. Insulin resistance due to pregnancy hormones tends to develop in pregnancy, possibly arising downstream of the insulin receptor; however, the β-cells in women who develop GDM are unable to increase insulin production sufficiently to keep glycaemic levels under control (Catalano *et al.*, 1993). GDM usually resolves within 6 weeks post-partum; however, women who develop GDM have a predisposition to developing Type 2 diabetes later on in life (Girling and Dornhorst, 2003).

Newborns to GDM mothers are often macrosomic and are prone to experience hypoglycaemia immediately after birth, as they have been exposed to high glucose levels *in utero*, and may therefore be producing too much insulin after birth without the corresponding high glucose from the mother (Barnes-Powell, 2007).

1.3.3 The diabetic milieu

Diabetes is essentially a disease of relative insulin deficiency, whether by insulin deficiency (Type 1) or insulin resistance (Type 2).

Insulin is found in the bloodstream at a constitutive level which regulates basal metabolism when blood glucose levels do not exceed 4mM; a higher level of insulin is released at glucose concentrations of >4mM (Henquin *et al.*, 2006) in a mechanism illustrated in Figure 1.12.

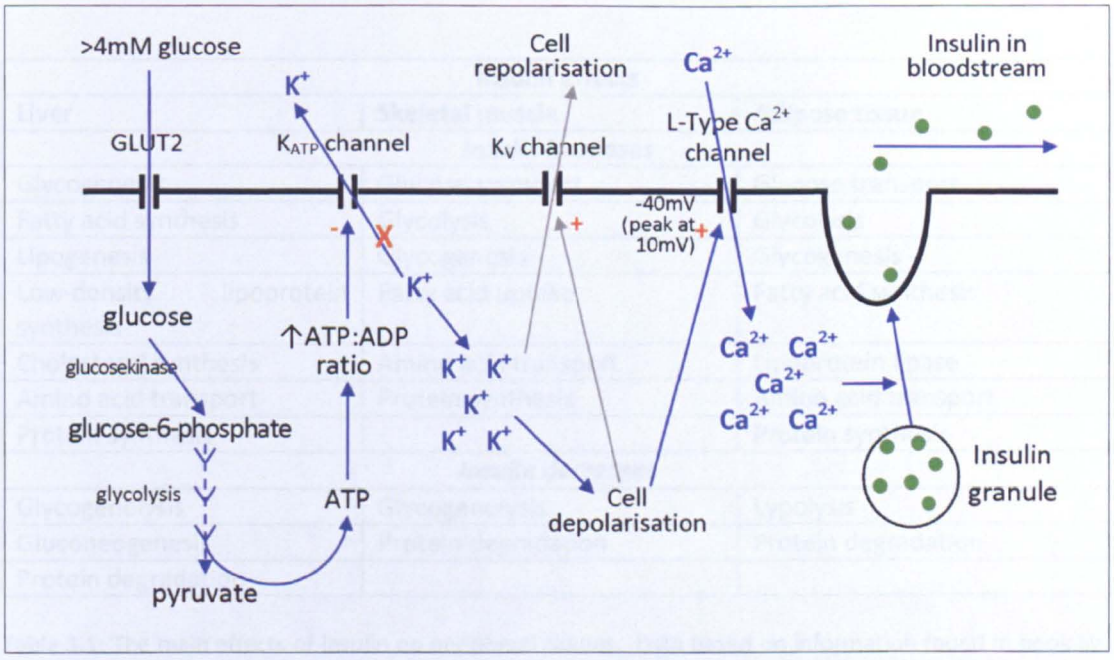


Figure 1.12: Insulin secretion mechanism. When extracellular glucose concentrations exceed 4mM, GLUT2 glucose transporters on β-cells in the islets of Langerhans transport glucose inside the cells, which then undergoes glycolysis. This increases the ATP:ADP ratio, closing ATP-sensitive potassium channels (K_{ATP} channel), causing the ions to accumulate intracellularly. This causes cell depolarisation, which begins at -40mV and peaks at 10mV, thereby opening L-Type Ca^{2+} channels. Ca^{2+} then accumulates inside the cell which mediates the release of insulin (green circles) from insulin granules to the bloodstream. The cell depolarisation then causes the activation of voltage dependent K^+ channels (K_V channels), which then inhibit further insulin release until the next depolarisation event. The effect of these depolarisation/repolarisation events lead to pulsatile insulin secretion. Blue arrows: pathways leading to insulin release, grey arrows: pathways leading to insulin release inhibition, arrow with + sign: activation of channel, arrows with red - sign: inhibition of channel. Figure is based on the reviews by Dunne (2000) and MacDonald and Wheeler (2003).

After its release into the circulation, insulin acts through the insulin receptor in the liver, skeletal muscle and adipose tissue. The insulin receptor autophosphorylates when bound to insulin, and subsequently phosphorylates insulin receptor substrates (IRSs). IRS-1 and IRS-2 then activate PI3K, MAPK, and CAP/cbl. PI3K then activates the PKC pathway, mammalian target of rapamycin (mTOR) and PKB. The MAPK pathway and mTOR signalling lead to increased protein synthesis, while increased cell survival, proliferation and differentiation are mediated by both the MAPK pathway and PKB. Increased glucose uptake through GLUT4

translocation to the cell membrane is mediated by CAP/cbl, PKC and PKB signalling. The metabolic effects of insulin are instead mediated by the PKB and PKC pathways (as reviewed by Leclercq *et al.* (2007)). A list of the metabolic effects of insulin in peripheral tissues is shown in Table 1.1.

Insulin effects		
Liver	**Skeletal muscle**	**Adipose tissue**
Insulin increases		
Glycogenesis	Glucose transport	Glucose transport
Fatty acid synthesis	Glycolysis	Glycolysis
Lipogenesis	Glycogenesis	Glycogenesis
Low-density lipoprotein synthesis	Fatty acid uptake	Fatty acid synthesis
Cholesterol synthesis	Amino acid transport	Lipoprotein lipase
Amino acid transport	Protein synthesis	Amino acid transport
Protein synthesis		Protein synthesis
Insulin decreases		
Glycogenolysis	Glycogenolysis	Lypolysis
Gluconeogenesis	Protein degradation	Protein degradation
Protein degradation		

Table 1.1: The main effects of insulin on peripheral tissues. Data based on information found in book by Watkins (2003).

At basal insulin levels, the insulin receptor signals to inhibit the actions of the opposing hormone glucagon, thus inhibiting lipolysis and gluconeogenesis and upregulating protein uptake. When glucose is present at high levels, the insulin receptor and IRS-2 activate the translocation of the GLUT4 glucose transporter to the cell membrane, thus upregulating glucose transport into the cell, as first hypothesized in 1980 (Cushman and Wardzala, 1980; Suzuki and Kono, 1980).

When considering the diabetic milieu, there are four important factors to consider: hyperglycaemia, which results from the defective glucose uptake, hyperinsulinaemia, as a result of fetal hyperglycaemia (in the fetus of a diabetic pregnancy) or due to insulin resistance (in the case of Type 2 diabetes), hypoglycaemia, as a result of hyperinsulinaemia in the Type 1 diabetic patient, and insulin resistance, the presenting feature in Type 2 diabetic patients.

As this thesis is mostly concerned with hyperglycaemia, this will be the focus in this section, although the mechanisms for and consequences of hyperinsulinaemia, hypoglycaemia and insulin resistance will also be discussed.

1.3.3.1 Hyperglycaemia: effects of high glucose on the vasculature

The diabetic condition is comprised of many characteristics, and one of the most widely known is hyperglycaemia, which can have both chronic and acute effects. This rise in blood glucose has several effects on the surrounding vasculature through various inter-connected mechanisms. Through these, hyperglycaemia acts as a pro-constrictor (Singh *et al.*, 2008; Fujimoto *et al.*, 2006; Boden *et al.*, 2007), pro-coagulatory (Kwaan, 1992; Boden *et al.*, 2007; Kario *et al.*, 1995), pro-inflammatory (Yang *et al.*, 2009; Sweet *et al.*, 2009), pro-angiogenic (Ettelaie *et al.*, 2008; Ejaz *et al.*, 2008; Liu *et al.*, 2007) and pro-permeability (Chiarelli *et al.*, 2000a; Sung *et al.*, 2006) agent. The pathways affected by hyperglycaemia are described here (Figure 1.13).

One of the classical mechanisms affected by high glucose is the PKC pathway. High glucose has been shown to cause *de novo* synthesis of diacylglycerol (DAG) in various tissues in the rat (Craven *et al.*, 1990; Derubertis and Craven, 1994; Shiba *et al.*, 1993; Inoguchi *et al.*, 1992), which in turn caused an increase in the production of PKC (Vasko *et al.*, 2009). This leads to an increase in the release of several growth factors, amongst which VEGF (Xia *et al.*, 2007; Kelly *et al.*, 2007). The PKC pathway has been the subject of various inhibition studies, and it has been shown that the diabetic changes to VEGF levels (Kelly *et al.*, 2007; Xia *et al.*, 2007) could be reversed by inhibiting this pathway.

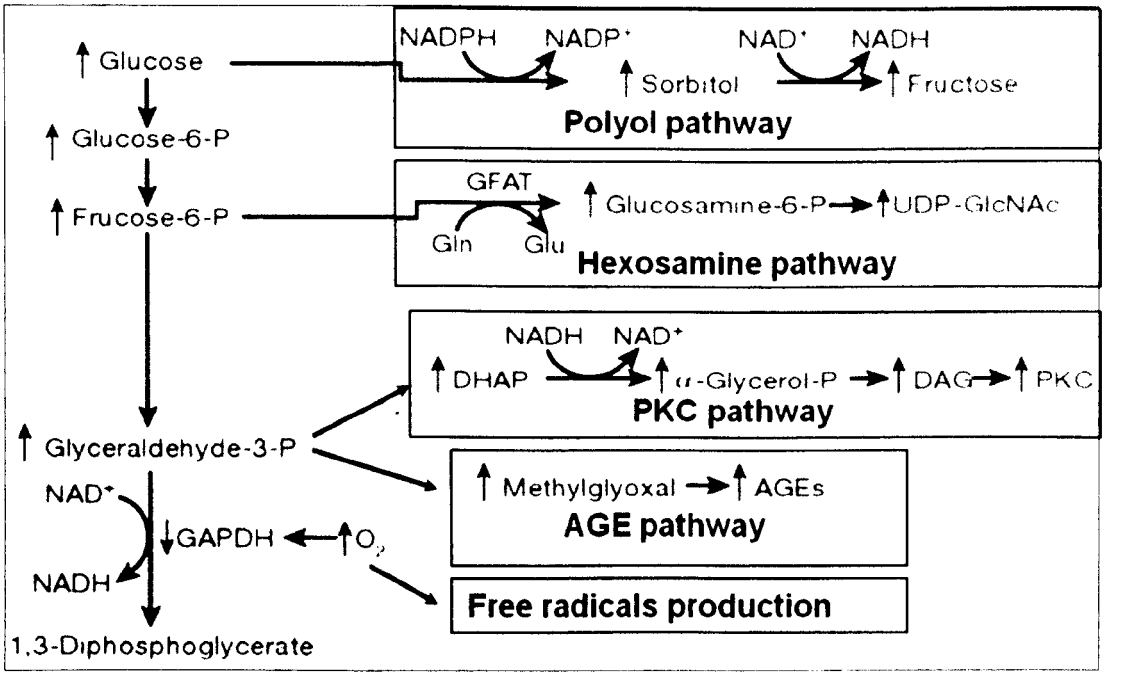


Figure 1.13: The five pathways altered as a consequence of high glucose. Abbreviations used: NADP(H): Nicotinamide adenine dinucleotide phosphate, NAD(H): Nicotinamide adenine dinucleotide, GFAT: glutamine:fructose-6-phosphate amidotransferase, Gln:glutamine, Glu: glutamate, UDP-GlcNAc: UDP-*N*-acetylglucosamine, DHAP: dihydroxyacetone phosphate, DAG: diacylglycerol, GAPDH: glyceraldehydes-3-phosphate dehydrogenase. Adapted by permission from Macmillan Publishers Ltd: [Nature] (Brownlee, 2001), copyright (2001).

Interestingly, the production of a key vasodilator, NO, is decreased in hyperglycaemic rats (Bohlen and Nase, 2001) and diabetic humans (Shiekh *et al.*, 2011). NO is normally produced by nitric oxide synthases (NOS), of which there are three members: eNOS and neuronal NOS (nNOS), which provide constitutive production of NO, and inducible NOS (iNOS), which when induced produces large quantities of NO. NOSs catalyse the oxidation of L-arginine to L-citrulline and NO. However, this reaction requires NADPH, and this molecule is decreased by the increased levels of NAD(P)H oxidase that are seen in hyperglycaemia and diabetes (see figure 1.14) (Guzik *et al.*, 2002) (Figure 1.14). Inhibitors of the PKC pathway can ameliorate this decrease in NO.

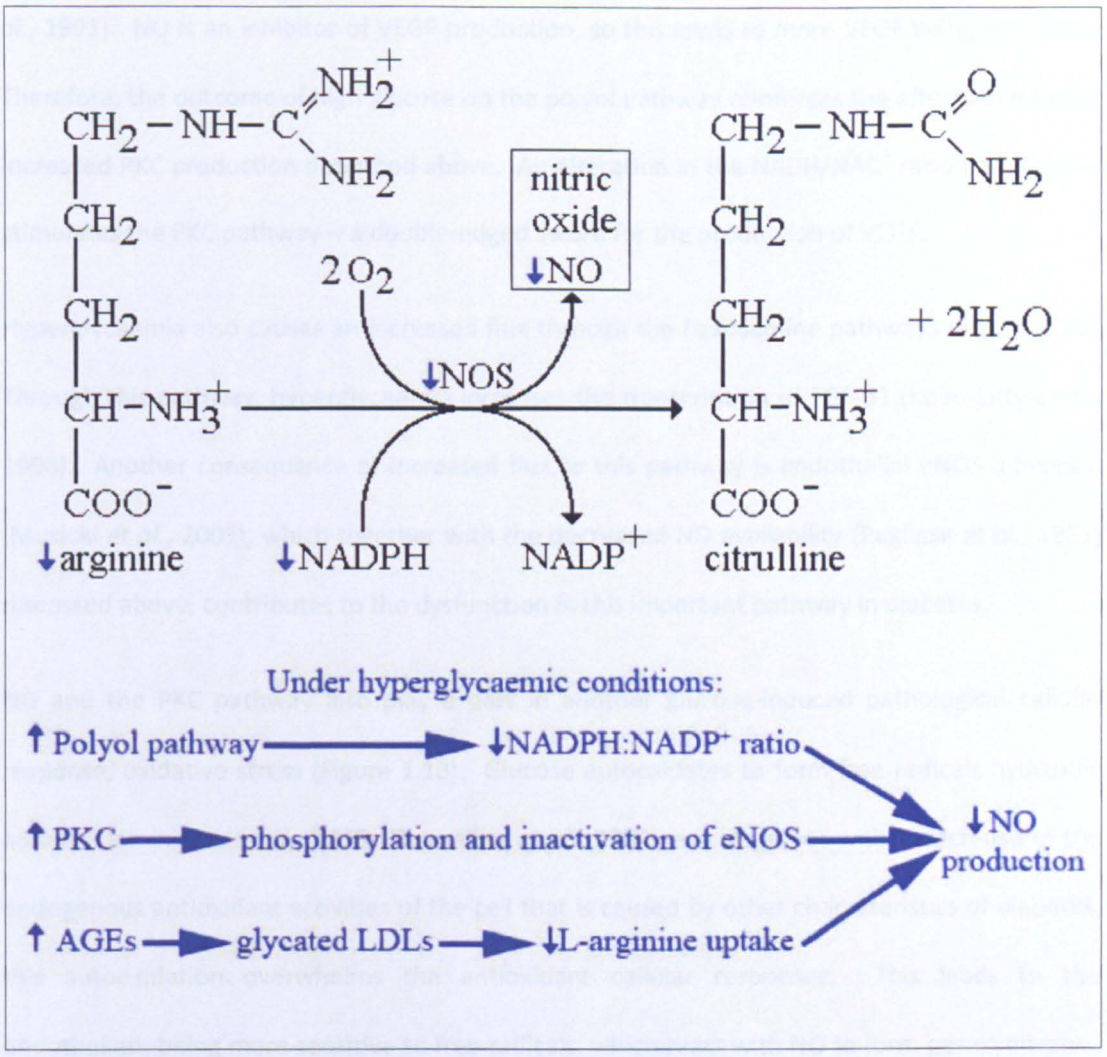


Figure 1.14: NO synthesis and effects of hyperglycaemia. NO (in rectangle) is produced along with citrulline from arginine, with the oxidation of co-enzyme NADPH to $NADP^+$ being a concurrent required step. Under hyperglycaemic conditions (in blue) the following are increased: the polyol pathway (leading to a decrease in the NADPH: $NADP^+$) (Bravi *et al.*, 1997), the PKC pathway (leading to phosphorylation and inactivation of eNOS) (Matsubara *et al.*, 2003), and advanced glycation end-products (leading to increased glycated low-density lipoproteins (LDLs), which results in decreased L-arginine cell uptake) (Posch *et al.*, 1999). All these changes lead to decreased NO production in response to hyperglycaemia.

The polyol pathway is also increased under conditions of hyperglycaemia (Figures 1.13 and 1.14). Too much glucose overwhelms the glycolytic pathway, and this causes an overproduction of fructose and sorbitol by aldose reductase and sorbitol dehydrogenase, respectively (Gabbay, 1973). The increase in these two sugars creates a pseudohypoxic environment, where there is an increase in the NADH/NAD^+ ratio (Ido *et al.*, 2004; Ido and Williamson, 1997). In this state, the endothelium increases its production of prostaglandins which act as vasoconstrictors, and decreases the production and availability of NO (Pugliese *et*

al., 1991). NO is an inhibitor of VEGF production, so this leads to more VEGF being produced. Therefore, the outcome of high glucose on the polyol pathway reinforces the effects due to the increased PKC production described above. An alteration in the NADH/NAD^+ ratio also directly stimulates the PKC pathway -- a double-edged sword for the production of VEGF.

Hyperglycaemia also causes an increased flux through the hexosamine pathways (Figure 1.13). Through this pathway, hyperglycaemia increases the transcription of TGF-β1 (Kolm-Litty *et al.*, 1998). Another consequence of increased flux to this pathway is endothelial eNOS inhibition (Musicki *et al.*, 2005), which together with the decreased NO availability (Pugliese *et al.*, 1991) discussed above, contributes to the dysfunction in this important pathway in diabetes.

NO and the PKC pathway also play a part in another glucose-induced pathological cellular response, oxidative stress (Figure 1.13). Glucose autooxidates to form free radicals hydroxilic anions (Dominguez *et al.*, 1998; Thornalley *et al.*, 1984), and together with a decrease in the endogenous antioxidant activities of the cell that is caused by other characteristics of diabetes, this autooxidation overwhelms the antioxidant cellular responses. This leads to the endothelium being more sensitive to free radicals, which react with NO to form peroxynitrates, one of the most damaging free radicals (Vareniuk *et al.*, 2008; Nishikawa *et al.*, 2000). The sensitivity to free radicals also directly switches on the PKC pathways (Xia *et al.*, 2007), and leads to sorbitol accumulation, as in the polyol pathway, and also leads to the production of nuclear factor κB (NFκB) (a pro-inflammatory transcription factor) (Nishikawa *et al.*, 2000).

All these changes can be seen under relatively short-duration hyperglycaemia (Lee *et al.*, 2009; Yang *et al.*, 2009). If however high glucose is sustained, the formation of advanced glycation end products (AGEs) occurs (Brownlee *et al.*, 1988a) (Figure 1.13). These are formed as glucose binds nonenzymatically and irreversibly to free amino acids and lipids (Brownlee *et al.*, 1988b). The functions of the proteins that contain AGEs can be altered, and as AGE formation is irreversible, they accumulate in cells. AGEs can form naturally as a person gets older, but it has been shown that this formation is greatly accelerated in diabetes mellitus, and that this is

directly proportional to glycaemic status (Stitt *et al.*, 1998). AGEs can increase the production of free radicals, therefore feeding into the oxidative stress mechanism detailed above. AGEs also can react with various intracellular proteins (Giardino *et al.*, 1994), which may alter their function, and the extracellular matrix (Brownlee *et al.*, 1986; Haitoglou *et al.*, 1992), which then leads to abnormalities in the structure of the affected vessels (Vlassara *et al.*, 2002; Brownlee, 1995a; Brownlee, 1995b; Zieman *et al.*, 2005; King and Brownlee, 1996). Also, AGEs can bind to their receptors (AGEs receptors), which then stimulate the production of NFκB and various cytokines (Schiekofer *et al.*, 2003; Yan *et al.*, 1994), leading to inflammation (Ettelaie *et al.*, 2008), and also inhibit the production of NO (Chakravarthy *et al.*, 1998; Bucala *et al.*, 1991).

These alterations working together have a powerful effect on the vasculature. The vasculature is prone to vasoconstriction and inhibition of vasodilation, as the endothelins, the major endothelial vasoconstrictors, are negatively regulated by NO. It has been shown that NO-dependent vasodilation, but not NO-independent vasodilation, is inhibited in hyperglycaemia (Williams *et al.*, 1998). NO is also part of the anti-coagulation cellular mechanisms, together with prostacyclin, thrombomodulin, and tissue plasminogen activator amongst others, and these mechanisms are decreased in diabetes, leading to a pro-coagulant state which increases the risk of stroke, amputation and myocardial infarction. In one study, a decrease in blood glucose to euglycaemia showed a decrease in tissue factor, which is a main initiator of coagulation (Boden and Rao, 2007). The risk of myocardial infarction has been shown to be increased after acute (30 minutes) hyperglycaemia in rats (Yang *et al.*, 2009). This fast acting effect of hyperglycaemia might be due to the fact that NO has a very short half-life, therefore high glucose can have rapid results that are seen in the immediate vicinity of the endothelium.

Atherosclerosis is a chronic inflammation of the arterial walls, and hyperglycaemia creates the conditions for this to occur by producing NFκB under oxidative stress, as described above. AGEs also seem to have a part in this inflammation, as studies in rats have shown that both AGE formation inhibitors and cross-link breakers reduce plaque area by 40% and 30% respectively

(Forbes *et al.*, 2004; Candido *et al.*, 2002). Atherosclerosis is the main cause of diabetic mortality and morbidity, and glucose seems to have a big role in it. Interestingly, it seems that long duration hyperglycaemia in rats predisposes the vasculature to increased inflammation, as bone marrow derived endothelial progenitor cells had a pro-inflammatory phenotype (Loomans *et al.*, 2009).

As for the pro-angiogenic and pro-permeability aspects of hyperglycaemia, VEGF and NO seem to be the main players. In a normal endothelial cell, if VEGF is produced at a basal rate it increases the production of NO by eNOS, and NO then feedbacks to inhibit further VEGF production (Ferrara, 2002; Kroll and Waltenberger, 1998; Ghiso *et al.*, 1999; Parenti *et al.*, 1998; Ferrara, 2004; Ziche *et al.*, 1997). However, in diabetes, this balance is lost. VEGF leads to a direct increase in permeability (Ferrara and Henzel, 1989), while inducible NOS produces large quantities of NO which also leads to increased permeability (Kroll and Waltenberger, 1999).

As some of the effects of glucose, especially on NO, which has a short life and is therefore short acting, are mechanisms to which cells respond rapidly, a short burst of hyperglycaemia might be enough to have a pathological effect on them. Several studies (Gerich, 2003; Fujimoto *et al.*, 2006) have been conducted on postprandial hyperglycaemia, and the effects of glucose are seen as quickly as 30 minutes after insult. It is therefore important to remember that it is not only the fasting glucose level that is important, but also the postprandial hyperglycaemic peaks.

The alteration of these mechanisms in hyperglycaemia is a main contributor to the diabetic phenotype, and in tissues that are insulin-independent for glucose uptake (such as the eye, kidney and nervous tissues), high glucose causes a range of pathologies. In the eye, hyperglycaemia can lead to retinopathy (Chakravarthy *et al.*, 1998; Ido *et al.*, 2004; Ido and Williamson, 1997), where excessive angiogenesis and permeability of newly-formed vessels causes visual problems. In the kidney, diabetic patients might experience nephropathy (Chiarelli *et al.*, 2000a; Derubertis and Craven, 1994; Kelly *et al.*, 2007; Xia *et al.*, 2007), where the kidneys become leaky to blood proteins and where there is an association with high blood

pressure due to the vasoconstriction described above (Balakumar *et al.*, 2009; Leehey *et al.*, 2000). In nerve endings, neuropathy might develop (Vareniuk *et al.*, 2008), where the blood supply is decreased due to the vasoconstriction caused by decreased NO (Cotter *et al.*, 2003), and sorbitol accumulation from the polyol pathway in the pericytes surrounding the cell cause osmotic stress (Sussman *et al.*, 1988). Therefore, hyperglycaemia is a key characteristic of diabetes, causing serious pathologies for the diabetic patient.

1.3.3.2 Hyperinsulinaemia

Hyperinsulinaemia is seen in two circumstances in diabetes: in Type 2 diabetes, where the aetiology of disease is insulin resistance, and thus greater and greater amounts of insulin are produced without effect (see section 1.3.3.4), and in GDM, where after 20 weeks gestation, the fetal pancreas responds to the maternal hyperglycaemia with fetal hyperinsulinaemia in order to try to restore an euglycaemic environment.

1.3.3.3 Hypoglycaemia

Hypoglycaemia is the main obstacle to tight glycaemic control (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998), as when it arises, it is a medical emergency. Hypoglycaemia is seen in Type 1 diabetics when incorrect insulin treatment delivers more insulin than the physiological needs of the body, and results in a blood glucose concentration which is too low to sustain the body, especially the brain, which needs a constant supply of glucose to function. A low level (<2.2mM) of glucose can lead to diabetic unawareness and diabetic coma, and can be reversed by taking a small quantity of carbohydrate or sugar.

The body's response to hypoglycaemia involves the release of glucagon and adrenaline, normally inhibited by insulin, to signal the liver to produce glucose (Schwartz *et al.*, 1987; Rizza *et al.*, 1979). Also, there is activation of the central sympathetic nervous system, which is manifested in a variety of symptoms including a pounding heart, hunger, sweating, anxiety and tremor (McAulay *et al.*, 2001). These symptoms are helpful in alerting the patient of their low glycaemic status. Whilst in poorly controlled diabetes featuring hyperglycaemia the threshold for these body's responses to hypoglycaemia is higher than normal, in patients with tight glycaemic control or frequent exposures to hypoglycaemia this threshold drops, leading to impaired awareness of the impending hypoglycaemia (White *et al.*, 1983).

In the occurrence of hypoglycaemia, blood is diverted away from 'non-essential' systems (such as the eyes and kidneys), and towards the brain and the liver (for production of glucose). It is thought that haemodynamic and haematological changes seen in response to hypoglycaemic episodes may be an aggravating factor in diabetic complications, by causing acute stress to the fragile diabetic microcirculation. Thus, hypoglycaemic episodes can result in capillary closure and micro-haemorrhages in the retina, as well as the rupturing of newly-formed vessels formed in response to retinal hypoxia (Frier and Hilsted, 1985; Frier *et al.*, 1987). All these factors contribute to advancing diabetic retinopathy.

1.3.3.4 Insulin resistance

In type 2 diabetes, relative insulin deficiency is caused by insulin resistance. Here, the insulin receptors are down-regulated (Tiwari *et al.*, 2007), usually through receptor degradation and its kinase activity decreasing, possibly due to serine/threonine phosphorylation. Thus, even though the β-cells of the pancreas of Type 2 patients are producing insulin, they are unable to compensate for this insulin resistance, and diabetes develops.

1.3.4 Microvascular complications – diabetic angiopathy

Tissues which are independent of insulin for glucose uptake show glucose toxicity under hyperglycaemia. Such tissues include endothelial cells and the pericytes that surround them. Over a prolonged time, pericytes are lost from around blood vessels, resulting in a loss of vessel integrity (the consequences of which (for the eye) are reviewed by Ejaz *et al.* (2008)), and vasoconstriction due to decreased NO production is seen. Another consequence of prolonged hyperglycaemia is an abnormal vessel wall, which displays an increased thickness due to overproduction of fibronectin and collagen type IV (Bollineni and Reddi, 1993; Koya *et al.*, 1997), and increased permeability and impaired architecture due to the effects of AGE receptors (RAGEs) signalling and oxidative stress (Brownlee *et al.*, 1984; Haitoglou *et al.*, 1992).

Here following are the main effects of this altered vasculature in the diabetic patient, and their pathophysiology.

1.3.4.1 The vasculature in diabetic retinopathy

Retinopathy is a leading cause of blindness in the UK, and most Type 1 diabetic patients will develop it within 20 years of diabetes onset (Fong *et al.*, 2003). Diabetic retinopathy affects mostly the vasculature of the retina in the eye, and hyperglycaemia seems to be the main causative player, as the severity and duration of high glucose are directly associated with the pathologic changes that cause retinopathy (Yanko *et al.*, 1983; Klein *et al.*, 1994). Retinopathy is characterised by an initial increase in permeability and blood flow to the retina together with altered composition and thickening of the basement membrane and loss of pericytes from the vessel walls. As well as these changes, microaneurysms are formed by retinal endothelial cell proliferation in vessels where pericytes have been lost. This environment results in retinal hypoxia, activating the expression of angiogenic factors such as VEGF that initiate neovascularisation. The main pathways that are disrupted in retinopathy in response to

hyperglycaemia are the PKC pathway and the formation of free radicals and AGEs, as reviewed by Cai and Boulton (2002).

1.3.4.2 The vasculature in diabetic nephropathy

Diabetic nephropathy is the most common cause of end stage renal failure. As with retinopathy, the changes seen in this disease seem to be directly related to glycaemic status (Mogensen, 1971).

Diabetic nephropathy is characterised by glomerular basement membrane thickening and mesangial expansion, as well as hyalinosis of both afferent and efferent arterioles, although it is the thickening and accumulation of extracellular matrix that is the central abnormality in the disease (Tervaert *et al.*, 2010). There is also evidence of podocyte damage and detachment (Petermann *et al.*, 2004). These changes result in a decrease in barrier function of the kidneys, leading to proteinuria and eventually may lead to end stage renal failure.

The main pathways that lead to these characteristics of diabetic nephropathy are accumulation of AGEs, increased oxidative stress and PKC activation, and increased production and expression of vasoactive growth factors in the glomerulus, as reviewed by He and King (2004). Indeed, suppression of these mechanisms has been shown to be protective against the development of diabetic nephropathy (Nakagawa *et al.*, 2006; Schrijvers *et al.*, 2006).

1.3.5 Diabetes management

In Type 1 diabetes, management of glycaemic levels with insulin or insulin analogues is currently the only method available to stabilise hyperglycaemic excursions. It has been shown in the DCCT trial (1993) that microvascular complications are reduced with strict control, although the incidence of hypoglycaemia then increases. In the last ten years, great advances

have been made in the field, with long- and fast-acting insulin analogues (Rys *et al.*, 2011), insulin pumps, and continuous glucose monitoring systems (Hoeks *et al.*, 2011) all helping to reduce incidences of hypoglycaemia whilst maintaining tight control.

Long- and rapid-acting insulin analogues are used to replicate the non-diabetic basal and post-prandial production of insulin. Long-acting insulin analogues are injected at regular intervals (usually once or twice daily) into the subcutaneous tissue. This insulin is then slowly absorbed into the bloodstream throughout the next 12-24h, therefore providing an insulin profile similar to basal insulin production in non-diabetics. Rapid-acting insulin analogues are instead used immediately prior to a meal, simulating the post-prandial rise in insulin release seen in non-diabetics.

Insulin pumps use fast-acting insulin, but they deliver it continuously to the body in smaller units, replicating the basal insulin profile. They then deliver a higher dose bolus immediately before a meal, replicating the post-prandial insulin peak. The advent of smaller and tubing-free 'patch' insulin pumps, the programming of different insulin delivering profiles matching differing schedules, and alarms sounding when hypo- or hyperglycaemia are detected, are all recent useful advances that aid convenience and safety in diabetes management (Selam, 2010).

Real-time continuous glucose monitoring systems are relatively new, but they could potentially provide an important and effective tool for improving glycaemic control, as the patient can see and immediately correct any hyperglycaemic excursion. However, studies still need to be performed to show the benefits of continuous glucose monitoring in certain patient groups (such as in pregnancy and with a history of severe hypoglycaemia) (Hoeks *et al.*, 2011). Together with insulin pumps, continuous glucose monitoring is leading to the development of closed-loop insulin delivering, or artificial pancreas, which will ultimately lead to optimal tight glucose control with reduced hypoglycaemia risk (Elleri *et al.*, 2011).

Recently, metformin (an oral drug which reduces hepatic gluconeogenesis and lipogenesis) treatment alongside insulin has increased, as studies have shown a potential benefit to Type 1 patients who are receiving large amounts of insulin, are overweight, or have an HbA_{1c} of over 64mmol/mol (Meyer *et al.*, 2002; Meyer and Guerci, 2003).

In Type 2 diabetes, often the first route of management is through an altered diet and exercise regime which aids weight loss in overweight Type 2 diabetic patients, helps regulate glycaemic levels, and decreases risk factors associated with cardiovascular disease (Sibal and Home, 2009), combined with metformin. However, if HbA_{1c} levels are above 56mmol/mol or microvascular symptoms are being exhibited, other oral drugs are then prescribed (such as insulin, sulphonylureas and thiazolidinediones) (Nathan *et al.*, 2006).

Tight glycaemic control in diabetes is important for managing macro and microvascular complications. There is a significant association between higher HbA_{1c} levels and increased macrovascular complications such as coronary artery disease (Selvin *et al.*, 2005a), peripheral arterial disease (Selvin *et al.*, 2006) and stroke (Selvin *et al.*, 2005b), and a 11mmol/mol decrease in HbA_{1c} also resulted in a 37% reduction in microvascular complications (Stratton *et al.*, 2000). However, glycaemic control is not the only avenue for macrovascular complications management, as hypertension and dyslipidaemia have also been shown to be associated with development of macro and microvascular disease in diabetic patients: the risk of myocardial infarctions increases 12% for every 10mmHg increase in systolic blood pressure (Adler *et al.*, 2000), and use of statins to decrease cholesterol significantly decrease the risk of cardiovascular disease (Ginsberg *et al.*, 2010). Thus, tight glycaemic, blood pressure, and lipid control are all important tools for management of diabetes.

1.4 Diabetic pregnancy and the placenta

1.4.1 Diabetes in pregnancy

Diabetes is a major complication of pregnancy, and constitutes a major morbidity risk factor for the fetus, with mortality rates for neonates four times those of normal pregnancies (Macintosh *et al.*, 2006). The mortality in this study was shown to result from an increased risk of congenital cardiac abnormalities and neural tube anomalies. Other complications in diabetic pregnancies include other congenital abnormalities, spontaneous abortion, stillbirth, neonatal hypoglycaemia, intra-uterine growth restriction (IUGR), respiratory distress, and, importantly for the purposes of this thesis, macrosomia (Sosenko *et al.*, 1979; Gale and Amiel, 2002). Further, recently a link was found between diabetes in pregnancy and metabolic syndrome in the offspring later in life (Dabelea, 2007).

Intensive management prior to conception and during pregnancy can reduce the risk of spontaneous abortion and congenital malformations (The Diabetes Control Complications Trial Research Group, 1996); however, even with tight control, some incidence of macrosomia remains (Evers *et al.*, 2002).

All types of diabetes (whether Type 1, Type 2 or gestational) require further interventions during pregnancy, as during gestation glycaemic homeostasis changes, and patients require more insulin (see section 4.1.3.) to achieve euglycaemia.

The diabetic pregnancy can be divided into two parts as far as complications are concerned: before 20 weeks and afterwards. The first 20 weeks post-conception are characterised by hyperglycaemia in the fetus, as insulin is not yet being produced by the fetal pancreas and maternal insulin only crosses the placenta in small amounts (Challier *et al.*, 1986), if at all (Keller and Krohmer, 1968). Therefore, the fetus is exposed to hyperglycaemia due to the transplacental glucose flux down the glucose concentration gradient. It is this early fetal hyperglycaemia that is thought to be responsible for the increase in congenital abnormalities

and spontaneous abortions seen in diabetic pregnancies (Hanson *et al.*, 1990), as glucose is thought to act as a teratogen here (Eriksson, 2009). Excessive glucose also acts as a growth inhibitor, and thus during the first trimester the pregnancy might be affected by IUGR.

In contrast, insulin is a growth promoting hormone. When the fetal pancreas begins production of insulin at 20 weeks gestation, hyperinsulinaemia might occur to try to achieve an euglycaemic environment. This theory is called the Pedersen hypothesis after the person behind it (Pedersen, 1954). The β-cells in the pancreas become hypertrophic to meet the demand of the hyperglycaemic environment (Jaffe, 1991), and the high insulin concentration causes the excessive deposit of fat, cellular glucose utilisation, and protein production in the fetus, which then leads to organomegaly (Langer, 2000), resulting in a macrosomic fetus. Macrosomia then often complicates the delivery of the infant by resulting in shoulder dystocia, operative delivery and brachial plexus injury (American College of Obstetricians and Gynecologists Committee, 1992). Macrosomia is present in 48.8-62.5% of diabetic pregnancies (Evers *et al.*, 2002).

Figure 1.15, below, is a flow chart illustrating the effects of maternal hyperglycaemia in pregnancy.

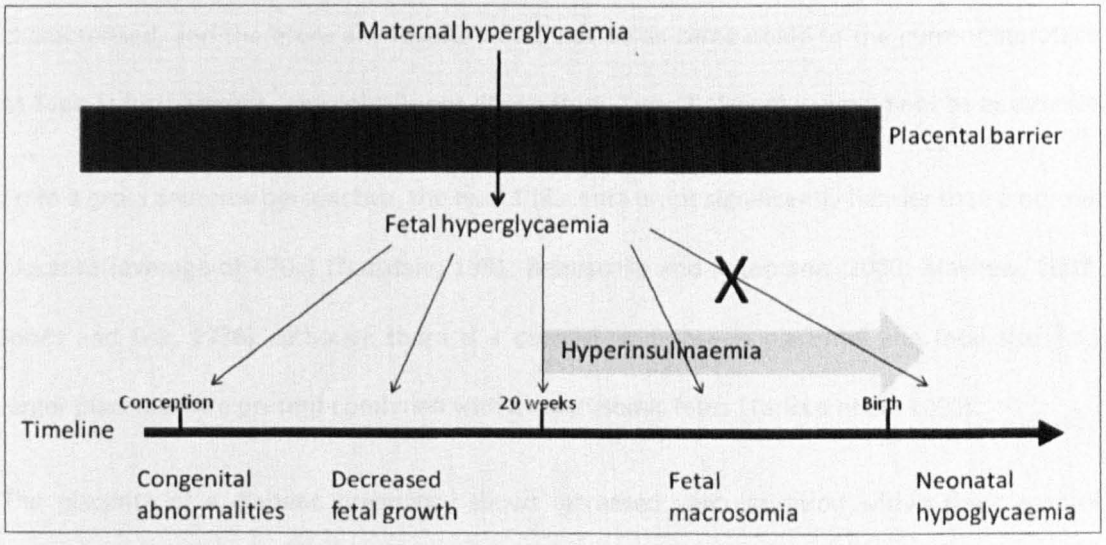


Figure 1.15: Temporal effects of maternal Type 1 diabetes on the pregnancy.

The fetus of the diabetic pregnancy might also be exposed to hypoxic conditions due to the maternal hyperglycaemia and fetal hyperinsulinaemia. Evidence for this comes from high erythropoietin (EPO) levels at birth, which correlate closely with both maternal HbA_{1c} levels in the month prior to delivery and fetal plasma insulin levels (Widness *et al.*, 1990). EPO is involved in the regulation of production of red blood cells, and normally it is produced only in the fetal kidneys (Zanjani *et al.*, 1981), but under hypoxic conditions it is produced in other tissues (Dame *et al.*, 2000; Davis *et al.*, 2003a). It is thought that this more ubiquitous (and hence higher) synthesis of EPO is an adaptation of the fetus to lower oxygen-carrying capacity of fetal blood.

We have seen here the effects a diabetic pregnancy may have on the fetus. In the next section, we shall detail the phenotype of the placenta of diabetic pregnancies.

1.4.2 The diabetic placenta

Type 1 placentae are the most studied placentae from diabetic pregnancies. This in itself makes it an ideal subject of study, as results can be compared to a well defined "diabetic phenotype". In contrast, placentae from Type 2 diabetic women have until recently not been thoroughly characterised, and therefore any results would not be as comparable to the current literature as Type 1; furthermore, any conclusions drawn from Type 2 placentae would not be as definite.

From a gross anatomy perspective, the type 1 placenta is not significantly heavier than a normal placenta (average of 470g) (Teasdale, 1981; Benirschke and Kaufmann, 2000; Mayhew, 2002; Jones and Fox, 1976), although there is a correlation between placental and fetal size, i.e., larger placentae are present combined with a macrosomic fetus (Taricco *et al.*, 2003).

The placenta of a diabetic pregnancy shows increased vascularisation within the placental tissue (72% increase in combined lengths of placental vessels (Leach *et al.*, 2004)). In addition, it has been found that more than 50% of Type 1 placental chorionic villous vessels are

significantly leakier to large macromolecules (76KDa) than normal placental chorionic villous vessels and show increased levels of endogenous VEGF (Leach *et al.*, 2004). The latter was linked to an increased proliferation index in the diabetic placenta.

The increase in vascularisation has been shown to be due to branching angiogenesis in GDM. This increase in this type of angiogenesis results in the vascular bed being less efficient, as it is structurally more complicated, with increased redundant connections between vessels in diabetic placentae compared to normal placentae (Jirkovska *et al.*, 2002). It is thought that this decreased efficiency of the placental vascular network might play a role in the fetal hypoxia seen in diabetic pregnancies.

In diabetic placentae, there is an increase in DNA content, which indicates more cellular material in the parenchymal tissue (Winick and Noble, 1967), and higher numbers of stromal fibroblasts and villous macrophages (Younes *et al.*, 1996). The trophoblast basement membrane has also been shown to be thicker in diabetic pregnancies compared to normal pregnancies, perhaps because of increased amounts of collagen type IV (Younes *et al.*, 1996).

The phenotype of the diabetic placenta depends partly on glycaemic control, which plays a large role in the abnormalities seen in the diabetic placenta. In tightly controlled pregnancies, for example, there are no significant differences in the number of abnormal villi recorded compared to non-diabetic pregnancies, whilst placentae from poorly controlled pregnant women contained a higher percentage of abnormal villi (Bjork and Persson, 1982).

Thus the human placenta in a diabetic environment undergoes structural and functional changes that may affect the fetus.

1.5 Hypothesis and aims

Hyperglycaemia is a main complication of diabetes, and in animal studies it has been shown to cause increased permeability of vascular beds. The placenta is a very sensitive organ to angiogenic and permeability stimuli, so that an increase in leakage of the endothelial lining of the placenta might adversely affect the fetus. It is known that one of the main complications of a pregnancy involving a diabetic mother is macrosomia of the baby.

Our working hypothesis was that hyperglycaemia causes an increased permeability in the vascular beds of the human placenta. To answer this research question, we developed aims:

- To establish an explant culture methodology as a suitable model to enable us to evaluate the effects of glucose on VEGF and VE-cadherin, two key molecules for assessing the permeability of the vascular beds.
- To analyse the effects of high glucose (15mM) on VEGF splice variants and VE-cadherin expression in the endothelium of normal and diabetic placental vessels by using the validated explant culture.
- To assess the effects of high glucose (15mM) on vascular leakage to a tetramethyl rhodamine isothiocyanate (TRITC) tracer in a dual perfusion model.

Chapter 2
Validation of chorionic villous explants

2.1 Introduction

2.1.1 General principles of chorionic villous explants culture

Chorionic villous explants provide a balance between whole-organ and cell culture experiments, providing the 3D architecture and *in* vivo environment of the former combined with the ease of use and flexibility of the latter. Use of chorionic villous explants provide an ideal methodology to study the placental architecture with short and long-term cultures, as explants remain viable for up to 12 days. There are a variety of explants culture methodologies used in the literature, each suited to each experimental set-up and experimental question. An explant culture which is suitable for trophoblast proliferation studies, for example, might not be suitable for VEGF studies in the endothelium. However, there are some principles that all explants cultures should aspire to fulfil. In a review of explant culture methodologies (Miller *et al.*, 2005), the authors elegantly summarised the four main requirements for judging whether an explants culture methodology is appropriate for a certain experimental question. These are: applicability, where the explants culture mimics an *in vivo* situation; validity, where the culture behaves like a 'living' system; consistency, where the explants culture gives consistent results; and generalisation, where we can compare results with other experimental systems and clinical situations. Miller *et al.* (2005) state in their review that the complete fulfilment of each criterion might not be possible, but that the culture method should be chosen to maximise the compliance to these criteria in light of the research question being asked.

Chorionic villous explants are most often taken from first and third trimester pregnancies. First trimester explants are used for the study of differentiation and invasion of extravillous trophoblast, whilst term placenta can be used for mature placental studies. Both allow the study of villous trophoblast proliferation and turnover. Third trimester explants can be incubated on a mesh support just below the gas-liquid boundary (Siman *et al.*, 2001), on the bottom of the well (Black *et al.*, 2004), or suspended from polystyrene blocks into the medium (Huppertz, unpublished). To allow first trimester trophoblast migration and differentiation, a

matrix (matrigel or collagen I) is added to the bottom of the well (Genbacev *et al.*, 1992), as trophoblast outgrowth grown on plastic are often contaminated with fibroblasts.

2.1.2 Trophoblast and hypoxia in explant cultures

The main focus of chorionic villous explant cultures until now has been trophoblast study. It is possible to isolate cytotrophoblast cells from the placenta; however, several investigators have shown that once the cytotrophoblast cells are put in cell culture, they quickly differentiate and fuse into a giant cell, which degenerates after 5 days (Morrish *et al.*, 1997; Handwerger and Aronow, 2003; Kliman *et al.*, 1986), therefore not allowing long-term study of trophoblast migration and differentiation. The duration of these cultures can be extended by addition of epidermal growth factor to the media (Johnstone *et al.*, 2005), but even this is not ideal, as it is exposing the cytotrophoblast to an external stimulus. Therefore, chorionic villous explants provide the ideal methodology for the study of trophoblast function.

One of the most studied variables when studying the trophoblast in placental explants is oxygen concentration. In the first trimester of gestation (up to ~11 weeks), the maternal blood does not yet bathe the blood lakes of the placenta, and therefore there is a hypoxic environment (estimated to be around 2-3% oxygen) (Foidart *et al.*, 1992; Jauniaux *et al.*, 2003). In both early and late gestation, hypoxia is thought to contribute to a variety of pathologies, including pre-eclampsia (Soleymanlou *et al.*, 2005). Therefore, hypoxia is a highly important factor in both physiologic and pathologic placental development, leading several groups to try to simulate it in *in vitro* placental explants. In some of these hypoxic studies, hyperoxic conditions (20% oxygen) were also compared to normoxic (8% oxygen) and hypoxic (1% oxygen) conditions (Royle *et al.*, 2009). As 20% oxygen is the level commonly seen in culture incubators, this gives an indication as to whether certain studies can be performed at this level, or if the molecule(s) or system studied is adversely affected by this hyperoxic environment. In the next section, we will discuss

how these previous placental explant cultures helped shape our two versions of chorionic villous explants.

2.1.3 Chorionic explant models in this study

2.1.3.1 Oxygen

The explant methodology needs to be carefully developed depending on what the experimental aims are. Explant cultures have been extensively used for studies focusing on the trophoblast layer. However, it has been shown that this layer undergoes significant changes in the initial period of culture, and then degenerates after a few days in culture (Siman *et al.*, 2001; Di Santo *et al.*, 2003). Therefore, explant cultures used for these experiments have been specially developed to preserve the trophoblast and allow its proliferation. We, however, wished to study the endothelium of the explants, a use of this methodology which has not been published before, and therefore a different explant culture might be more suited for our experiments.

Our aim was to study VEGF and VE-cadherin expression in the endothelium. Therefore, we needed an explant culture which minimised VEGF production. The normal oxygen concentration in the intervillous space *in utero* is around 8% (45-50mmHg) (Fujikura and Yoshida, 1996). Studies on hypoxia in placental explants have repeatedly shown that VEGF expression is lowest in conditions of hyperoxia (20% oxygen), whether as excreted VEGF in the media of first trimester (Munaut *et al.*, 2008) and third trimester explants (Padavala *et al.*, 2006), or in the placental tissue of third trimester placental explants itself at 0h (before culture) (Sokolov *et al.*, 2008). Of course, the study by Munaut *et al.* (2008), which shows very low levels of VEGF both at 0h (before culture) and at 48h, uses first trimester placentae, which have very different angiogenic and permeability characteristics than full term placenta and a very different oxygen environment (2-4% oxygen). The study by Sokolov *et al.* (2008) shows an expression area for VEGF levels in syncytiotrophoblast and vascular endothelial cells to be 3.11%

in the tissue. These decreases in VEGF would be advantageous for our studies, as it would lower the baseline VEGF and therefore make any increase due to glucose more noticeable. Therefore, our explants were incubated in 20% oxygen.

2.1.3.2 Excision artefacts

A possible complication is that VEGF might be expressed on excision of the explants from the placenta, as this process technically creates a wound, and VEGF is expressed in wound healing. However, a study of hypoxia on the expression of inflammation and wound healing molecule TNFα and anti-angiogenic soluble VEGF receptor sVEGFR-1 found that 20% oxygen minimised expression of these wound healing molecules (Royle *et al.*, 2009), and so in theory the wound healing effect might not be relevant.

2.1.3.3 Flow and shear stress

One of the methodologies tested, hereafter called the 'free-floating methodology', involves the explants being submerged in media in a universal tube, and agitated on a roller. The explants in this methodology are free-floating in the media (hence the name), and this simulates the condition *in vivo* where the chorionic villi are floating in the maternal blood lakes, with blood flowing over the explants as it enters the intervillous space from the spiral arteries and is drained from this space by maternal veins.

In vivo, the endothelium adapts remarkably well to changes in flow and shear stress. In response to a change in flow, endothelial cells produce powerful vasoactive molecules (such as prostacyclin and nitric oxide), resulting in vascular relaxation due to inhibition of smooth muscle cell contraction (as when the endothelium is in steady state). If the flow then returns to normal, this process is reversible, due to the short half life of these signalling molecules and the nearby

presence of other signalling molecules that allow the switching off of these second messengers. If, however, the altered flow and shear stress persist, transcription factors that regulate flow-responsive gene transcription are activated, which leads to changes in the phenotype of the cell and more permanent adaptation to the altered environment (Davies, 1997). We hypothesise that these mechanisms will allow our free-floating explants to adapt to the flow in the agitated tube, allowing the replication of *in vivo* flow in our experimental set-up.

In the case that the adaptation of the endothelium to the shear stress created by agitation were not appropriate, we also chose to validate another technique, the stationary methodology, where the explants are floating in a well of a twelve-well culture plate, with a metallic mesh (with 700μm pores) underneath to avoid the trophoblast cells migrating to the well's bottom wall.

2.2 Aims and objectives

The aim of this chapter was to validate a placental chorionic villous explants methodology to study its vasculature, in respect to two important molecules for blood vessels, VE-cadherin and VEGF. In order to achieve this aim, we performed two protocols, one with (free-floating methodology) and one without (stationary methodology) simulation of blood flow.

2.3 Methods

2.3.1 Materials

The source and catalogue number for all materials mentioned in this chapter are listed in Appendix 1.

2.3.2 Placenta selection

Consent for use of the placentae in our study was sought if the donors met the following criteria: healthy pregnancy with no complications, non-smoker, 18-35 years of age, BMI 20-25, no medications, gestational age ≥37 weeks. Women gave informed consent as they came in for their scheduled term elective Caesarean sections. Any pregnancy (for example, diabetes, pre-eclampsia, pregnancy hypertension) and/or placental complication (for example, placenta praevia, placental abruption, twins) was excluded from the study. Each placenta was transported to the laboratory for excision of explants as soon it was delivered.

2.3.3 Explant excision

The placentae were cooled on ice during the excision of explants. Explants (11mg) (n=5 per experiment, 6 explants per placenta) were excised from different cotyledons, taking care not to include maternal and fetal tissue and cutting from the central part of the cotyledon. Villous tissue taken was free of calcification and infarcts. Explants were washed in ice-cooled PBS to remove blood. The explants were processed within 30 minutes of delivery. Half the explants were used for the free-floating methodology, and half for the stationary methodology.

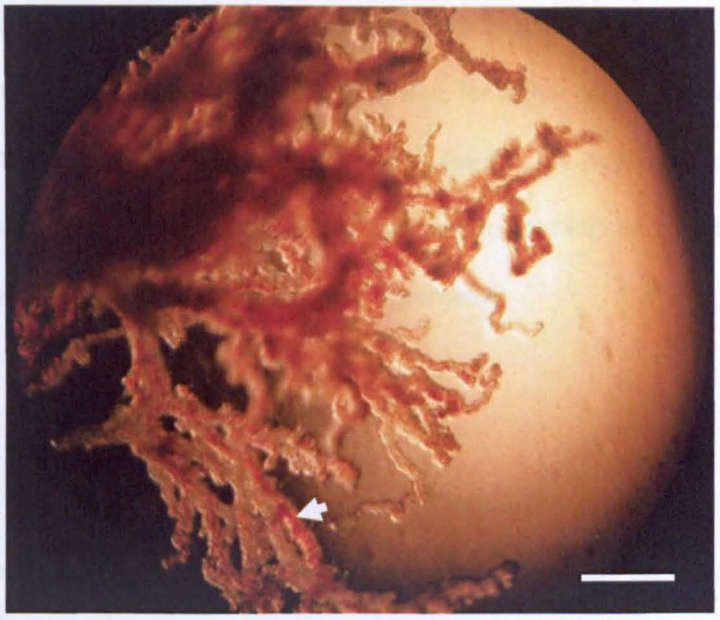


Figure 2.1: Micrograph of a chorionic villous explant floating in PBS. Visible are the branches of chorionic villous trees, and in one villous, some remaining blood is visible (arrow). Magnification: ×40, scale bar: 500μm.

2.3.4 Free-floating methodology

Explants (n=5, 3 explants per placenta) were incubated in 5ml M199 medium (which had been briefly oxygenated) containing 5% (v/v) Fetal Calf Serum, 100U/ml penicillin, 100mg/ml streptomycin, 2μg/ml Fungizone with continual agitation (45 rpm) to simulate blood flow, for either 0h, 1h, 4h or 24h. After this period of incubation, the explants were removed and fixed in 1% (w/v) paraformaldehyde (PFA, pH7.2) for 30 minutes to allow cross-linking to occur, before being quickly washed in cold PBS and inserted in a drop of optimum cutting temperature compound (OCT) and snap frozen with liquid nitrogen cooled isopentane to minimise freeze damage.

2.3.5 Stationary methodology

Explants (n=5, 3 explants per placenta) were incubated in 2ml M199 medium (which had been briefly oxygenated) containing 5% (v/v) Fetal Calf Serum, 100U/ml penicillin, 100mg/ml

streptomycin, 2μg/ml Fungizome with a net support (pore size 700μm) in a twelve-well plate, to avoid the explants to adhere to the bottom of the well, for either 0h, 1h, 4h or 24h. After this period of incubation, the explants were removed and fixed in 1% (w/v) PFA for 30 minutes to allow cross-linking to occur, before being quickly washed in cold PBS and inserted in a drop of OCT and snap frozen with liquid nitrogen cooled isopentane.

2.3.6 Fixation before excision control

In order to evaluate whether the phenotype seen in the t=0h control was due to the excision and washing process, control cotyledons from placenta (n=1) were subjected to a fixation before excision protocol, and then the phenotype of the resulting tissue analysed compared to the 0h control.

Whole placentae cotyledons were excised from the organ alongside the decidual septa, so as not to disrupt the structure of the lobule. The cotyledons were quickly washed in cold PBS to remove most blood, and then fixed with 1% (w/v) parafolmaldehyde at 4°C for 4h. The cotyledons were then excised into explants as described in section 2.3.3, and the explants were then washed in PBS to remove further blood, and then fixed in 1% PFA for a further 30 minutes, washed in PBS, and then inserted in a drop of OCT and snap frozen with liquid nitrogen cooled isopentane.

2.3.7 Immunofluorescence protocol

Frozen sections were cut to 7μm-thick slices with a cryostat (CM1510, Leica) and positioned on microscope slides, which were removed from the cryosectioning machine and allowed to airdry. An ImmEdge pen (hydrophobic pen) was then used to draw around each section, to keep the liquids on the section. A 20μl drop of 0.1% (w/v) PFA in PBS was added to each section for 5

minutes, to fix the section, and then removed. A 20µl drop of 0.1% (v/v) Triton, 0.1% (w/v) BSA in PBS was then added and left for ten minutes at room temperature in a covered humidity tray, to permeabilise cell membranes so the antibodies would be able to enter cells, and then removed. A 20µl drop of blocking solution (1:20 dilution of normal human serum in PBS) was then added for 30 minutes at room temperature in a covered humidity tray. After removal, 20µl of the primary antibody (mouse anti-human VE-cadherin (555661, BD Biosciences): 5µg/ml, mouse anti-human VEGF (MAB293, R&D Systems): 5µg/ml, negative control: 0.1% (w/v) BSA in PBS) was added, and the slides placed in a covered humidity tray for an overnight incubation at 4°C. After the overnight incubation, two quick washes with PBS with standard pipette were performed, followed by three 15 minute washes with 0.1% (w/v) BSA in PBS. A 20µl drop of goat anti-mouse IgG fluorescein isothiocyanate (FITC) secondary antibody (F-0257, Sigma-Aldrich, 20µg/ml) was added and kept at room temperature for 2 hours in darkness. The same washes as after the primary antibody were then repeated in aluminium foil covered Coplin jars. One ten minute wash with PBS only was then performed, followed by one 2-5 minute wash with distilled water. The slides were then carefully dried, mounted with vectorstain and sealed with nail varnish. The slides were then allowed to settle overnight before analysis.

2.3.7.1 Vessel identification verification

In order to confirm that the counting of vessels was performed in an accurate fashion, some separate slides were incubated with endothelial marker PECAM-1 (5µg/ml, SC-1505-R, Santa Cruz) primary antibody and goat anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC) secondary antibody (T-6778, Sigma-Aldrich). This allows the endothelium to be shown in the TRITC but not FITC channel. Micrographs of both channels were taken, but only the micrographs in the FITC channel were initially analysed. After vessels were identified in the FITC channel, the accuracy of the identification was checked in the TRITC micrographs.

2.3.8 Freeze damage and morphology evaluation

One slide (containing one section) of each explant was exposed to 0.1% (w/v) toluidine blue in water for 30 seconds before being thoroughly washed with water. The slide was then immediately analysed under the microscope (Nikon LaboPhot-2 fluorescence microscope, Nikon Instruments) to check the gross morphology of the explants. Toluidine blue stains nucleic acids and all proteins a blue-purple colour, and allows detection of freeze damage to the tissue.

Haematoxylin and Eosin (H&E) staining was also performed to allow structural detail to be evaluated. Slides were rehydrated in tap water, submerged for 5 minutes in Harris haematoxylin, and washed in a bath of running tap water until the water cleared. They were then dipped in acid alcohol for 10 seconds, washed until there were no streaks on the slides, and dipped in Lithium Carbonate/Scott's Tap Water. Slides were then submerged in 1% Eosin for 5 minutes, and washed again in tap water. A series of dehydration steps were then performed, by dipping the slides in 50%, 70%, 90% and 100% alcohol for 10 seconds each. Finally, slides were dipped in fresh and finishing xylene for 2 minutes each, and mounted with di-n-butyle phthalate in xylene (DPX).

2.3.9 Microscopy and analysis of slides

A Nikon fluorescence microscope (Nikon LaboPhot-2 fluorescence microscope, Nikon Instruments) was used to visualise the immunoreactivity of the placental tissue. Micrographs were obtained with a Nikon Coolpix 995 digital camera. Using Adobe Photoshop 6.0, each micrograph was analysed by using selective random sampling (adapted from systematic random sampling (Mayhew, 1991), by superimposing a grid onto each picture and then assessing the immunoreactivity in every 5^{th} square (see Figure 2.2). To calculate the percentage of vessels showing immunoreactivity to VEGF or VE-cadherin, vessels were counted using the

unbiased forbidden line counting technique; for each time point between 150-200 vessels were counted to avoid over-sampling.

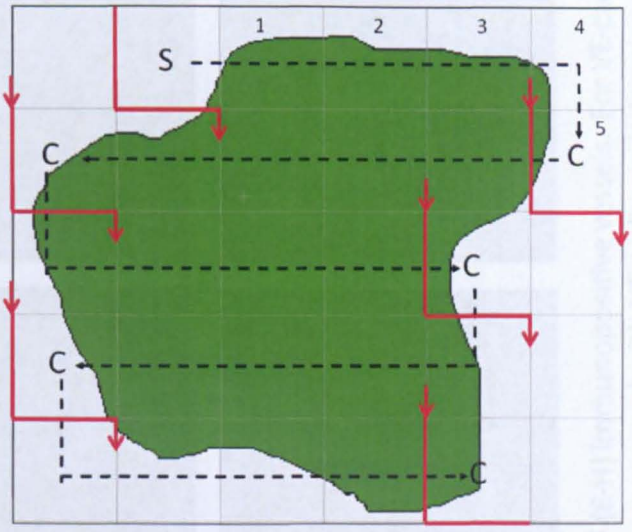


Figure 2.2: Selective random sampling. A grid was superimposed on the micrograph, and counting was started from the start point square (S), chosen as the upper left area of tissue. Every 5th square on the grid was counted (C) after the start point. When the square at the limit of the tissue (square 4 in this example) was reached, the counting continued in the square immediately below it. To avoid counting vessels twice, the forbidden lines (in red in this diagram) method was used, where vessels on the forbidden line were not counted. Selective random sampling was also used when counting under the microscope, and to choose which micrographs to take, except instead of squares we used fields of view.

We attempted to sub-categorise the vessels stained with VEGF and VE-cadherin, by dividing the vessels immunopositive to VEGF into three intensity levels, intensity 1 being the weaker staining and intensity 3 being the brightest staining, and by dividing VE-cadherin immunopositive vessels by differentiating between diffuse cytoplasmic immunoreactivity (location 1), partially diffuse cytoplasmic location and partially continuous junctional location immunoreactivity (location 2) and continuous junctional location immunoreactivity (location 3) (Figure 2.3).

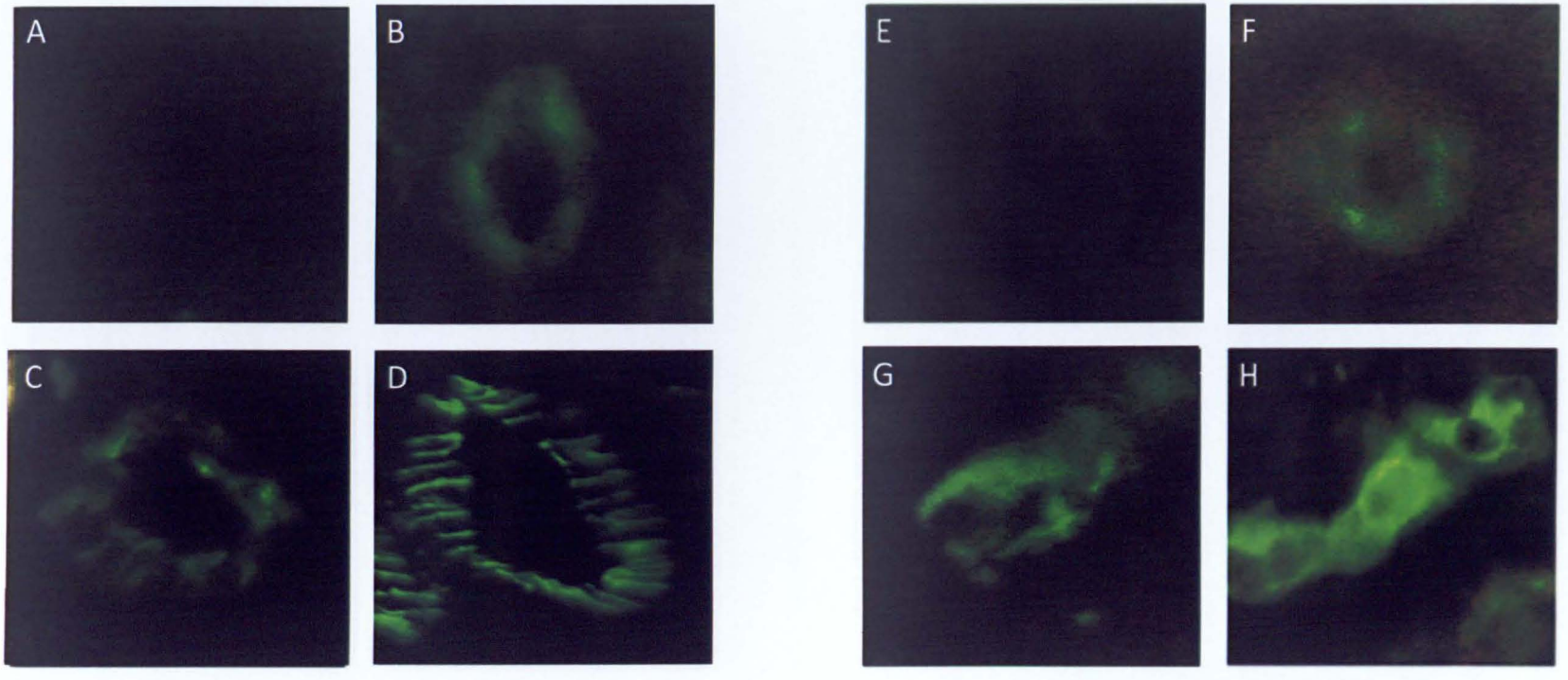


Figure 2.3: Micrographs showing sub-categories of VE-cadherin (A-D) and VEGF (E-H) immunopositive vessels. For VE-cadherin, micrograph A shows a VE-cadherin immunonegative vessel, micrograph B shows a vessel with VE-cadherin in location 1 (fully cytoplasmic location), micrograph C shows a vessel with VE-cadherin in location 2 (partly cytoplasmic and partly junctional locations), and D shows a vessel with location 3 (fully junctional location) VE-cadherin. For VEGF, micrograph E shows a VEGF immunonegative vessel, micrograph F shows intensity 1 VEGF immunoreactivity, micrograph G shows intensity 2 VEGF immunoreactivity, and micrograph H shows intensity 3 VEGF immunoreactivity.

2.3.9.1 Statistical testing

GraphPad Prism version 5.01 statistical software was used to analyse results. Parametric testing was not appropriate due to the low n numbers. Kruskal-Wallis non-parametric testing was therefore used. However, the only non-parametric post-test to the Kruskal-Wallis available in Prism, Dunn's post-test, has inherent low power (Demsar, 2006). More powerful post-tests, such as Holm's procedure, were not available. Therefore, in order to avoid a high rate of Type 2 statistical errors (false negative), whenever the Kruskal-Wallis test detected a significant difference between the groups, the Mann-Whitney U test was used to determine significance between relevant sub-groups. This could produce Type 1 statistical errors (false positives); however, the use of the Mann-Whitney U test was justified by the low power of Dunn's post-test, and the lack of more suitable tests in the statistical software available to us.

Although data was analysed using non-parametric testing, the results in this and subsequent experimental chapters are presented in histograms showing means ± standard deviation, normally used when presenting data analysed in parametric tests; this was for simplicity of presentation.

2.4 Results

2.4.1 Morphology of the explants

All explants from both methodologies and at all timepoints were free of ice crystal damage, as detected using Toluidine blue staining (Figure 2.4A-B).

Furthermore, all explants from both methodologies and at all timepoints had a normal morphology as evaluated by H&E staining. Both the vascular tissue and the trophoblast appeared intact with no detachment or appearance of large vacuoles or necrosis (Figure 2.4C-D)

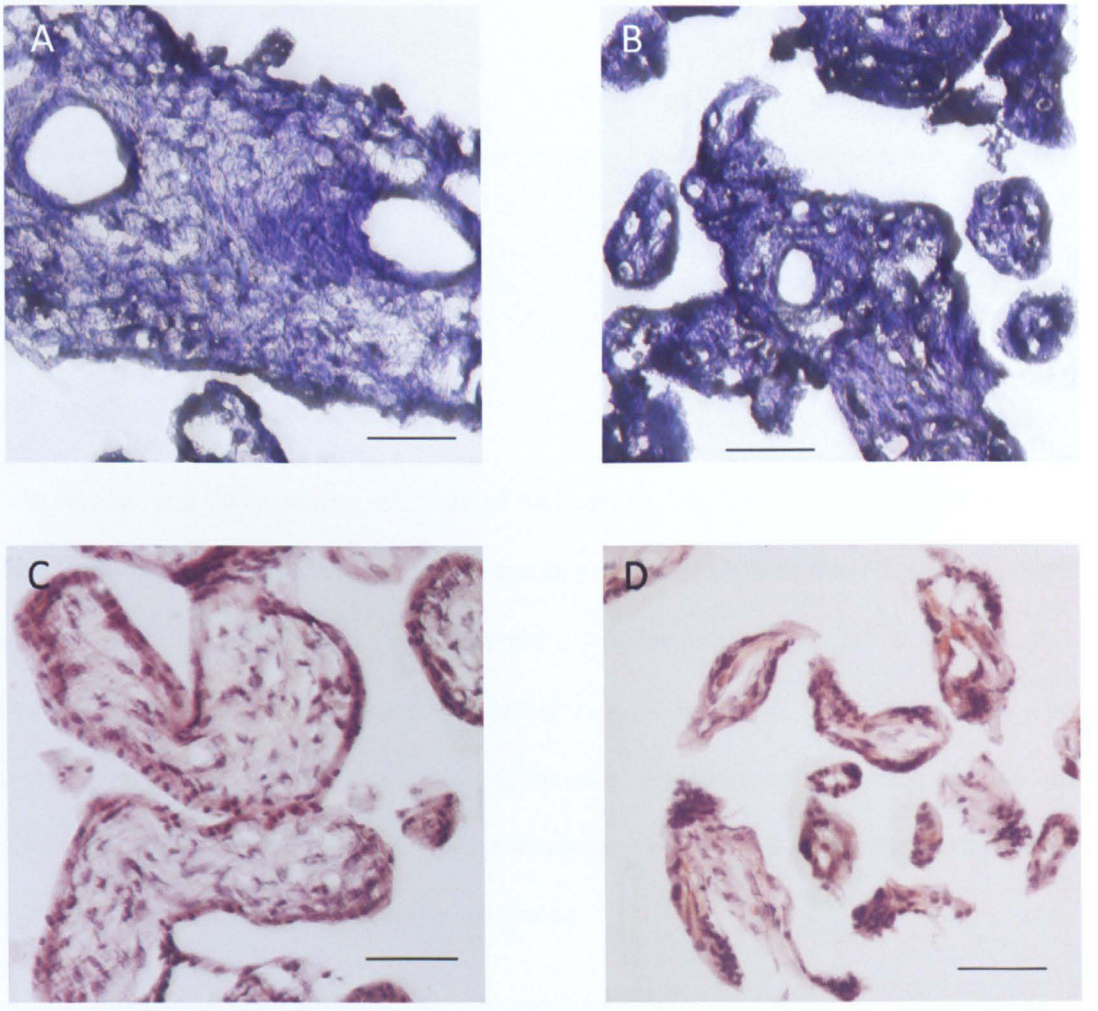


Figure 2.4: Toluidine blue and H&E staining of placental chorionic villous explants. A: Toluidine blue micrograph of a stem villous showing no freeze damage to the tissue. B: Toluidine blue micrograph of intermediate and terminal villi showing no freeze damage to the tissue. C: Micrograph of a placental explant slice stained with H&E, showing structural detail of intermediate villi. D: Micrograph of a placental explant slice stained with H&E, showing intermediate and terminal villi. The absence of freeze damage and the normal morphology of the placental chorionic villous explants confirms that the chorionic villous explant methodology is suitable for our investigations. Magnification: x200. Scale bar: 50um.

2.4.2 Vessel identification and negative controls

PECAM-1 immonureactivity aided the identification of the placental vasculature. Confidence in identifying immunonegative vessels was obtained by identifying vessels in the FITC channel, and then confirming that the observation was indeed a vessel by PECAM-1 immunoreactivity in the TRITC channel (Figures 2.5A-B).

Negative controls (without primary antibodies) showed no non-specific immunoreactivity of the secondary antibody (Figures 2.5C-D).

2.4.3 Fixation before excision results

Toluidine blue and H&E staining of slices of explants derived from whole cotyledons fixed before the excision process revealed a large proportion of vessels with blood trapped in them (Figure 2.6A-B). These vessels would therefore not be suitable for pseudo-quantitative immunofluorescence analysis, hence this protocol was conducted on only one placenta – an extremely low n=1. In addition, due to the high amount of blood remaining in the vasculature, counts could not be performed; thus, direct comparison between this tissue and 'fixation after excision' explants (i.e., t=0h) could not be conducted.

However, qualitative immunofluorescence analysis revealed that in 'fixation before excision' explants, vessels that did not contain trapped blood showed a normal phenotype of fully junctional VE-cadherin immunopositive vessels (Figure 2.6C) and very few vessels immunopositive for VEGF (Figure 2.6D).

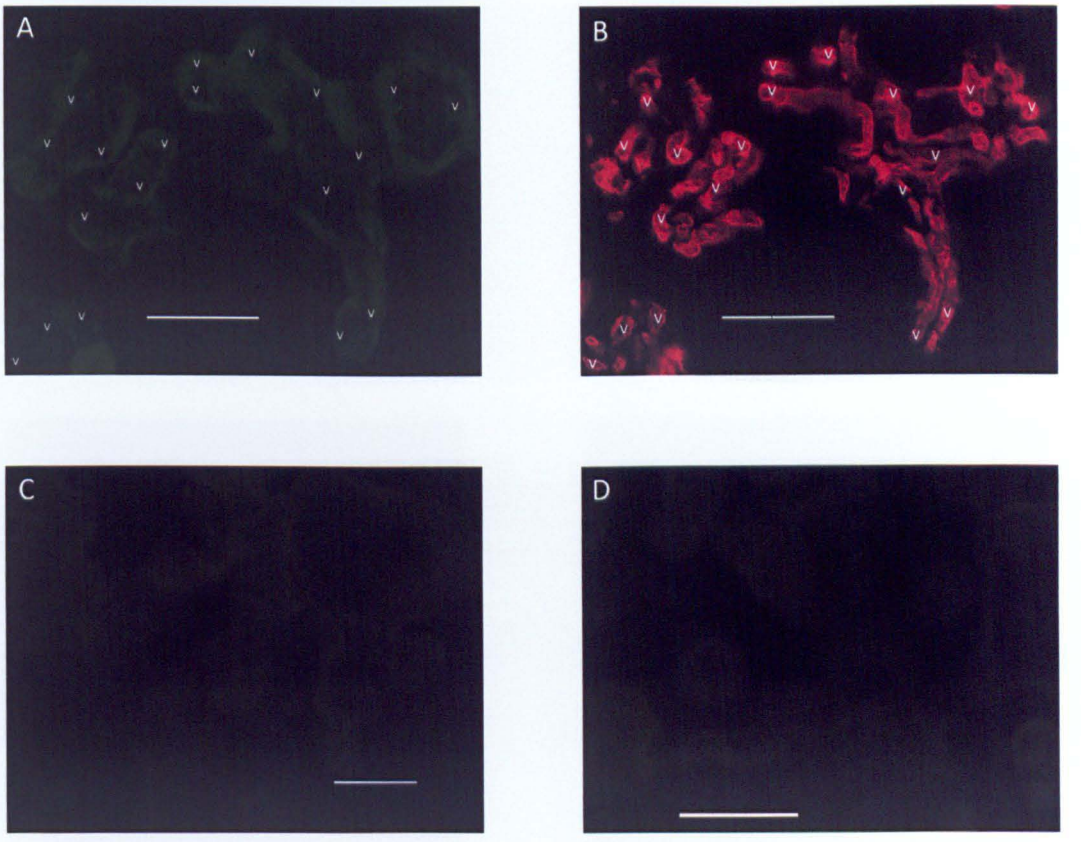


Figure 2.5: Vessel identification with PECAM-1, and negative controls. Assessment of total number of vessels was performed by counting all visible vessels (v) in a given field of view in the FITC channel (A), and subsequently the presence of vessels in that area was verified by looking in the same field of view in the TRITC channel (B) and confirming vessel location with polyclonal PECAM-1 immunoreactivity. The tissue in A and B was treated exclusively with TRITC-labelled secondary antibody, and therefore A resembles an immunonegative control. C: Negative control for VE-cadherin immunoreactivity, showing no non-specific immunoreactivity. D: Negative control for VEGF immunoreactivity, showing no non-specific immunoreactivity. v: vessels. Magnification: x200. Scale bar: 100um.

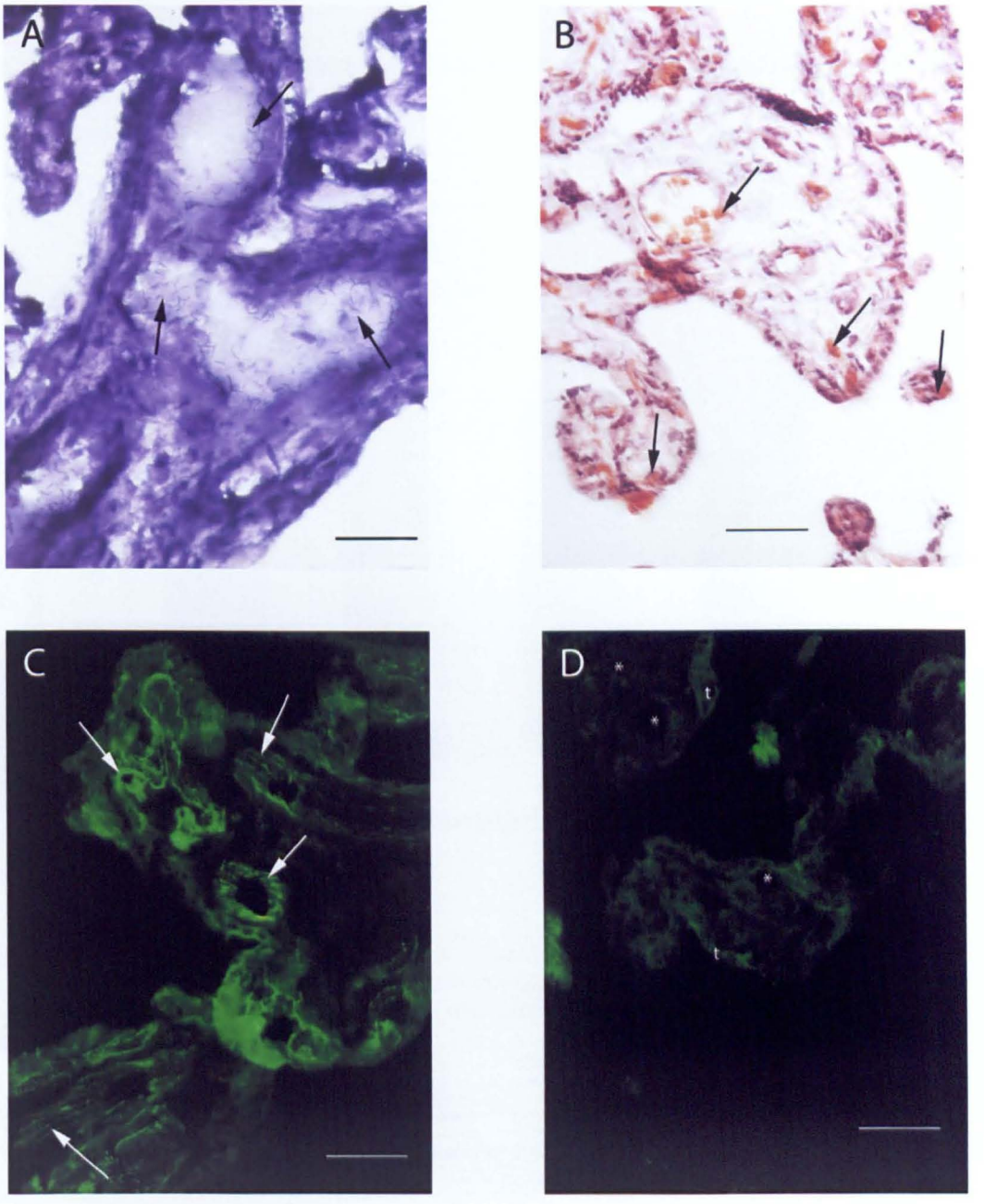


Figure 2.6: Micrographs of tissue fixed before excision. A: Toluidine blue staining of tissue, showing no freeze damage but red blood cells (light purple) trapped in the vasculature of a stem villus. B: H&E staining of tissue, showing normal morphology of tissue but red blood cells (intense red) trapped in the vasculature of stem, intermediate and terminal villi. C: A majority of vessels in the fixed before excision tissue showed VE-cadherin immunoreactivity in location 3. D: A majority of vessels in the fixed before excision tissue showed no VEGF immunoreactivity. whilst there was some trophoblast VEGF immunoreacitivity (t). Black arrows: red blood cells, white arrows: VE-cadherin location 3 immunoreactivity, asterisks: VEGF negative vessels, t: VEGF immunopositive trophoblast. Scale bar: 50 um.

2.4.4 Total number of immunopositive vessels

2.4.4.1 Percentage staining for VE-cadherin

Figure 2.7 shows the percentage of vessels immunopositive for VE-cadherin in free-floating and stationary methods.

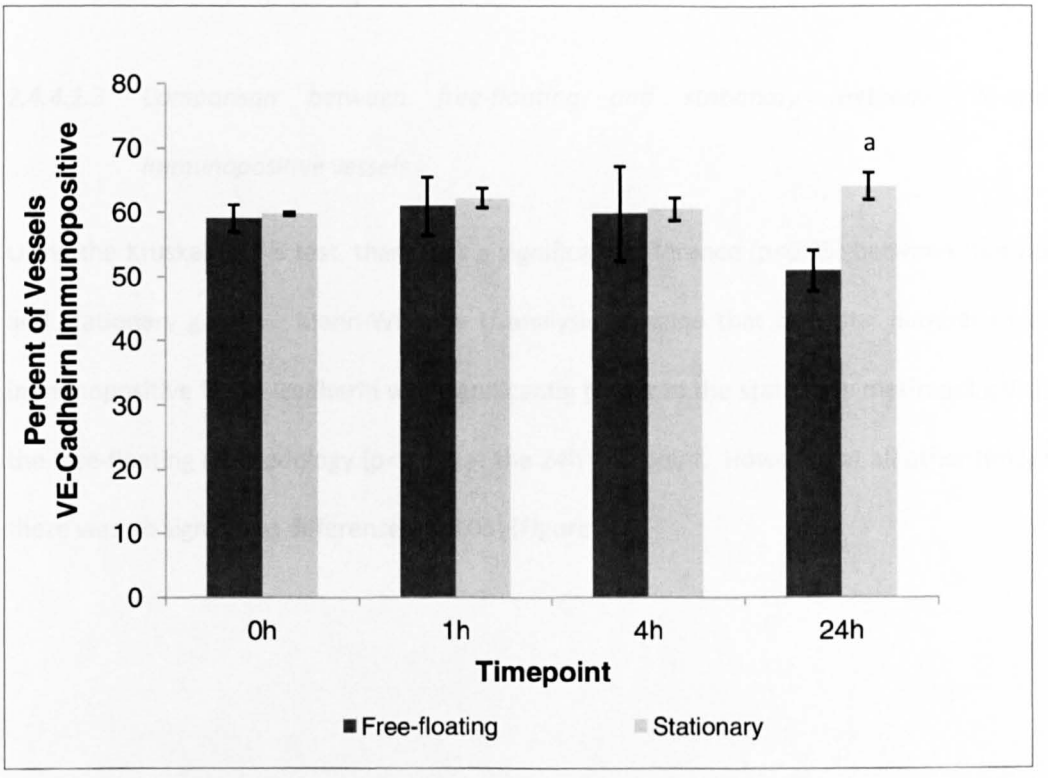


Figure 2.7: VE-cadherin immunopositive vessels – free-floating vs. stationary methods. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=5), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups.
a: $p<0.05$ relative to 24h free-floating.

2.4.4.1.1 Percentage staining for VE-cadherin in free-floating method

The total number of vessels immunoreactive to VE-cadherin were about 59% at 0h (pre-incubation), 61% at 1h, 60% at 4h, and 51% at 24h (Figure 2.7, dark grey bars). Kruskal-Wallis statistical analysis resulted in no significant difference between any of the groups ($p>0.05$).

2.4.4.1.2 Percentage staining for VE-cadherin in stationary method

The total number of vessels immunoreactive to VE-cadherin was about 60% at 0h (pre-incubation), 62% at 1h, 60% at 4h, and 64% at 24h (Figure 2.7, light grey bars). Kruskal-Wallis statistical test resulted in no significant difference between the groups ($p>0.05$).

2.4.4.1.3 Comparison between free-floating and stationary methods, VE-cadherin immunopositive vessels

Using the Kruskal-Wallis test, there was a significant difference ($p<0.05$) between free-floating and stationary groups. Mann-Whitney U analysis revealed that the total number of vessels immunopositive for VE-cadherin was significantly higher in the stationary methodology than in the free-floating methodology ($p<0.05$) at the 24h timepoint. However, at all other timepoints, there was no significant difference ($p>0.05$) (Figure 2.7).

2.4.4.2 Percentage staining for VEGF

Figure 2.8 shows the percentage of vessels immunopositive for VEGF in free-floating and stationary methods.

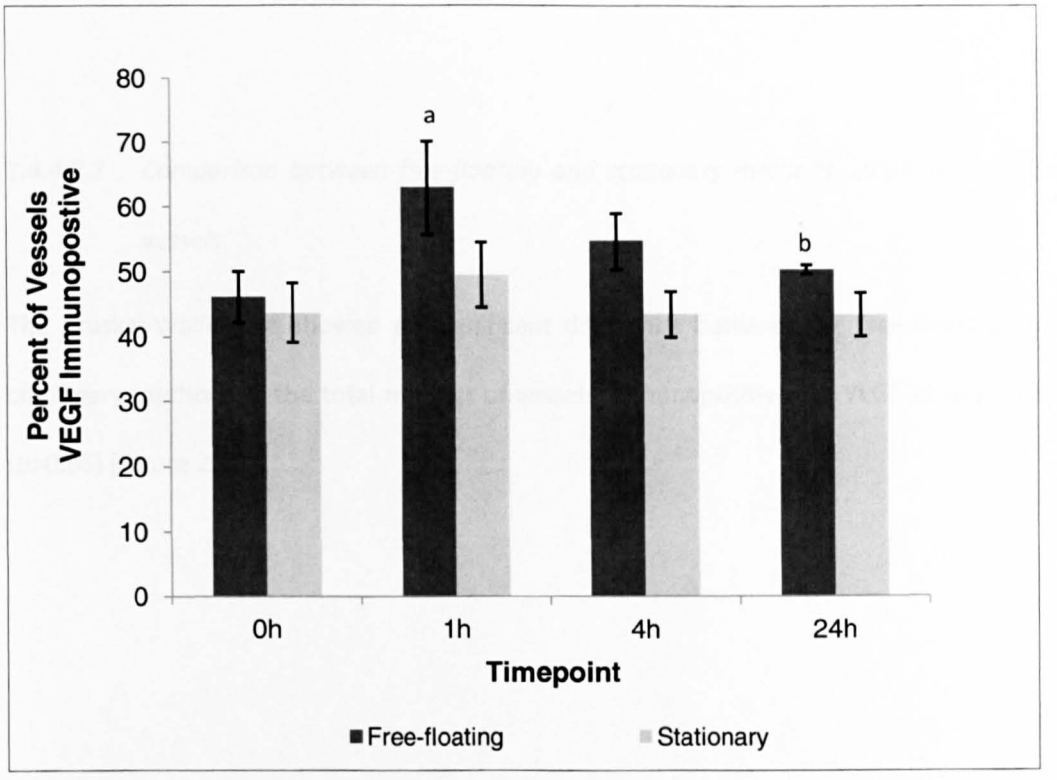


Figure 2.8: VEGF immunopositive vessels – free-floating vs. stationary methods. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=5), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups.
a: $p<0.05$ relative to 0h, b: $p<0.05$ relative to 1h free-floating.

2.4.4.2.1 Percentage staining for VEGF in free-floating method

The total number of vessels immunoreactive to VEGF was about 46% at 0h (pre-incubation), 63% at 1h, 55% at 4h, and 50% at 24h (Figure 2.8, dark grey bars). Kruskal-Wallis statistical test resulted in a significant difference between the groups ($p<0.05$) (Figure 2.8); Mann-Whitney U test was used to show that the significant difference was between 1h and 0h and between 1h and 24h. Thus VEGF is highest after 1h and then declines (although there is no significant difference between 1h and 4h, perhaps because of the intra-timepoint variation (error bars) at 1h and 4h).

2.4.4.2.2 Percentage staining for VEGF in stationary method

The total number of vessels immunoreactive to VEGF was about 44% at 0h (pre-incubation), 50% at 1h, 43% at 4h, and 43% at 24h (Figure 2.8, light grey bars). Kruskal-Wallis statistical analysis resulted in no significant difference between any of the groups ($p>0.05$).

2.4.4.2.3 Comparison between free-floating and stationary methods, VEGF immunopositive vessels

The Kruskal-Wallis test showed no significant difference between the free-floating and the stationary methods in the total number of vessels immunopositive for VEGF at any timepoint ($p>0.05$) (Figure 2.8).

2.4.5 Location/intensity of immunopositive vessels

2.4.5.1 Percentage staining for VE-cadherin

Figure 2.9 shows the percentage of immunopositive vessels in locations 1 and 3 (wholly cytoplasmic and wholly junctional locations, respectively) in free-floating and stationary methods.

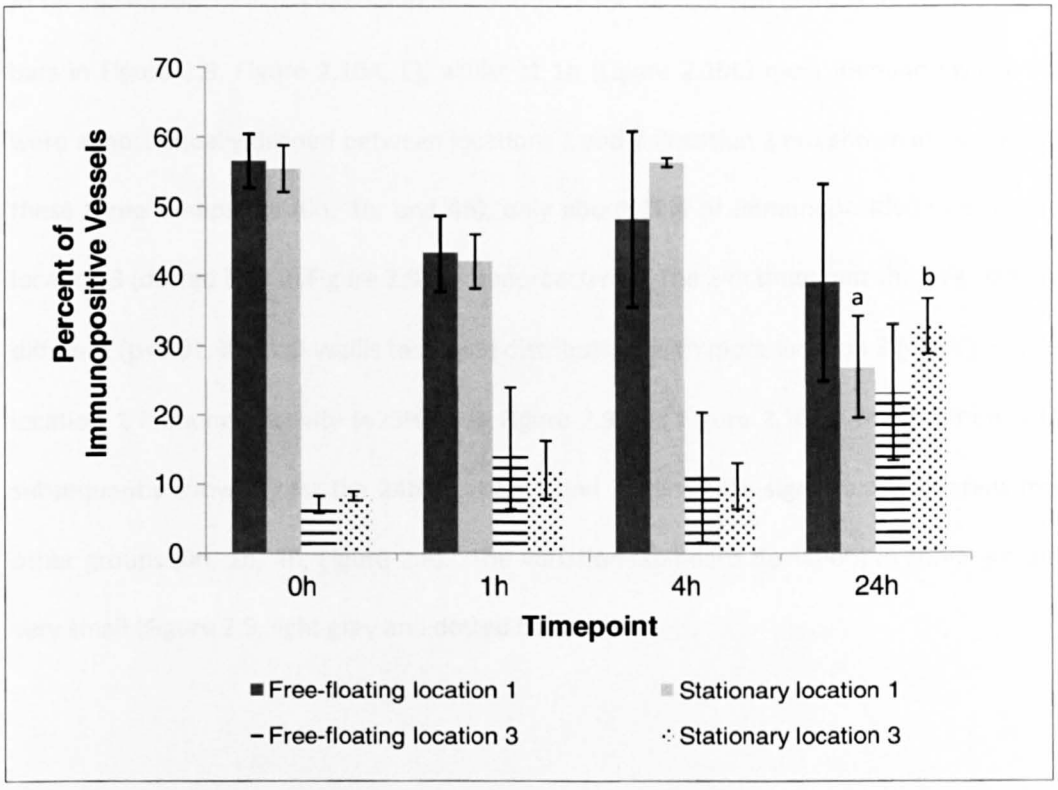


Figure 2.9: Location of VE-cadherin in immunopositive vessels – free-floating and stationary methods. Location 2 data not shown as not physiologically relevant. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=5), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups. a: $p<0.05$ relative to 0h, 1h, 4h stationary method location 1, b: $p<0.05$ relative to 0h, 1h, 4h stationary method location 3.

2.4.5.1.1 Percentage staining for VE-cadherin in free-floating method

At all timepoints, most (≈40-60%) vessels immunopositive for VE-cadherin were grouped in location 1 (diffuse immunoreactivity not localised to the junctional space) (dark grey bars in Figure 2.9 and Figure 2.10A, B, D, F), and the fewest (≈5-25%) vessels were in location 3 (beads-on-a-string VE-cadherin immunoreactivity localised exclusively to the junctional space) (striped

bars in Figure 2.9). There was great variation (standard deviation), but no significant difference ($p>0.05$, n=5) was detected using the Kruskal-Wallis test between the various timepoints (Figure 2.9, dark grey and striped bars).

2.4.5.1.2 Percentage staining for VE-cadherin in stationary method

At 0h and 4h most (≈55%) vessels immunopositive for VE-cadherin were at location 1 (light grey bars in Figure 2.9, Figure 2.10A, E), whilst at 1h (Figure 2.10C) most immunopositive vessels were almost equally divided between locations 1 and 2 (location 2 not shown in Figure 2.9). At these three timepoints (0h, 1h, and 4h), only about 10% of immunopositive vessels showed location 3 (dotted bars in Figure 2.9) immunoreactivity. The 24h timepoint shows a significantly different ($p<0.01$, Kruskal-Wallis test, n=5) distribution, with more location 3 (≈35%) and fewest location 1 immunoreactivity (≈25%), see Figure 2.9 and Figure 2.10G. Mann-Whitney U test subsequently showed that the 24h location 1 and 3 were both significantly different from all other groups (0h, 1h, 4h, Figure 2.9). The variation (standard deviation) in some groups was very small (Figure 2.9, light grey and dotted bars).

2.4.5.1.3 Comparison between free-floating and stationary methods, VE-cadherin locations

There were no significant differences between the two methodologies at any timepoint ($p>0.05$, Kruskal-Wallis test, n=5). However, the error bars for the free-floating methodology were very large (5.45%-14.17%) compared to the stationary methodology (0.62%-7.29%), see Figure 2.9.

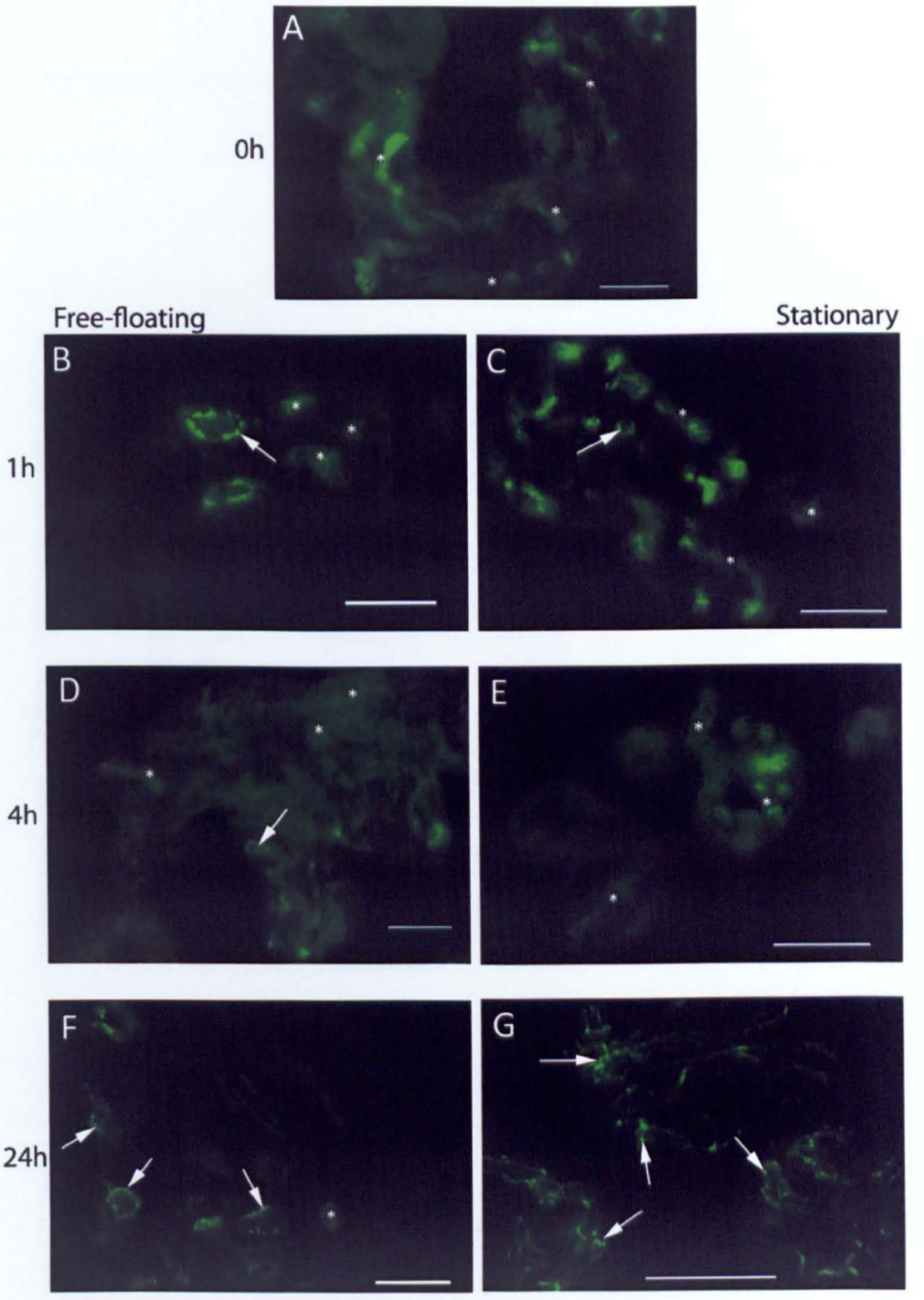


Figure 2.10: Representative micrographs of VE-cadherin immunolocalisation in placental explants at different timepoints. A: 0h explants show mostly location 1 (asterisks) immunoreactivity. B: 1h free-floating explants show mostly wholly non-junctional location 1 (asterisks) immunoreactivity with few wholly junctional location 3 (arrows) immunoreactivity. C: 1h stationary explants show a similar VE-cadherin profile as 1h free-floating explants: mostly location 1 (asterisks), with few location 3 (arrows). D: 4h free-floating explants show mostly wholly cytosolic (asterisks) VE-cadherin localisation, with few wholly junctional (arrows) localisation. E: 4h stationary explants show a similar pattern as the 0h and 1h explants, and 4h free-floating explants. F: 24h free-floating explants show more location 3 (arrows) immunoreactivity than the other free-floating groups, although the difference was not significant (variation not shown). G: 24h stationary explants (high magnification) showing significantly fewer vessels with wholly cytosolic VE-cadherin (asterisks) and significantly more vessels with wholly junctional (arrows) VE-cadherin. In short, all timepoints exhibit a similar profile, except the 24h timepoint, which displays more junctional VE-cadherin (arrows). The variation between explants is not shown here, but is shown in Figure 2.9. Arrows: VE-cadherin location 3 immunoreactivity, asterisks: VEGF negative vessels. Magnification: x200. Scale bar: 100um.

2.4.5.2 Percentage staining for VEGF

Figure 2.11 shows the percentage of immunopositive vessels showing intensity 1, 2 and 3 immunoreactivity in free-floating and stationary methods.

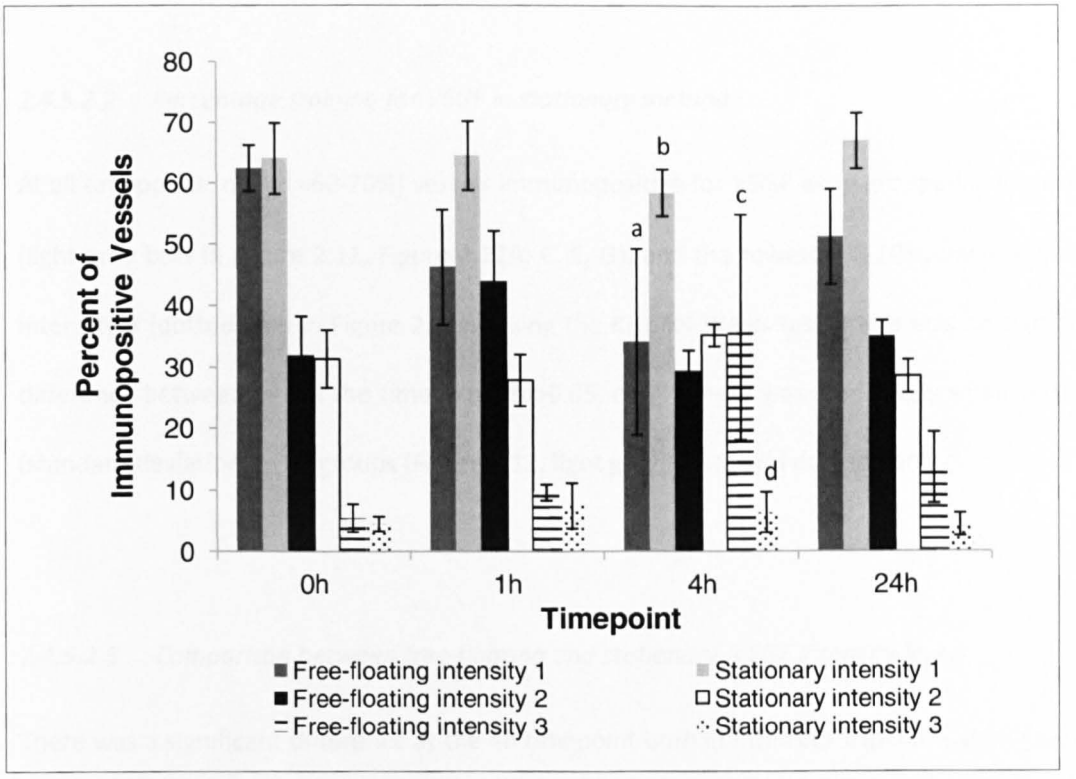


Figure 2.11: Intensity of VEGF immunopositive vessels – free-floating and stationary methods. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=5), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups. a: $p<0.05$ relative to 0h intensity 1, b: $p<0.05$ relative to 4h free-floating intensity 1, c: $p<0.01$ relative to 0h intensity 3, d: $p<0.01$ relative to 4h free-floating intensity 3.

2.4.5.2.1 Percentage staining for VEGF in free-floating method

At 0h, 1h and 24h, most (≈45-60%) of vessels immunopositive for VEGF were grouped in intensity 1 (dark grey bars in Figure 2.11, and Figure 2.12A, B, F), and the fewest (≈5-15%) immunopositive vessels were in intensity 3 (striped bars in Figure 2.11). At 4h, the immunopositive vessels were almost evenly distributed among the three intensity levels (Figure 2.11 and Figure 2.12D); there was a significant difference between 0h and 4h at intensities 1 and 3 ($p<0.05$ and $p<0.01$, respectively, Mann-Whitney U test, n=5). There was no significant

difference ($p>0.05$, Mann-Whitney U test, n=5) between any other timepoints, perhaps due to the great variation (standard deviation) in some groups, especially at the 4h timepoint (Figure 2.11, dark grey, black and striped bars).

2.4.5.2.2 Percentage staining for VEGF in stationary method

At all timepoints, most (≈60-70%) vessels immunopositive for VEGF were grouped in intensity 1 (light grey bars in Figure 2.11, Figure 2.12A, C, E, G), and the fewest (≈5-10%) vessels were in intensity 3 (dotted bars in Figure 2.11). Using the Kruskal-Wallis test, there was no significant difference between any of the timepoints ($p>0.05$, n=5) There was a relatively small variation (standard deviation) in all groups (Figure 2.11, light grey, white and dotted bars).

2.4.5.2.3 Comparison between free-floating and stationary, VEGF intensity levels

There was a significant difference at the 4h timepoint both in intensity 1 ($p<0.05$, dark grey and light grey bars in Figure 2.11) and intensity 3 ($p<0.05$, Mann-Whitney U test, n=5; striped and dotted bars in Figure 2.11, Figure 2.12D, E). In addition, the error bars for the free-floating methodology were very large (1.32%-18.33%, dark grey, black and striped bars in Figure 2.11) compared to the stationary methodology (1.89%-5.74%, light grey, white, and dotted bars in Figure 2.11).

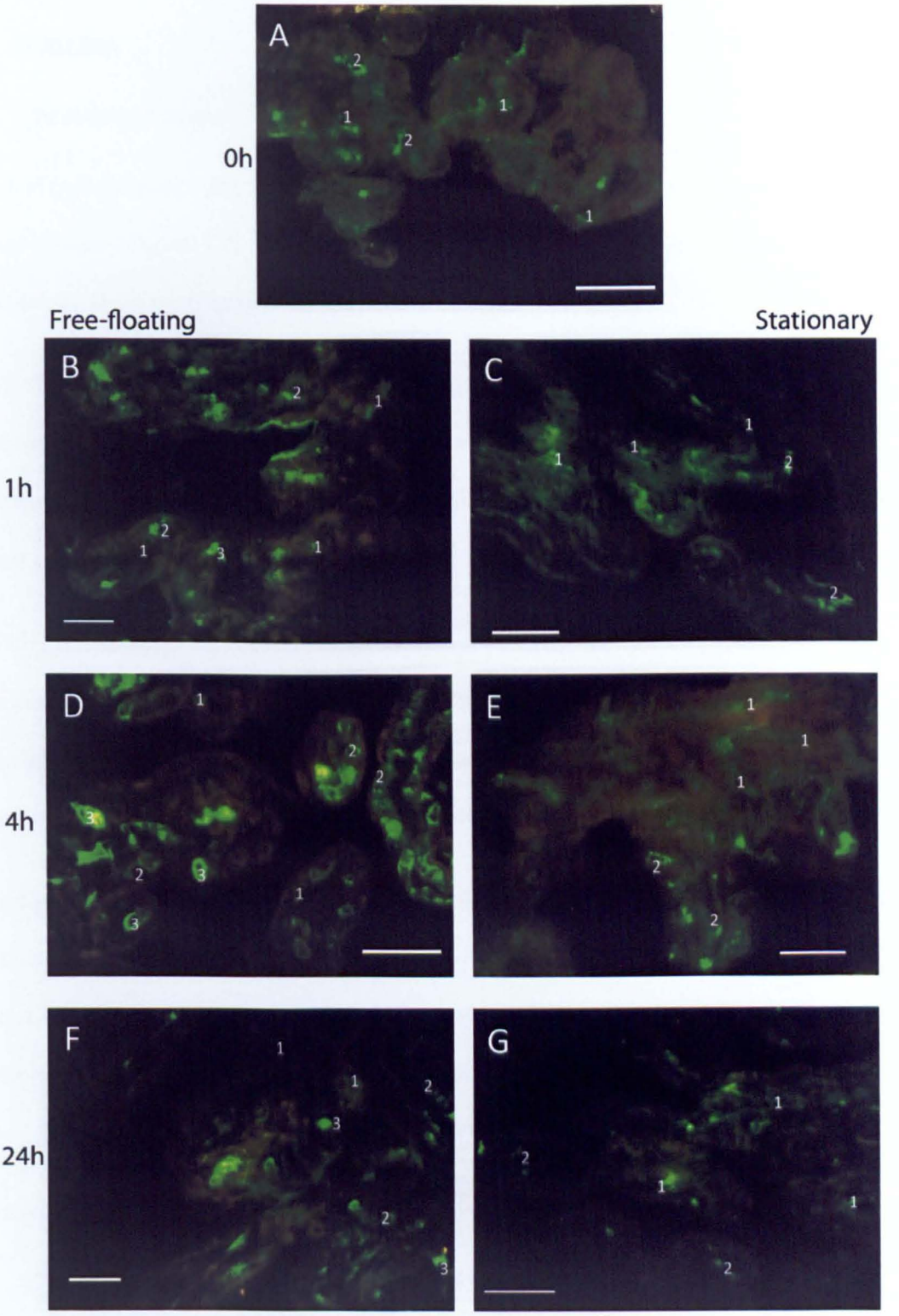


Figure 2.12: Representative micrographs of VEGF immunoreactivity in placental explants at different timepoints. A: 0h explants showing intensity 1 and 2 VEGF immunoreactivity. B: 1h free-floating explants showing mostly vessels at intensity 1 and 2, with few vessels at intensity 3, C: 1h stationary explants showing mostly intensity 1 and 2 VEGF immunoreactivity, D: 4h free-floating explants showing an even distribution between intensity 1, 2, and 3 VEGF immunopositive vessels, E: 4h stationary explants, showing vessels with mostly intensity 1 and 2 VEGF, F: 24h free-floating explants showing a similar profile to 1h free-floating explants, G: 24h stationary explants showing a similar profile to 0h, 1h, and 4h stationary explants. In short, the 4h free-floating group shows a higher intensity of VEGF immunoreactivity than all the other groups, which show similar profiles between them. 1: Intensity 1 immunopositive vessels, 2: Intensity 2 immunopositive vessels, 3: intensity 3 immunopositive vessels. Magnification: x200. Scale bar: 100um.

2.5 Discussion

2.5.1 Summary of results

In all freshly cut explants (0h), the junctional integrity marker VE-cadherin was present in about 60% of vessels (Figure 2.7). In both the free-floating methodology and the stationary methodology, there were no significant differences between any groups.

For VE-cadherin, the variation in the free-floating groups, in both total number of vessels and the location of staining, is higher than in the stationary groups. In the localisation of immunoreactivity analysis, free-floating and stationary methodologies gave about the same number of vessels in each location (Figure 2.9).

For VEGF, an important molecule for the processes of increased permeability and angiogenesis, there are about 45% VEGF immunopositive vessels at 0h (pre-incubation). In the free-floating methodology, there was a difference between 0h and 1h, but no further difference between any other groups (Figure 2.8). Therefore, the 1h timepoint is unusable for further experiments

The intensity of staining in the free-floating methodology also showed a big intra-group variation, as shown by the standard deviation. Following the stationary methodology, in contrast, there was no significant difference between the various groups and the 0h control, and the intra-group variation was lower (Figure 2.11).

Therefore, the best methodology to use for our subsequent glucose studies on explants (Chapters 3 and 4) is the stationary methodology.

2.5.2 Context of our explant culture validation

In our explant studies, the endothelium was the main and exclusive focus. In contrast, most other explants studies currently in the literature focus exclusively on the trophoblast. This limited our approach, whereby quantitative techniques currently used in molecular biology,

such as Western blotting and enzyme-linked immunosorbent assay (ELISA), which would present global data (trophoblast and endothelium) could not be used. As the endothelium represents a relatively minor percentage of the total explant, the results from these techniques would not be a true representation of the endothelium. The trophoblast, in comparison, represents a bigger percentage of the total mass of the explants, and thus, the results from quantitative techniques give a better description of the trophoblast. In this thesis we have instead used a pseudo-quantitative technique, by counting immunopositive vessels following immunofluorescence, to attempt to quantify our results.

The total number of VEGF immunoreactive vessels is around 45% at 0h (Figure 2.8). Explants which were fixed before being cut showed the expected profile of high VE-cadherin and low VEGF (Figure 2.6C-D), but their usefulness was hampered by the excessive blood found in the vessels (Figure 2.6A-B) as a result of less effective washing of whole cotyledons as opposed to individual explants, and therefore more repeats were not performed. These experiments revealed that excision prior to fixation might be resulting in increased VEGF; nevertheless, more repeats would be necessary to confirm this.

In the free-floating vessels, the number of vessels VEGF immunopositive increased from 45% at 0h to a maximum of around 63% at the 1h timepoint, before dropping to 54% and 50% at 4h and 24h (Figure 2.8). This pattern implies that the explant methodology itself is having an effect on the levels of VEGF, and therefore the free-floating methodology is completely unusable for experiments concerning VEGF production. However, even if the stationary methodology is used, the excision artefacts are still present, but seem to be less prevalent and more stable, and therefore more suitable for VEGF studies.

In addition, measuring the intensity of staining has an inherent flaw, as it only shows vessels which have already been shown to express VEGF, and not vessels which are affected due to duration of culture. Therefore, the results from analysing the intensity levels can only tell us

how a test condition affects the excision artefacts of the placental vessels, and not how it would affect the vessels *in vivo*.

This explant validation should serve as a warning for other laboratories working with explants. Anybody using them should always validate the explant methodology, showing how the technique itself affects the molecule that is being analysed, for the time point chosen. In particular, the effect of the methodology on molecules known to be expressed during wound healing, such as cytokines, should be analysed.

For our experiments, due to the nature of the research question (whether high glucose is able to have an effect with acute 4h insults) we expect to have small changes to the vascular expression of VEGF-A splice variants and junctional integrity; having a large variation, therefore, is not appropriate, even if it may be a more physiological environment. Therefore the free-floating methodology could not be used for our purpose, and the stationary methodology was chosen.

An unexpected result was the presence of VEGF in the 0h (pre-incubation) explants (Figure 2.12A). As explained in section 2.1.3.1, Sokolov *et al.* (2008) had used 0h explants in their methodology, and found only 3.11% of syncytiotrophoblast and endothelium immunopositive for VEGF, as compared to our ≈45%. Unfortunately, in their paper, there is not enough detail in the Methods section to reproduce their methodology. The authors only state that they fixed their explants in formalin and embedded their explants in paraffin prior to their immunohistochemical analysis. The difference in VEGF localisation may be due to the authors excising their explants after fixation, therefore eliminating the wounding that our tissue experienced. Additionally, and more importantly, the paraffin step with high temperatures used by Sokolov *et al.* (2008) may have induced antigen denaturing, which is not experienced in frozen histochemistry protocols (as our experiments used).

Although the explant methodology is not perfect, it has several useful characteristics. It allows for long duration experiments which would be impossible with a perfusion technique. It is a relatively inexpensive methodology, and allows for easy manipulation of the media in which the explants are incubated. The stationary method provides a substantial improvement to the free-floating methodology for our experiments, as it gives the least variation in VE-cadherin immunoreactivity and in intensity of staining throughout the experiment for VEGF, and is thus the chosen methodology for further experiments.

With respect to Miller's criteria for acceptable explants cultures (Miller *et al.*, 2005), the stationary methodology fits consistency, as the variation is not too great, and generalisation, as this methodology can be compared to others easily. It also partially fits the criteria of validity, as the placental explants experience excision artefacts just as any 'living' system would if it were excised from its organ. We have also tried to maximise applicability; however, we felt that the benefits from having an oxygenated environment (see section 2.1.3.1) and having a stationary media instead of replicating flow, thereby deviating somewhat from mimicking the *in vivo* situation, were essential for answering our research question investigating the effects of glucose on VEGF expression.

2.5.3 Shear stress in free-floating explants

In the free-floating methodology, we tried to simulate the *in vivo* environment of flow over the explants. However, we observed a very high variation in the number of vessels expressing VEGF and junctional localisation of VE-cadherin.

Endothelial cells are reported to adapt very quickly to shear stress and not lose functionality (Davies, 1997). However, it has been observed *in vitro* that individual endothelial cells react with a large variation to various stimuli, as there is heterogeneity in shear stress distribution, and there are large variations between cells of average cell stress concentration. There is

variation in the location of flow sensing structures on endothelial cells, which are involved in the sensitivity of the response to flow, and therefore the stress concentration is different in different parts of the same cell, leading to different responses to flow in different cells (Davies *et al.*, 1995). The difference in response between endothelial cells has been shown in both acute and chronic responses. As an example of the former, some endothelial cells in a monolayer and *in vivo* had a strong intracellular calcium mobilisation, a key cellular mechanism for initiating changes in response to flow and to shear stress, whilst others needed a much greater increase in flow to achieve the same end result (Falcone *et al.*, 1993). Chronically, it has been shown that gene induction of vascular cell adhesion molecule-1 (VCAM-1) protein, an inflammatory molecule, is heterogeneous in decreased shear stress in rabbit arteries (Walpola *et al.*, 1995).

These studies help explain our large variability in the free-floating methodology; there was an overall response and adaptation to changes in flow and shear stress, but this adaptation showed variability within explants. Also, in our model, the explants were subjected to a uniform flow, but whether this was physiologically accurate and recreated the *in vivo* direction of flow is unknown.

A reason why there is an increase in VEGF at 1h in the free-floating methodology could be that following the excision procedure the endothelium is not intact. The adaptability of the endothelium to shear stress requires intact endothelium, and therefore the wound healing effect that we are observing might worsen the variability and effects to the flow that we have observed.

The variation in the various groups (standard deviation) was very high in the free-floating methodology, more than in the stationary methodology, especially regarding the intensity levels (Figures 2.9 and 2.11). This might be related to the individual placentae reacting to the rolling agitation inherent in the free-floating technique. Whilst this methodology was used to simulate flow, an unexpected result of the explants culture (the excision artefacts mentioned in

section 2.5.2) meant that the explants were subjected to a non-physiological wounding effect, as *in vivo* the placenta may be less likely to experience severe wound healing in normal pregnancies. The resulting wounded explants might therefore be unable to withstand the stresses of flow when subjected to it in the free-floating methodology. That the increased VEGF was due to the excision process was shown by its absence in the qualitative analysis of the 'fixation before excision' results (section 2.4.3 and Figure 2.6D).

Normal placental tissue (non-wounded) from different individuals might respond very similarly to physiological and pathological environment (hence the perfusion model being a very suitable model for experimentation in the environment of the placenta). However, once a tissue is injured in a situation that would not normally occur *in vivo*, intrinsic differences in individual placentae might become more obvious, affecting the results.

2.5.4 Excision artefacts and 'wound response'

Although it is better than the free-floating methodology, as it has less variation, the stationary methodology still has some problems to consider whilst using it, the main one being the explants' high VEGF and low VE-cadherin immunoreactivity. In previous studies, VE-cadherin in the endothelium of perfusion-fixed placentae reached about 90% of total vessels (Babawale *et al.*, 2000; Leach *et al.*, 2004). In our explant model, this percentage at 0h (pre-incubation) is around 60%. This difference can be explained by the initial cutting of the placentae to excise the explants, whilst the perfusion-fixed placentae are not cut prior to fixation. The cutting might thus have created an inflammation at the site of the 'wound', which the tissue tries to respond to with a wound healing response. VE-cadherin delocalisation from the endothelial junctional space is a feature of inflammation in vessel remodelling and wound healing (Albuquerque and Flozak, 2002), as is VEGF upregulation in the vessels affected by wounding.

The main factor altering 'wound healing' in our explants compared to *in vivo* wounds is the lack of blood in the explants, as all blood is washed off immediately after excision. Therefore, there is no coagulation cascade (for a review describing the coagulation cascade and the classic wound healing process, see Clark (1993)), as there are no platelets, neutrophils or monocytes in placental tissue. Macrophages are present in the villous mesenchyme (Hofbauer cells) and recently they have been shown to play a role in trophoblast differentiation, mesenchyme development, and importantly, gestational inflammatory responses (Tang *et al.*, 2011); however, in our explant model, the mesenchyme did not seem to be involved, and it was the endothelium that showed VEGF immunoreactivity.

If an adapted wound healing process is responsible for the high VEGF seen in our explants, what is the mechanism in the endothelium of our explants? Clues to this question are found in endothelial cell culture models that test wound healing, as in this model there is no blood involved either.

In cell culture, wounding and subsequent healing is most often replicated by scratching a pipette tip on an endothelial monolayer, creating a linear area devoid of any cells. Another methodology is to freeze an area of a monolayer, and this again creates an area without any cells. The repopulation of endothelial cells into the empty area is then monitored. Using these techniques, it was shown that there is an increase in DNA transcription to lead to cell migration; this was seen in cells close to the margin of the 'wound' but not those elsewhere. By 10h, expression of endothelin-1 (ET-1), an indicator of endothelial proliferation, was already prevalent, and therefore VEGF might be present as well. Endothelial cells migrate into the wound by 24h, and express molecules such as VEGF and ET-1, indicators of endothelial proliferation (Akimoto *et al.*, 2002). Wound healing then continues for days after the wound was created.

In our explant model, we observed endothelial VEGF production early in wound healing, being present even at time 0h immediately after the creation of the wound in normal tissue. This is

similar to the situation observed in cell culture, and might occur because there are no blood constituents which take over the initial wound healing response and inflammation *in vivo*. However, our explant model shows differences from wound healing in endothelial cell culture, as the endothelial production of VEGF and VE-cadherin disappearance from the junctions (indicative of wound healing processes) seems to decrease in our explants at 24h, instead of the continuing wound healing at and after 24h seen in cell culture. We hypothesise that this is because in our explants there is the additional 3D chorionic villous architecture which, in the absence of the classical wound healing effect seen *in vivo* involving blood constituents and the immune response (macrophages, platelets, fibroblasts etc.), signals to inhibit the wound healing response and restore junctional integrity.

2.5.5 Caveats to wounding hypothesis

Although we hypothesised that wound healing may have caused the higher levels of VEGF seen in the explants, other explanations are possible. VEGF is known to be present and be an important mediator of endothelial proliferation. Whilst we cannot exclude that this is occurring in our explants, we have taken steps in our set-up to try to minimise this risk. We have placed the explants in metal nets, allowing the tissue to be freely suspended in the media, without an extracellular matrix containing collagen I or fibrin, which introduces an oxygen gradient and induces *in vitro* endothelial cell proliferation (Bocci *et al.*, 2001; Cenni *et al.*, 2011). Perhaps the best evidence that our explants are unlikely to be undergoing proliferation is the lack of any oxygen gradient. Proliferation and angiogenesis are processes that are driven by hypoxia (see section 1.1.4), and are inhibited by normoxic/hyperoxic conditions, such as those in which we incubated our explants (see section 2.1.3.1).

Another possibility is that the endothelium is producing VEGF as a survival factor, to prevent apoptosis induced by wounding (see section 1.2.1.3). However, VEGF is only a survival factor

when present at low doses. At higher doses, and in conjunction with TGF-β, it acts as an apoptotic agent in human and bovine cells (Ferrari *et al.*, 2006). Indeed, again in human and bovine cells, apoptosis is shown to occur in angiogenesis, where ECs need to be pruned in the newly formed vessels (Pollman *et al.*, 1999). We only tested our explants for VEGF, and not other cytokines; thus, we are unable to say with certainty whether the VEGF present is acting in this instance as a survival factor, or as an inflammatory response.

Furthermore, both proliferation and anti-apoptosis hypotheses are unable to explain why after 24h there was a reduction of VEGF present in the endothelium of explants. For proliferation, VEGF needs to remain present until the remodelling is completed, and for the pro-survival hypothesis, a decrease in VEGF would lead to apoptosis of tissue which was not seen (Figure 2.4).

2.5.6 Recovery period

The temporal pattern of downregulation of VEGF and localisation of VE-cadherin suggest a 24h recovery period in the explants in this study. If the 'wound healing' hypothesis is shown to be correct, further studies could use the first 24h of culture as a recovery period, and the subsequent period for the actual experiments. However, in order for this recovery period to be useful, further validation would be needed in order to observe whether there is a plateau in the temporal pattern of VEGF and VE-cadherin beyond 24h, to establish whether the recovery period is indeed 24h long, or if it is ongoing after this timepoint.

However, continued incubation beyond 24h was not performed in our study. This, along with the caveats to the 'wound healing' hypothesis, negated the usefulness of a recovery period and therefore no such period was designed into subsequent chapters.

2.6 Summary

Due to the lower number of VE-cadherin immunopositive vessels at 24h, the intensity of VEGF immunopositive vessels at 4h, and the high variation in most of the timepoints, the free-floating methodology is not suitable for the study of VEGF and VE-cadherin in placental explants.

The stationary methodology is the preferred methodology, but care should be taken to consider the excision artefact effect that seems to be a player in this methodology.

Chapter 3
The effect of 15mM glucose on the explant culture: analysis of VE-cadherin and VEGF at two different timepoints

3.1 Introduction

3.1.1 Effects of glucose on VE-cadherin and VEGF expression

In Chapter 1, the general effects of hyperglycaemia in the vasculature of the diabetic milieu were discussed. This chapter focuses on the effects of high glucose on two molecules important for vascular integrity and permeability, namely VEGF and VE-cadherin.

3.1.1.1 Effects of glucose on VEGF

Diabetic patients, even those with short duration exposure to the diabetic condition (such as children, duration of diabetes 2-4 years), show increased levels of plasma VEGF (Chiarelli *et al.*, 2000b). Furthermore, in this study patients who had poor control (defined as an HbA_{1c} level higher than 86mmol/mol) had significantly higher VEGF levels than those with lower HbA_{1c} levels, indicating that glucose is responsible for this increase. A study by Sorensen *et al.* (2005) on Type 1 diabetics, comparing 48h of good glycaemic control with poor glucose control, found that the good glucose control group (which had a median blood glucose of 6.3mM) had lower plasma VEGF concentration than the poor glucose control (median blood glucose: 15.9mM). What is remarkable about this paper is that it is a paired study, where each subject examined was subjected to both good control and poor control, thereby eliminating individual differences between different subjects.

Possible mechanisms behind this hyperglycaemia-induced VEGF increase have been studied. One of the major players in VEGF actions is NO. VEGF action is normally coupled with NO, and when acting together, endothelial cell structure and function is maintained. When VEGF increases, NO is produced, which then feeds back to decrease VEGF production. However, although VEGF is increased in diabetes, NO is decreased. That this uncoupling occurs is shown in a study by Nakagawa *et al.* (2006), where it was found that 30mM glucose for 24h inhibited NO production in response to VEGF in bovine arterial endothelial cells and that proliferation

was increased in these cells under these conditions, compared to a 5mM glucose control; the presence of an exogenous NO donor blocked this proliferation. VEGF's effects on proliferation upon uncoupling of VEGF-NO were mediated by the activation of MAPK1/3 by the VEGFR-2 receptor (Nakagawa *et al.*, 2006), whilst inhibition of VEGFR-1 signalling can account for the decreased NO production (Ahmad *et al.*, 2006; Bussolati *et al.*, 2001). Nakagawa (2007) explains in his review that there are several mechanisms by which high glucose might impair NO production, including inhibition of eNOS activation, scavenging of NO by glucose itself, and quenching of NO to form peroxynitrate by oxidative stress mechanisms.

Another study which links oxidative stress to high glucose production of VEGF was conducted by Chen *et al.* (2004). In this study, incubation of HUVECs with 25mM glucose for 24h caused the upregulation of haeme oxygenase (HO), a marker of oxidative stress. HO activity then increased VEGF mRNA, and this VEGF increase was partly blocked by an HO inhibitor. However, this paper found that there is an increase of eNOS activity, as opposed to a decrease. Although these studies have different findings regarding NO production, they are in agreement regarding glucose increasing VEGF expression.

TGF-β2 has been shown to have different effects on VEGF production depending on glucose concentration: after 5 days euglycaemic (5.5mM glucose) incubation, human retinal pericytes exposed to 0.1ng/ml TGF-β2 significantly increased VEGF expression, and exposing to 1ng/ml and higher concentrations of TGF-β2 decreased VEGF expression compared to no TGFβ2 exposure, whilst incubation under 18mM glucose indicated no significant difference in VEGF expression between no TGFβ2 and any TGF-β2 concentrations (Vidro *et al.*, 2008). Importantly, TGF-β2 has been shown to be increased in rat models of Type 1 diabetes (Lamers *et al.*, 2007).

The renin angiotensin aldosterone system (RAS) also seems to be important in the production of VEGF under hyperglycaemia (Feliers and Kasinath, 2010). This is a system that regulates blood pressure and blood volume, and that produces angiotensin. However, angiotensin's

precursor is produced in the liver and cleaved in the lungs and renin is produced in the kidney. Thus, this model is only relevant in whole body systems, and not in *in vitro* placental explants.

3.1.1.2 Effects of glucose on VE-cadherin

Although not as much evidence exists for the direct effects of glucose on VE-cadherin as there is for VEGF, a limited number of papers have investigated this topic.

The presence of advanced glycation end products (AGEs) have been shown to disrupt VE-cadherin junctions. A 3h incubation with bovine serum albumin modified with AGEs has been shown to decrease the junctional integrity of HUVECs, and to decrease VE-cadherin protein levels (Otero *et al.*, 2001). However, AGE formation is a long term process, and therefore the effects due to AGEs would only manifest themselves after long duration exposures to glucose.

Shorter-term effects of glucose have shown that VE-cadherin is initially affected by glucose, but in the long-term VE-cadherin localisation to the junctions is restored, possibly as an adaptation to the altered environment. Maillard and Leach (2002) found that a 2h exposure to high glucose (25mM) resulted in significantly more HUVECs with discontinuous VE-cadherin junctions; however, there was no difference in total VE-cadherin expression. The VE-cadherin localisation to the junctional space was recovered after 2 days under 25mM glucose insult, when there was no longer a significant difference between the cells incubated under euglycaemic and hyperglycaemic conditions.

That longer duration (days as opposed to hours) has no effect on VE-cadherin localisation was also confirmed by both Matsui-Hirai *et al.* (2011), who found no difference between HUVECs incubated under 22mM glucose and those incubated under 5.5mM glucose for three days, and by Singh *et al.* (2011), who found no difference in VE-cadherin localisation and total protein in glomerular endothelial cells incubated in 25.5mM glucose for 2, 7 and 14 days. However, there are also contradictory results: Rangasamy *et al.* (2011), found that phosphorylated VE-cadherin

(found in non-junctional VE-cadherin) is increased in human retinal endothelial cells incubated for 72 hours under 30.5mM glucose compared to those incubated under 5.5mM glucose.

3.1.2 How is 'wound healing' affected by glucose?

We saw in Chapter 2 (see sections 2.1.3.2 and 2.5.4) that there are excision artefacts (decreased junctional VE-cadherin and increased VEGF) in our explants, which might be attributed to a wounding response. Therefore, it is important to consider how glucose affects this.

It is well known that in diabetes wound healing is often impaired, leading to chronic conditions such as diabetic foot ulcers. These conditions are thought to be a consequence of high glucose levels. It has been shown in a study of 63 diabetic patients that ulcer wound healing times were significantly longer for patients with higher HbA_{1c} values than in patients with lower HbA_{1c} values (Markuson *et al.*, 2009). High HbA_{1c} values are of course representative of chronic hyperglycaemia.

Our explant culture, however, does not model chronic effects of glucose. It is an acute model, where we are exposing the explants to 15mM glucose for only 4 or 24h, and most studies agree that the development of complications is due to the chronic effects of glucose, as reviewed by Fowler (2008). McMurry (1984) describes the stages of wound healing affected by glucose in diabetes, which can be ameliorated by either insulin administration or the reduction of glucose levels as: granulocyte phagocytosis, chemotaxis, and killing of bacteria, synthesis of protocollagen and collagen, fibroblast proliferation, and capillary ingrowth. Importantly though, one study (Weringer *et al.*, 1981) suggested that, rather than the hyperglycaemia *per se* causing the dysfunction in vessels ingrowth, it was the decreased insulin availability which caused this phenotype. Metalloproteinases that are involved in clearing the way for inflammatory

molecules in wound healing have also been shown to be affected by 25mM glucose (Death *et al.*, 2003).

Our acute glucose insult studies described in this chapter may reveal, rather than confound, effects of high glucose on post-wounding events.

3.1.3 Effects of hyperglycaemia on explant cultures

Hyperglycaemia is a key characteristic of diabetes, and, as detailed in Section 1.3.3.1, has effects on the production of VEGF, and thus, on the permeability of the endothelial layer and VE-cadherin localisation. We wished to see if we could observe a change in VEGF and VE-cadherin localisation within a single insult with a mild hyperglycaemic hit (15mM glucose) of 4h and 24h duration. The 15mM glucose was chosen as a blood glucose level that is seen in Type 1 diabetic pregnancies where the mother is exposed to 15mM postprandial glucose for 4h before return to normal (Kerssen *et al.*, 2004; Kyne-Grzebalski *et al.*, 1999) (see section 4.1.3 and Figure 4.3 for more detail).

The 4h timepoint, as analysed in Chapter 2, was an ideal short duration timepoint to use. Studies conducted on postprandial hyperglycaemia (Gerich, 2003; Fujimoto *et al.*, 2006) revealed that glucose can cause fast-acting responses which can be seen from 30 minutes onwards. The 4h we chose would allow data collection of any effects of glucose on the production of VEGF and localisation of VE-cadherin.

The 24h timepoint was chosen to represent a single but long duration insult, to observe whether a longer insult worsened the phenotype observed at 4h. Cell culture studies have revealed that glucose exposure leads to increased VEGF production and VE-cadherin disruption (Chen *et al.*, 2004; Nakagawa *et al.*, 2006; Rangasamy *et al.*, 2011); whether chorionic villous explants respond similarly requires elucidation.

In previous literature of glucose studies with human tissue, there has often been an osmolarity control in the form of mannitol. We wished to test this 'negative' control to see if it is an effective control. We wanted to test this for two reasons. Firstly, the normal range of blood osmolarity is 275-299mOsm/L, the biggest contributor to this value being sodium levels, which contribute around 270mOsm/L. The glucose component contributes the same value as its concentration in mM (i.e., in a normal adult, around 5mM, so it contributes around 5mOsm/L). Therefore, adding 10mM glucose, or indeed mannitol, to the media only increases its osmolarity by 10mOsm/L, which is a physiologically insignificant addition. Secondly, mannitol has long been used in medicine to increase permeability and open up endothelial junctions, which is very relevant to our experiment (Infanti, 2008; Hartwell and Sutton, 1993). The concentration of mannitol used in these experiments was extremely high (in the molar values), and it is assumed that this has an osmotic effect, thus mannitol being used so often as an osmotic control. However, mannitol is also an effective oxygen free radicals scavenger, which has been shown to have important vascular effects (Fu and Jiao, 1995; Magovern *et al.*, 1984; Paterson *et al.*, 1989). Therefore, mannitol may not be a good osmotic control in this situation, especially at the concentration we are using it, and may indeed have an effect itself and this will be examined.

3.2 Aims and objectives

The first aim was to observe whether a short duration (4h) of 15mM glucose insult in chorionic villous explants affected the production of VEGF and localisation of VE-cadherin.

The second aim was to observe whether 15mM glucose for 24h in chorionic villous explants was as effective as the long duration hyperglycaemic studies in cell culture models at increasing VEGF expression, and whether VE-cadherin junctional integrity remained stable at this timepoint.

The third aim was to establish whether mannitol was a good osmolarity control for our explants, as it is in cell culture methods.

The fourth aim was to discern whether the excision artefacts observed in Chapter 2 affected our results.

3.3 Methods

All the materials and methods were as in section 2.3 (with the same donor demographic requirements as those outlined in section 2.3.2 and with the exclusion of the free-floating methodology), with the following exceptions:

3.3.1 Explant culture

The explant culture was performed for 4h and 24h as specified in Chapter 2's 'Stationary methodology' (section 2.3.5) (n=5 placentae, 6 explants per timepoint); these made up our euglycaemic group. In parallel to these, 6 explants (n=5 placentae) per timepoint were cultured in media containing an added 10mM glucose (bringing the total glucose concentration to 15mM glucose), and 6 explants per timepoint were cultured in media containing 10mM mannitol.

3.3.2 Analysis

As in Chapter 2, total number of vessels immunoreactive to VE-cadherin and VEGF were counted. The vessels showing immunoreactivity were then split into three categories. For VE-cadherin, location 1 was wholly cytoplasmic VE-cadherin, location 2 was partly junctional and partly cytoplasmic VE-cadherin, and location 3 was wholly junctional VE-cadherin.

For VEGF, counts were made by qualitative scoring of intensity of fluorescence. All scoring was performed by a single researcher (myself); furthermore, analysis of each n number was performed in a single sitting. Criteria used: intensity 1 was weak immunofluorescence, intensity 2 was medium immunofluorescence, and intensity 3 was strong immunofluorescence.

GraphPad Prism version 5.01 statistical software was used to perform Kruskal-Wallis test with Mann-Whitney U test between relevant groups. The reasoning behind these statistical tests has been discussed in section 2.3.9.1. Unless otherwise specified, all the statistics shown throughout Chapter 3 were performed with the Mann-Whitney U test (n=5). Where statistical significance is present, this indicates that a previous Kruskal-Wallis test (n=5) had shown a significant difference between the wider groups.

3.4 Results

3.4.1 Total number of VE-cadherin and VEGF immunoreactive vessels

3.4.1.1 VE-cadherin

In any given group (euglycaemic, hyperglycaemic and mannitol incubations), there were no significant differences between either short duration (4h) or long duration (24h) insult in the number of vessels showing VE-cadherin immunoreactivity (Figure 3.1). The mannitol group always revealed a significantly lower number ($p<0.01$ for euglycaemia and $p<0.05$ for hyperglycaemia) of VE-cadherin immunopositive vessels (Figure 3.1). However, intra-experimental differences were not found between the glycaemic groups ($p>0.05$).

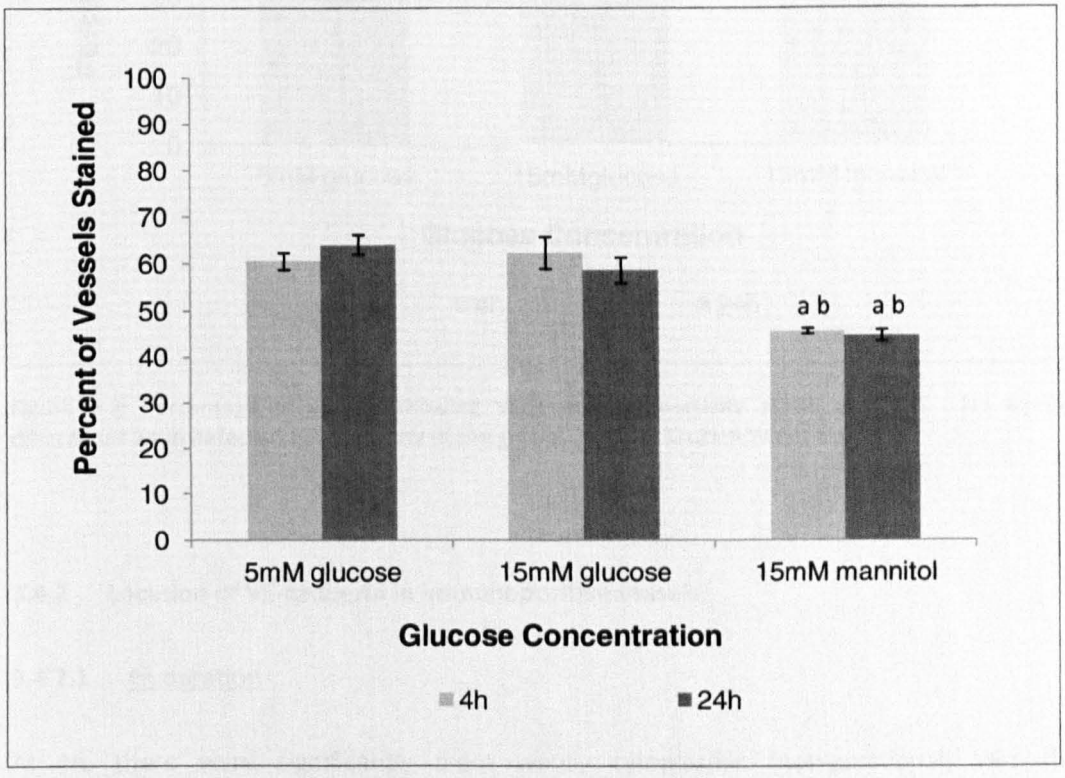


Figure 3.1: Percentage of vessels showing VE-cadherin immunoreactivity at 4h and 24h. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=5), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups.
a: $p<0.01$ relative to euglycaemia, b: $p<0.05$ relative to hyperglycaemia.

3.4.1.2 VEGF

The number of vessels showing VEGF immunoreactivity did not differ ($p>0.05$, Kruskal-Wallis test, n=5) between experimental groups or duration of insult (Figure 3.2).

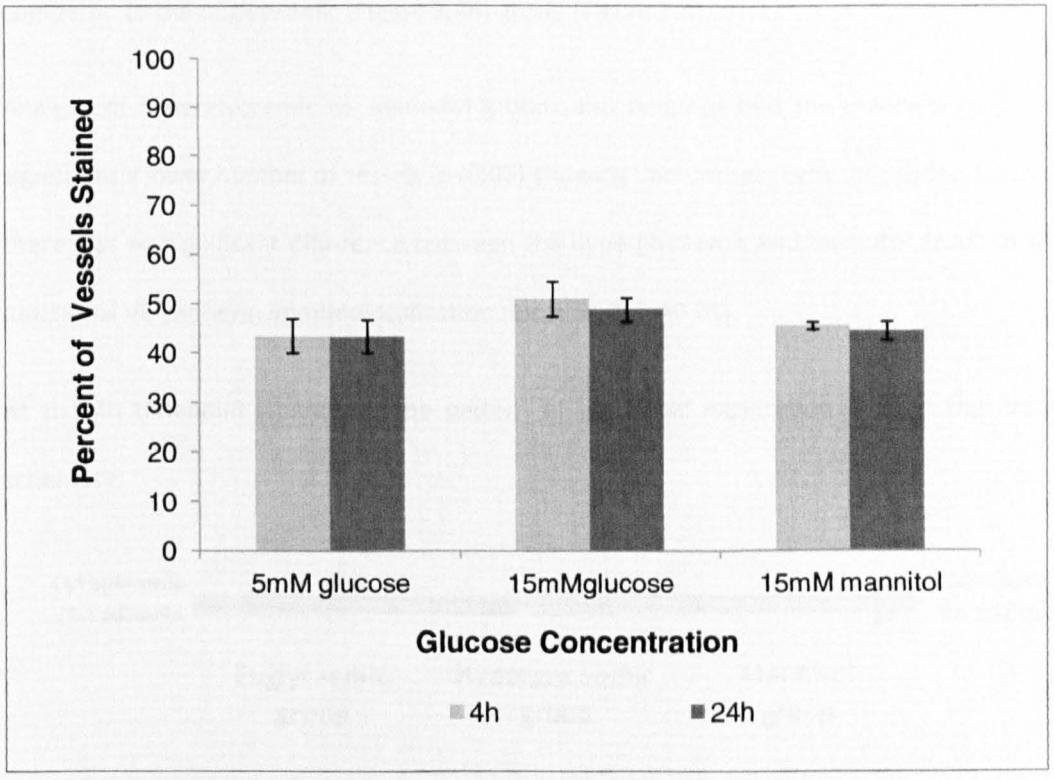


Figure 3.2: Percentage of vessels showing VEGF immunoreactivity at 4h and 24h. No significant differences were detected between any of the groups ($p>0.05$, Kruskal-Wallis test, n=5).

3.4.2 Location of VE-cadherin in immunopositive vessels

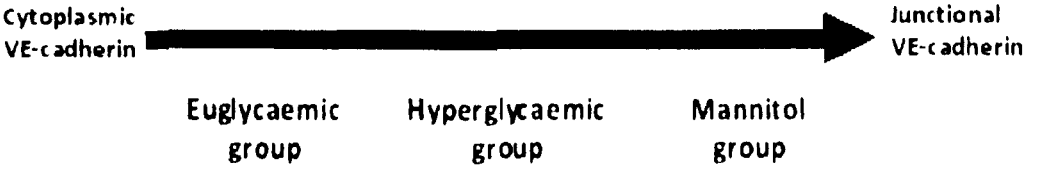
3.4.2.1 4h duration

At 4h, there were significantly more wholly cytoplasmic (non-junctional) VE-cadherin immunoreactive (location 1) vessels in euglycaemic conditions than in hyperglycaemic conditions ($p<0.05$) (Figure 3.3), whilst the number of vessels showing exclusively junctional VE-cadherin immunolocalisation (location 3) in the euglycaemic (Figure 3.4B) and hyperglycaemic (Figure 3.4C) groups was not significantly different ($p>0.05$) (Figure 3.3).

The number of vessels in the mannitol group (Figure 3.4D) showing wholly cytoplasmic localisation (location 1) of VE-cadherin was significantly lower than that in the euglycaemic group ($p<0.01$), and there were also significantly more vessels ($p<0.05$) showing wholly junctional (location 3) VE-cadherin immunoreactivity in the mannitol (Figure 3.4D) group compared to the euglycaemic (Figure 3.4B) group (Figure 3.3).

Analysis of hyperglycaemic vs. mannitol groups also revealed that the mannitol group had a significantly lower number of vessels ($p<0.05$) showing immunoreactivity at location 1; however, there was no significant difference between the hyperglycaemic and mannitol group in wholly junctional VE-cadherin immunolocalisation (location 3) ($p>0.05$).

At the 4h timepoint, therefore, the pattern of junctional localisation is as in the following schematic:



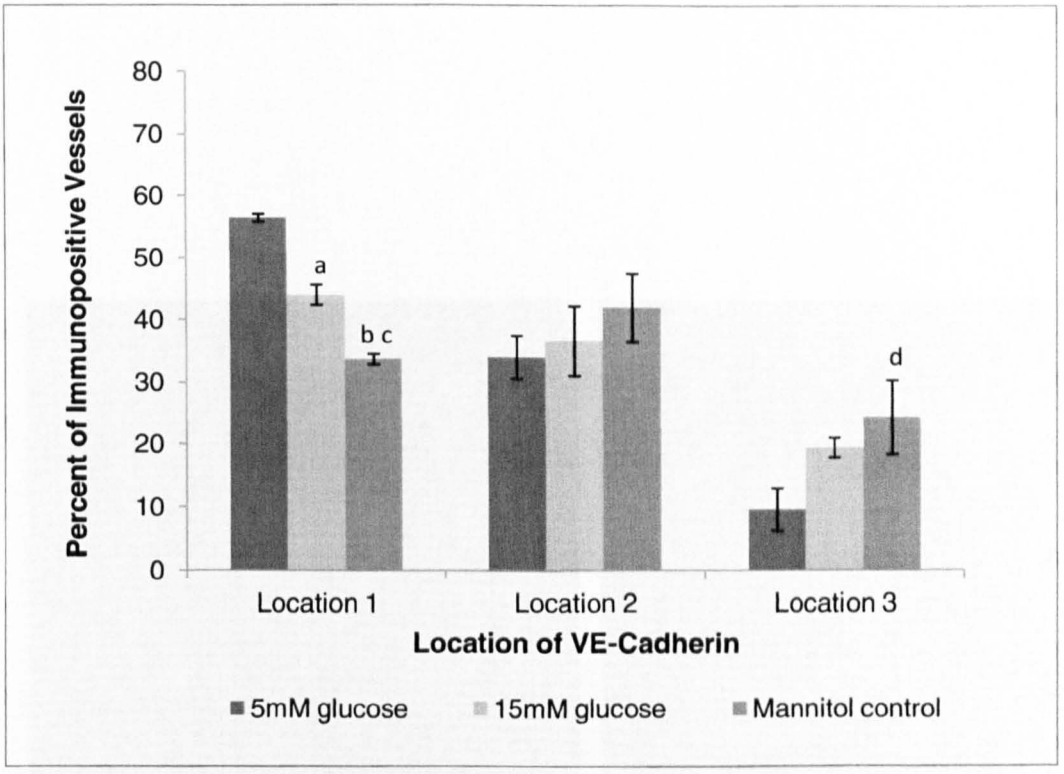


Figure 3.3: Effect of 4h 15mM glucose insult on VE-cadherin immunolocalisation. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=5), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups.
a: $p<0.05$ relative to 5mM glucose location 1, b: $p<0.01$ relative to 5mM glucose location 1, c: $p<0.05$ relative to 15mM glucose location 1, d: $p<0.05$ relative to 5mM location 3.

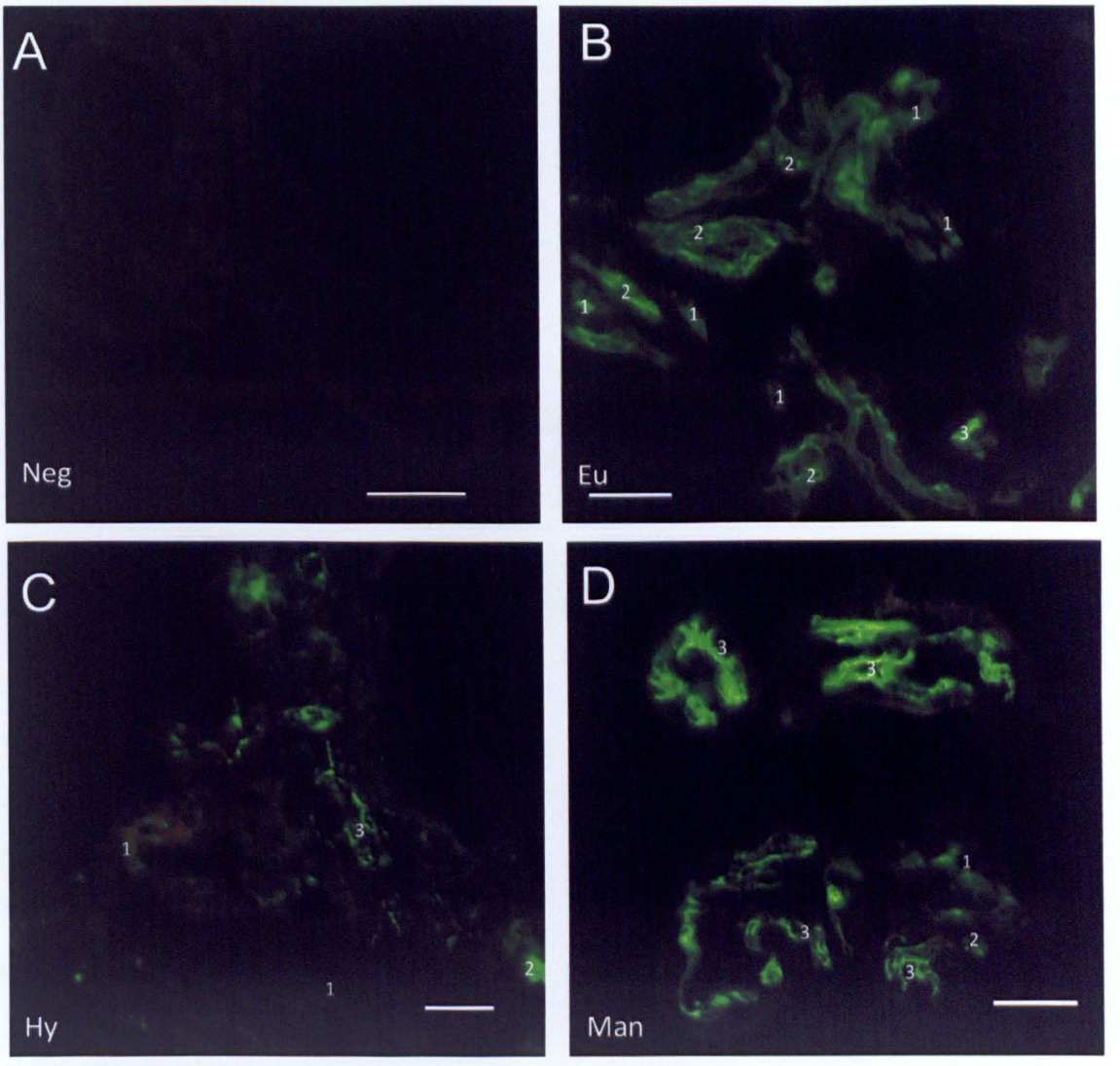


Figure 3.4: Representative micrographs of VE-cadherin immunoreactivity after 4h incubation. A: negative control showing no non-specific immunoreactivity. B: 5mM glucose explants showing more vessels with location 1 VE-cadherin immunoreactivity than both other groups (C and D), and fewer location 3 VE-cadherin immunoreactivity than the mannitol group (D). C: 15mM glucose explants showing fewer location 1 VE-cadherin immunoreactivity than the euglycaemic group (B) but more than the mannitol group (D). D: mannitol explants showing fewer vessels in location 1 immunoreactivity than both other groups (B and C), but more location 3 VE-cadherin immunoreactivity than the euglycaemic group (B). Neg: negative control, Eu: euglycaemia, Hy: hyperglycaemia, Man: mannitol, 1: VE-cadherin location 1, 2: VE-cadherin location 2, 3: VE-cadherin location 3 immunoreactivity. Magnification: x200. Scale bar: 50um.

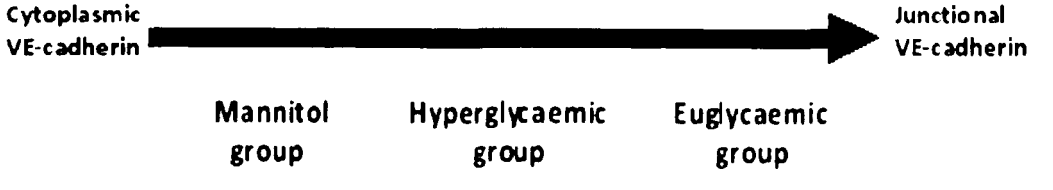
3.4.2.2 24h duration

After 24h culture in hyperglycaemia, there were significantly higher numbers of vessels with wholly cytoplasmic VE-cadherin immunolocalisation (location 1) compared to the 24h euglycaemic group ($p<0.05$) (Figure 3.5). Junctional expression of VE-cadherin was also downregulated in hyperglycaemia; there were significantly more exclusively junctional (location 3) VE-cadherin immunolocalisation in the euglycaemic (Figure 3.6B) group than in the hyperglycaemic (Figure 3.6C) group ($p<0.05$) (Figure 3.5).

Mannitol (Figure 3.6D) insult resulted in significantly more vessels showing exclusively cytoplasmic VE-cadherin immunolocalisation than the euglycaemic group ($p<0.01$); conversely, there were significantly more vessels with wholly junctional (location 3) VE-cadherin immunolocalisation in the euglycaemic group compared to the mannitol group ($p<0.01$).

Comparison of hyperglycaemia with mannitol revealed no significant difference in the number of vessels showing wholly non-junctional (location 1) immunolocalisation ($p>0.05$); however, there was a significant difference in the number of vessels showing wholly junctional (location 3) VE-cadherin immunolocalisation, with the hyperglycaemic group having more vessels than the mannitol group at this location ($p<0.05$) (Figures 3.5).

At the 24h timepoint, therefore, the pattern of junctional localisation is as in the following schematic:



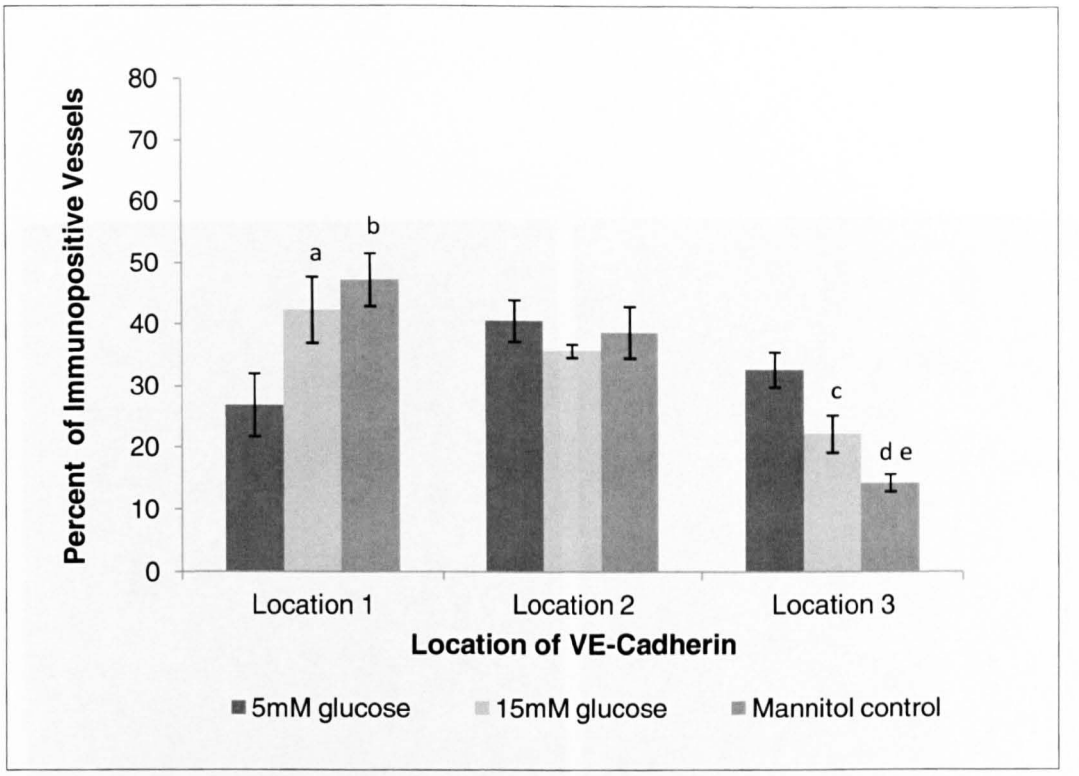


Figure 3.5: Effect of 24h 15mM glucose insult on VE-cadherin immunolocalisation. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=5), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups.
a: $p<0.05$ relative to 5mM glucose location 1, b: $p<0.01$ relative to 5mM glucose location 1, c: $p<0.05$ relative to 5mM glucose location 3, d: $p<0.01$ relative to 5mM glucose location 3, e: $p<0.05$ relative to 15mM glucose location 3.

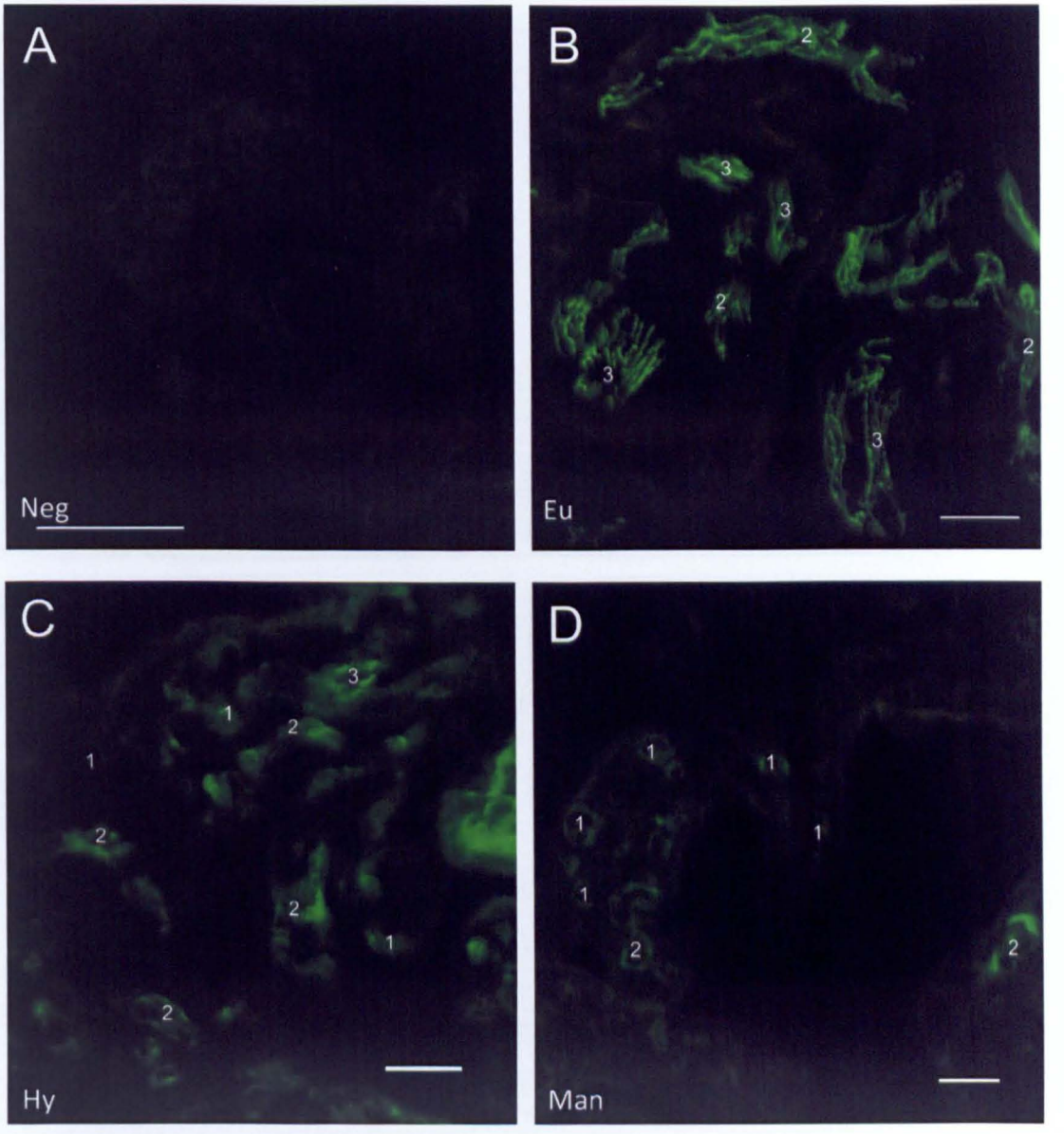


Figure 3.6: Representative micrographs of VE-cadherin immunoreactivity after 24h incubation. A: negative control showing no non-specific immunoreactivity, B: explants incubated in 5mM glucose showing location 2 and 3 VE-cadherin, C: explants incubated in 15mM glucose showing more wholly non-junctional vessels (location 1) and fewer wholly junctional (location 3) vessels than the euglycaemic group (B), but more location 3 VE-cadherin immunoreactivity than the mannitol group. D: explants incubated in mannitol, showing more wholly non-junctional (location 1) but fewer wholly junctional VE-cadherin immunoreactivity than both other groups (B and C). Neg: negative control, Eu: euglycaemia, Hy: hyperglycaemia, Man: mannitol, 1: location 1 immunoreactive vessels, 2: location 2 immunoreactive vessels, 3: location 3 immunoreactive vessels. Magnification: x200. Scale bar 100um.

3.4.3 Intensity of VEGF immunofluorescence

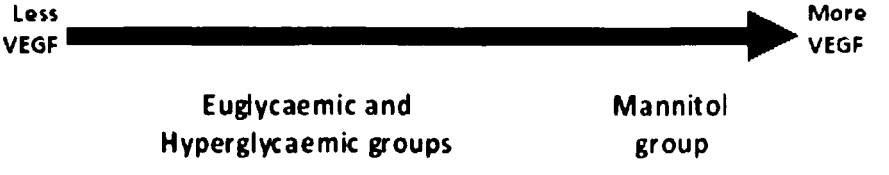
3.4.3.1 4h duration

At 4h, there was no significant difference between the VEGF immunoreactivity of euglycaemic and hyperglycaemic groups for any intensity level ($p>0.05$) (Figure 3.7).

There was a difference in VEGF immunoreactivity between the euglycaemic (Figure 3.8B) and mannitol (Figure 3.8D) groups, where there were fewer vessels grouped in intensity 1 ($p<0.05$), more vessels in intensity 2 ($p<0.05$) but no significant difference in intensity 3 ($p>0.05$), in the mannitol group compared to the euglycaemic group.

Comparing the mannitol group with the hyperglycaemic (Figure 3.8C) group also revealed differences, with the mannitol group showing significantly fewer vessels grouped in intensity 1 ($p<0.05$) and more vessels in intensity 2 ($p<0.05$) immunoreactivity, whilst no significant difference was seen between these two groups in intensity 3 ($p>0.05$) (Figure 3.7).

At the 4h timepoint, therefore, the pattern of junctional immunoreactivity is as in the following schematic:



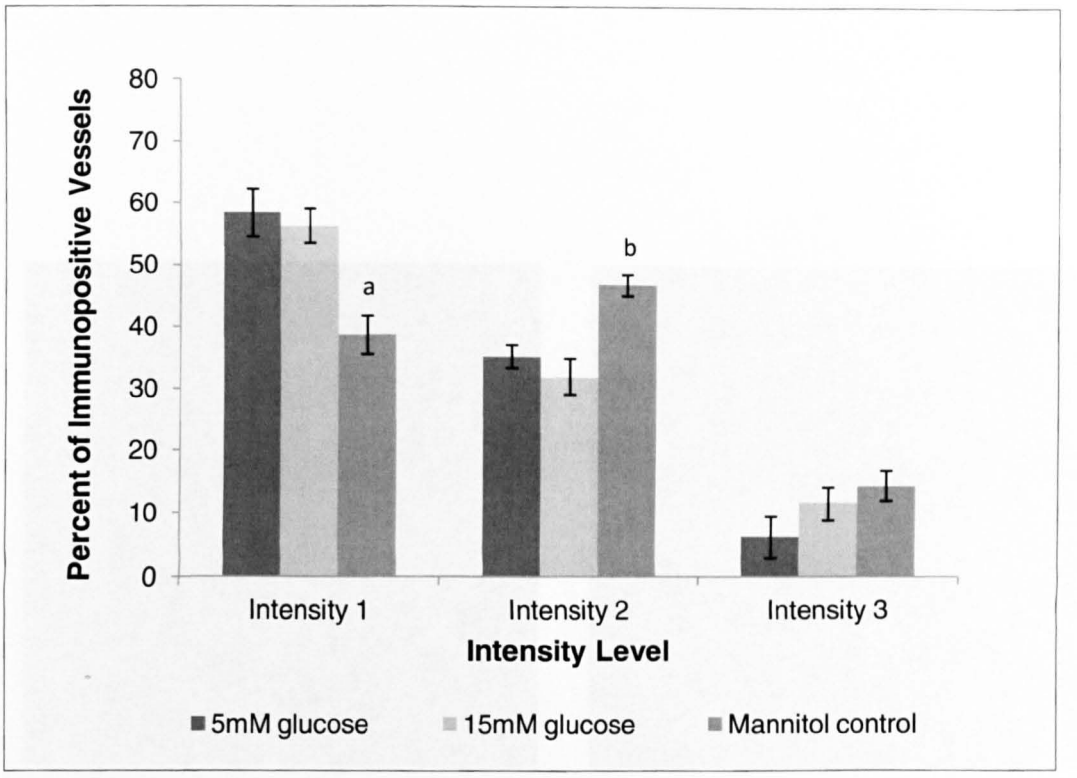


Figure 3.7: Effect of 4h 15mM glucose insult on VEGF intensity levels. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=5), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups. a: $p<0.05$ relative to 5mM glucose and 15mM glucose intensity 1, b: $p<0.05$ relative to 5mM glucose and 15mM glucose intensity 2.

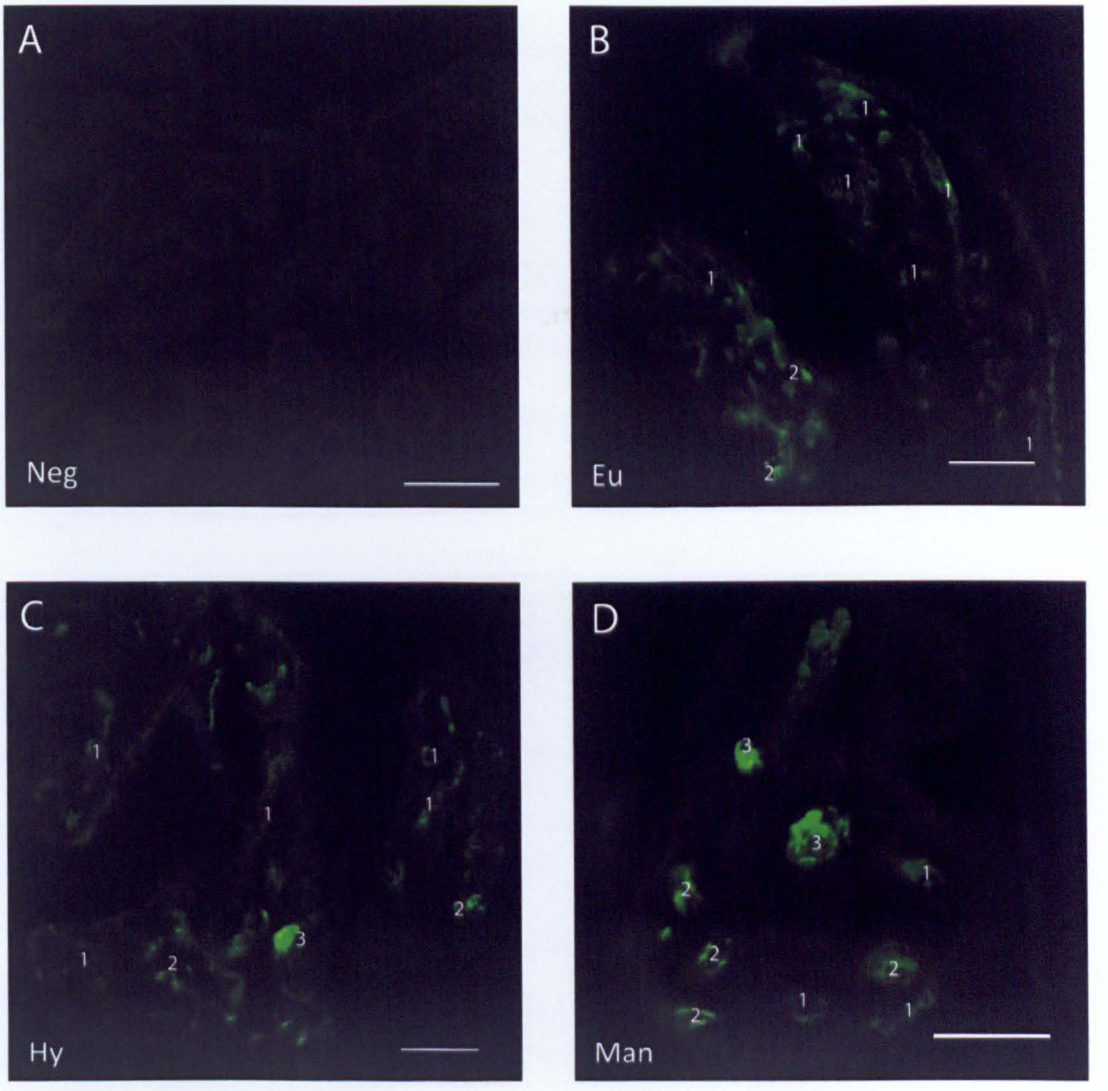


Figure 3.8: Representative micrographs of VEGF immunoreactivity following 4h incubation. A: negative control showing no non-specific immunoreactivity, B: explants incubated in 5mM glucose media showing most VEGF immunopositive vessels with intensity 1 immunoreactivity. C: explants incubated in 15mM glucose media showing more vessels with VEGF intensity 1 and fewer with VEGF intensity 2 than the mannitol group (D). D: explants incubated in mannitol media showing fewer vessels with intensity 1 and more vessels with intensity 2 than both euglycaemic (B) and hyperglycaemic (C) groups. Neg: negative control, Eu: euglycaemia, Hy: hyperglycaemia, Man: mannitol, 1: intensity 1 VEGF immunopositive vessels, 2: intensity 2 VEGF immunopositive vessels, 3: intensity 3 VEGF immunopositive vessels. Magnification: x200. Scale bar: 100um.

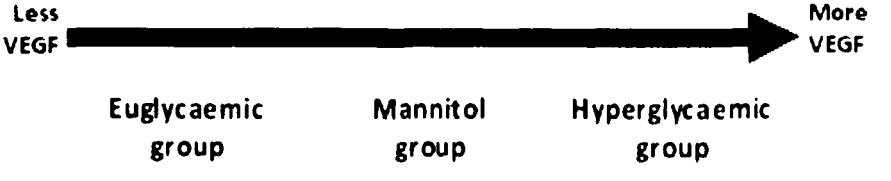
3.4.3.2 24h duration

At 24h, there was a significant difference between the euglycaemic and hyperglycaemic groups, with the hyperglycaemic group showing a significantly lower percentage of vessels with intensity 1 VEGF immunoreactivity ($p<0.05$) and significantly higher percentage of vessels with intensity 3 VEGF immunoreactivity ($p<0.01$) compared to the euglycaemic group, whilst there was no significant difference in intensity 2 between these two groups ($p>0.05$) (Figure 3.9).

There was a significant difference between the euglycaemic (Figure 3.10B) and mannitol (Figure 3.10D) groups, with significantly fewer vessels showing intensity 1 ($p<0.05$) VEGF immunoreactivity in the mannitol group, but no significant differences in the number of vessels showing intensity 2 and intensity 3 (both $p>0.05$) VEGF immunoreactivity.

The hyperglycaemic (Figure 3.10C) and mannitol groups showed a significant difference in the number of vessels showing intensity 3 VEGF immunoreactivity ($p<0.05$), but no significant difference in intensities 1 and 2 (both $p>0.05$) (Figure 3.9).

At the 24h timepoint, therefore, the pattern of VEGF immunoreactivity is as in the following schematic:



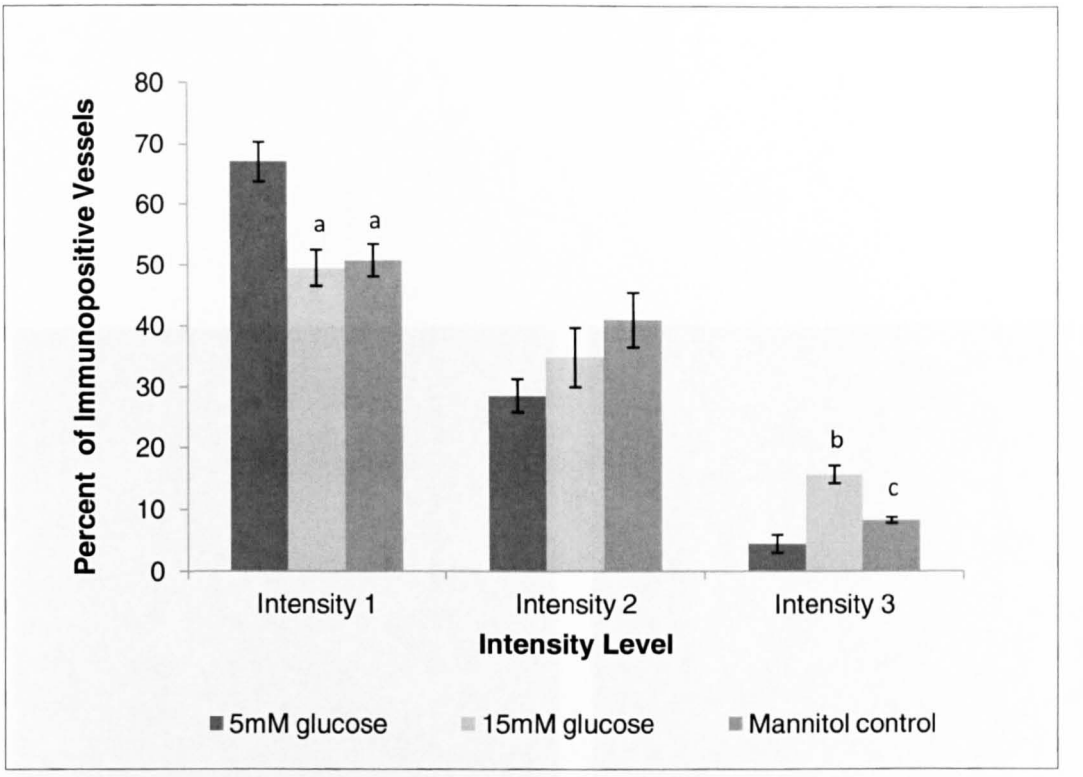


Figure 3.9: Effect of 24h 15mM glucose insult on VEGF intensity levels. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=5), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups. a: $p<0.05$ relative to 5mM glucose intensity 1, b: $p<0.01$ relative to 5mM glucose intensity 3, c: $p<0.05$ relative to 15mM glucose intensity 3.

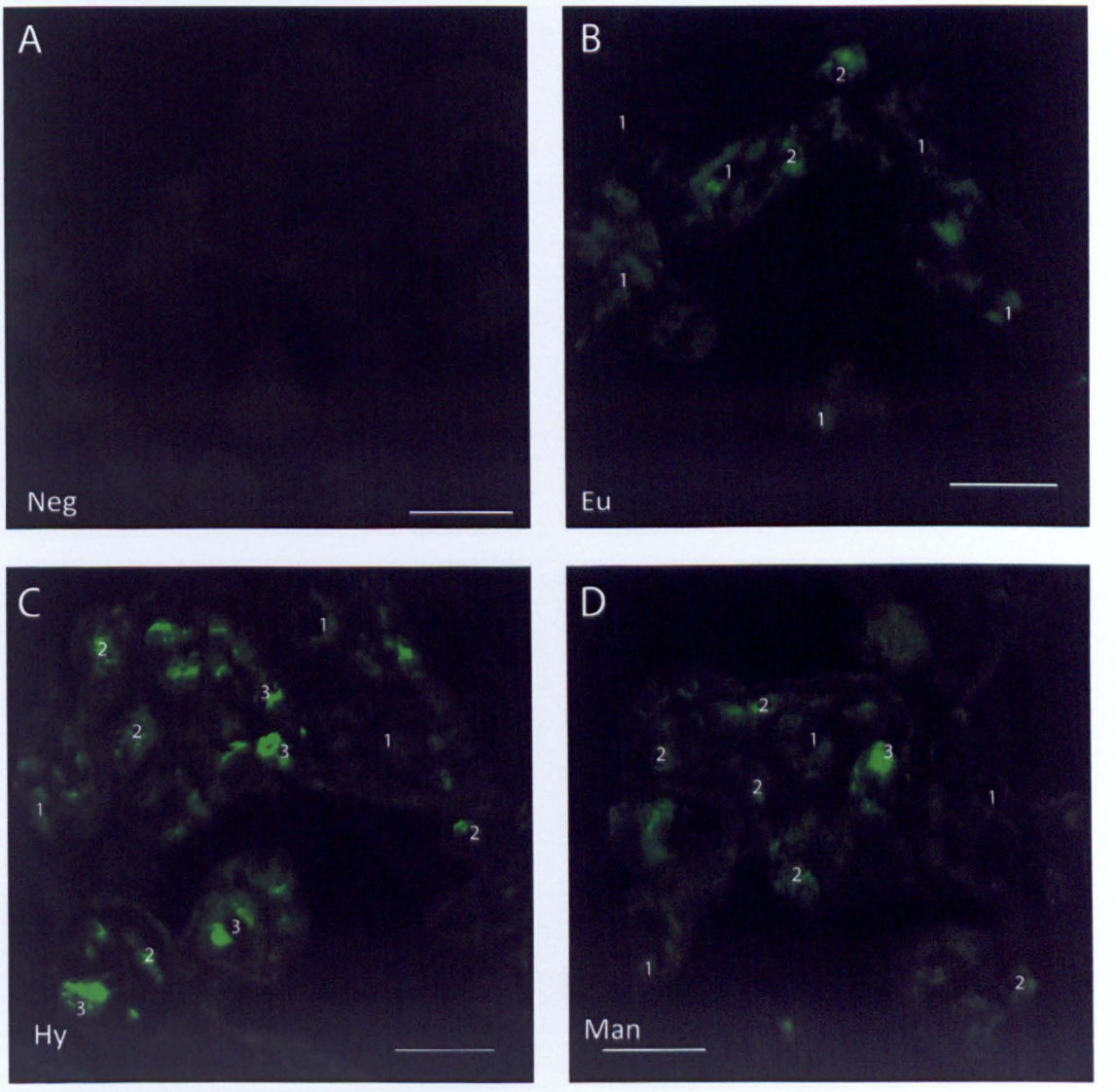


Figure 3.10: Representative micrographs of VEGF immunoreactivity following 24h incubation. A: negative control showing no non-specific immunoreactivity, B: explants incubated in 5mM glucose media showing mostly intensity 1 VEGF immunoreactivity. C: explants incubated in 15mM glucose media showing fewer VEGF intensity 1 vessels than the euglycaemic group (B) but more VEGF intensity 3 vessels than both euglycaemic (B) and mannitol (D) groups. D: explants incubated in mannitol media showing fewer VEGF intensity1 and more VEGF intensity 3 vessels than the euglycaemic group (B), and fewer VEGF intensity 3 vessels than the hyperglycaemic group (C). Neg: negative control, Eu: euglycaemia, Hy: hyperglycaemia, Man: mannitol, 1: intensity 1 VEGF immunoreactivity, 2: intensity 2 VEGF immunoreactivity, 3: intensity 3 VEGF immunoreactivity. Magnification: x200. Scale bar: 100um.

3.5 Discussion

3.5.1 Summary of results

There was no significant difference ($p>0.05$) in the total number of VE-cadherin (Figure 3.1) and VEGF (Figure 3.2) immunopositive vessels between 5 and 15mM glucose at 4h. However, the mannitol osmolarity 'control' showed significantly fewer vessels immunopositive for VE-cadherin ($p<0.01$ for mannitol vs. euglycaemia and $p<0.05$ for mannitol vs. hyperglycaemia, Figure 3.1), which indicates that mannitol was having a non-osmotic effect, possibly affecting the excision artefacts.

A metabolic effect of mannitol is also seen in the localisation analysis. The location data reveal that not only is mannitol affecting the number of vessels immunopositive for VE-cadherin, it is also changing the junctional localisation of VE-cadherin, as more vessels have the 'beads on a string' pattern of immunolocalisation characteristic of junctional VE-cadherin (location 3) at 4h than the euglycaemic control ($p<0.05$), but not the hyperglycaemic group ($p>0.05$). However, when looking at wholly non-junctional VE-cadherin (location 1) there was a significant difference ($p<0.05$ between euglycaemic vs. hyperglycaemic and hyperglycaemic vs. mannitol, and $p<0.01$ between euglycaemic vs. mannitol) between all groups, revealing the VE-cadherin junctional profile of the high glucose group to be intermediate between the euglycaemic group and the mannitol group (Figures 3.3-3.4). Mannitol was also having a non-osmotic effect on VEGF at 4h, as there were fewer intensity 1 vessels in the mannitol group compared to both euglycaemic ($p<0.05$) and hyperglycaemic ($p<0.05$) groups. There were no significant differences ($p>0.05$) in the intensity levels between euglycaemic and hyperglycaemic groups at 4h for VEGF (Figures 3.7-3.8).

At 24h, there was no significant difference ($p>0.05$) in total number of vessels immunoreactive to either VE-cadherin (Figure 3.1) or VEGF (Figure 3.2) between 5mM glucose and 15mM glucose, whilst the mannitol group had significantly fewer ($p<0.01$ and $p<0.05$ respectively) VE-

cadherin immunopositive vessels, indicative of a non-osmotic effect on wound healing. However, there were significantly fewer vessels ($p<0.05$) showing 'beads on a string' VE-cadherin immunolocalisation (location 3) in hyperglycaemic compared to euglycaemic conditions; the mannitol incubation had significantly fewer vessels ($p<0.01$ compared to euglycaemia and $p<0.05$ compared to hyperglycaemia) showing wholly junctional VE-cadherin than either of the other groups (Figures 3.5-3.6). VEGF intensity levels revealed that the hyperglycaemic group had a significantly higher number of vessels at the highest intensity level (intensity 3) than both other groups ($p<0.01$ vs. euglycaemia and $p<0.05$ vs. mannitol), indicating a higher expression of VEGF in hyperglycaemia (Figures 3.9-3.10).

Not taking into consideration the mannitol 'control', VE-cadherin location in the hyperglycaemic group was significantly different from the euglycaemic group at both 4h ($p<0.05$ for location 1, Figures 3.3 and 3.4B-C) and at 24h ($p<0.05$ for location 1 and $p<0.01$ for location 3, Figures 3.5 and 3.6B-C). The percentage of vessels showing intensity 1 and intensity 3 VEGF immunoreactivity was significantly different between euglycaemic and hyperglycaemic groups at 24h ($p<0.05$ for intensity 1 and $p<0.01$ for intensity 3, Figures 3.9 and 3.10B-C) but not at 4h ($p>0.05$, Figures 3.7 and 3.8B-C).

3.5.2 Effect of short duration glycaemic insult

These studies show that at 4h there was no significant difference in the number of vessels showing VEGF immunoreactivity, neither in the total number of vessels nor in the intensity of surface expression. One such short duration of hyperglycaemic insult appears not to be sufficient to cause disturbances in vascular VEGF expression. This observation agrees well with the induced VE-cadherin dynamics seen in these explant studies. Immediately after excision (as seen in Chapter 2), 7-8% of vessels showed exclusively junctional VE-cadherin (Figure 2.9). After 4h in solution, the percentages remained similar for euglycaemia (9%) but were higher

(19%) in the presence of 15mM glucose (Figure 3.3). The lack of VEGF response could go somewhat to explain this phenomena; increases here would lead to a reduction in junctional occupancy of VE-cadherin (Underwood *et al.*, 2002; Esser *et al.*, 1998). The data suggests 15mM glucose may even be playing a protective role in the early post-wound stages; however, the validity of this hypothesis and the factors involved remain to be investigated.

In diabetic patients, the usual timecourse of postprandial increases in glucose levels lasts for 3-4h, which then reverts down to more acceptable levels until the next meal, when the blood glucose increases again (see Chapter 4, Figure 4.3). The only case where this would not be true would be if there were undiagnosed diabetes without treatment. Therefore any study that extends for a longer time period (over 24h) would be relevant to undiagnosed diabetics, who might be subject to prolonged periods of hyperglycaemia, but not pregnant women, who are tested for gestational diabetes in their pregnancy if at risk. Pregnant women are thought to be more diligent in their insulin regime than their non-pregnant counterparts (Kyne-Grzebalski *et al.*, 1999), but as their glucose homeostasis changes throughout the pregnancy, there are times where their blood glucose may rise above acceptable limits, until they realise this fact and they change their insulin regime.

The 4h timepoint is very important physiologically, and therefore we have continued to use it in this thesis, to test other hypotheses.

3.5.3 24h duration

There is clear evidence in the literature, using bovine and human endothelial cell culture methodologies, showing that a 24h exposure to high glucose should have an effect on VEGF expression (Nakagawa *et al.*, 2006; Chen *et al.*, 2004), whilst 24h or longer incubation in high glucose has been shown by some studies not to have any effect on VE-cadherin (Matsui-Hirai *et al.*, 2011; Maillard and Leach, 2002; Singh *et al.*, 2011). The main drawback to these studies is

that they use cell culture methodologies, and therefore the physiological reproduction of the effects of the surrounding tissue is not analysed. Our studies in chorionic villous explants maintain the physiologic 3D architecture of the villi in our setup, therefore adding an essential layer of complexity to our experiment. Research with chorionic villous explants similar to ours has shown that the trophoblast surrounding the villi degenerates in the first few days in culture (Siman *et al.*, 2001). Whilst this does not directly affect the vessels we are analysing, the degenerating trophoblast may be releasing substances into the media that may affect the VEGF production in the endothelium of the vessels, as they are subjected to the same media as the trophoblast. This might explain the fact that in our explant model, we observe a decrease in VE-cadherin localisation to the junctional space in hyperglycaemic insult compared to euglycaemic incubation, in contradiction to the cell culture studies mentioned above. However, different VEGF splice variants might be affected, which would not be shown with the antibody used in this chapter. This is investigated in Chapter 4.

3.5.4 Effects of mannitol on the explants

In this chapter, we have tested the efficacy of mannitol as an osmotic control for glucose, as it used in cell culture experiments. The significant differences found between the mannitol group and both normal and glucose groups suggest otherwise. Our data, although on the surface unexpected, confirm recent change in consensus of opinion regarding the role of mannitol.

Mannitol has been used for years in medicine as a hyperosmotic agent in humans, by perfusing the vasculature with high (1.4-1.6M) concentrations to open the blood-brain barrier and relieve intra-cranial pressure (ICR) (Hartwell and Sutton, 1993). However, a recent review of four papers published in the last ~10 years, has shown that mannitol infusion is actually less effective at relieving ICR than hypertonic saline (Infanti, 2008). The author of this review does not provide hypotheses for why this might be, only the evidence. A possibility could be that

mannitol acts as a reactive oxygen species (ROS) scavenger, and therefore aids the integrity of the endothelial junctions in the blood-brain barrier, and therefore cannot affect ICR.

A study by Huo *et al.* (2009) also suggests an effect on the endothelium by mannitol. This study, which used a much lower mannitol concentration (100mM), was shown *in vitro* to block the effects of ROS in a rabbit corneal epithelial cell model. This study was also investigating wound healing in a pig cornea organ culture model, and indeed mannitol was shown, putatively through its ROS scavenger activity, to inhibit wound healing through a decrease in DNA synthesis (Huo *et al.*, 2009), and therefore, our mannitol group might be affecting the wound healing, explaining why the mannitol group is not acting as an osmolarity control in our results.

We see in our explant culture model that exposure to mannitol resulted in a significant decrease in endothelial VE-cadherin immunoreactivity in comparison with normal and glucose groups at both 4h and 24h timepoints. Mannitol may in this case be inhibiting wound healing, as seen in the rabbit corneal model described above. This overall decrease in VE-cadherin would have an inhibitory effect on wound healing given the role VE-cadherin plays in endothelial barrier function and as a survival factor (Carmeliet *et al.*, 1999; Lampugnani *et al.*, 1995), thus disruption of this endothelial barrier plays a role in early angiogenic signalling. Down-regulation of the molecule would have profound effects on the endothelium, thus mannitol even at 10mM levels may be injurious to human placental microvessels.

As mannitol is putatively affecting the wound healing response, and not acting as an osmotic control as wished, it proved unsuitable for this study.

3.5.5 Excision artefacts

As discussed in Chapter 2, there might be a 'wound healing' artefact caused by the excision process in our explant model. This can be seen in the observed total number of vessels expressing VE-cadherin and VEGF, being 60% and 45%, respectively, at 0h (Figures 2.7 and 2.8).

This is different from what is seen *in vivo* or indeed in non-wounded tissue. A study by Leach *et al.* (2004) shows that in a non-wounding methodology (placental perfusion), 92.9% of non-diabetic vessels show VE-cadherin immunoreactivity, whilst only 5% of non-diabetic vessels show VEGF immunoreactivity.

In our explants about 60% of vessels did not show any VEGF immunoreactivity. This is compared to 95% in the intact tissue of Leach et al (2004), and therefore it matches well with a profile affected by the excision process. This theory proposes that VEGF is produced when explants are cut, and the absence of VEGF in some vessels in euglycaemic explants may indicate that these were vessels which had not been cut in the excision process, whilst the VEGF immunopositive vessels had been affected by excision artefacts.

No further decrease can be seen in the total number of vessels immunopositive to VEGF between the euglycaemic and hyperglycaemic groups, and VE-cadherin localisation indicates that the mannitol group (which as seen above is likely being affected by the wound healing process) shows a different profile than the hyperglycaemic group. Therefore, it seems unlikely that the 15mM glucose group is being affected by the wound healing response significantly more than the euglycaemic group.

3.5.6 Critique of analytical approaches used

The data has been analysed in two ways in both this chapter and Chapter 2: (1) the percentage of vessels immunopositive for VE-cadherin and VEGF, and (2) the location/intensity of staining in immunopositive vessels. The intensity of fluorescence is useful as a semi-quantitative measure of how much VEGF is present. The sub-cellular location of VE-cadherin is important for understanding the contribution to cell-cell junctional adhesiveness. We believe this method to be of use for the VE-cadherin immunoreactivity, as location 1 (wholly cytoplasmic and non-junctional) VE-cadherin is contributing as little to cell-cell adherence as a cell which is not

expressing any VE-cadherin, as neither have the junctional VE-cadherin necessary for cell-cell adhesion and regulation of permeability. However, there is less of an argument for the use of intensity data for VEGF, as there is no biological basis for the classification of the three intensity levels. Furthermore, wound healing may confound the intensity data for VEGF, as in a non-wounded vessel only very little basal VEGF would be expressed; therefore, the fact that some 45% of vessels are expressing VEGF may be due to the wound healing. If glucose is having an effect on wound healing, the intensity of immunoreactivity would change, but it would not affect the non-wounded vessels; if, however, glucose was having an effect independent of wound healing, the total number of vessels immunoreactive to VEGF would change, as non-injured vessels would express VEGF. We see in this chapter that glucose is not having an effect on 'wound healing' caused by the excision artefacts, as demonstrated by the lack of change in VEGF intensity (Figures 3.7 and 3.9). Nor is glucose having an effect independent of 'wound healing', as shown by the unchanged total number of VEGF immunopositive vessels (see Figure 3.2).

From this point onwards (Chapters 4 and 5), only the total number of vessels immunopositive to VEGF are counted, regardless of their intensity.

3.5.7 4h vs. 24h

All groups (euglycaemic, hyperglycaemic and mannitol incubations) showed no significant difference between 4h and 24h when analysing the total number of vessels immunopositive with VEGF. Any chronic effects of glucose have therefore not yet set in. Glucose is known to have effects on the vasculature through increased advanced glycation end-products (AGEs), but these are not formed until after weeks of sustained hyperglycaemia, certainly not by 24h (Dawnay and Millar, 1997). For technical reasons, our explant culture cannot be continued for

the long-term duration it would take to develop AGEs (maximum culture duration 12 days (Miller *et al.*, 2005)), and therefore these chronic effects of glucose cannot be studied.

As the 4h and 24h glucose timepoints are similar to each other, one can conclude that any undergoing mechanism at 4h is still in action at 24h. However, as discussed above, it is the 4h timepoint that is physiologically relevant, as glycaemic levels are unlikely to be elevated constantly for 24h, whilst 4h periods of 15mM glucose are likely to be present in diabetic pregnancies (see introduction of Chapter 4).

3.5.8 Other glycaemic studies in placental explants

Other studies involving glucose in placental explants have studied mainly glucose deprivation and glucose consumption.

Interestingly, glucose deprivation seems to have more profound effects than hyperglycaemic insult. Studies involving placental growth hormone production, a hormone that has a positive correlation with birth weight (which is often increased in diabetic pregnancies) and is produced exclusively by the syncytiotrophoblast, have found that glucose deprivation increased production of the hormone in 24h explants culture, whilst 25mM glucose had no effect (Fuglsang *et al.*, 2008; Patel *et al.*, 1995). Another study has found TNF-α to be decreased in response to 25mM, but not 15mM glucose in placental explants (Coughlan *et al.*, 2001). This result is important for our explant culture, as TNF-α is involved in inflammation and is produced in endothelial cells, and could thus be affecting the wound healing response we observe in our explants. Also, this highlights the importance of choosing the right glucose concentration to test, as different concentrations may have different results. We chose 15mM glucose, as this represents a mild glycaemic insult which is likely to occur even in well controlled women in pregnancy.

Importantly, glucose consumption in term explants was studied (Di Santo *et al.*, 2003). The glucose consumption in term placental explants was 0.135μmol/g/min for a 4h incubation and 0.102μmol/g/min for a 24h incubation. This means our 11mg explants consumed 0.12mM glucose by 4h, and 0.54mM glucose by 24h. This would make a negligible difference from the starting concentration of 5mM and 15mM glucose.

3.5.9 VEGFb in explants

The antibody we used in this chapter was a pan-VEGF antibody, which does not distinguish between the two splice variants, VEGFa and VEGFb (for naming convention, see section 4.1.2). Therefore, even though we see no significant difference in the total number of vessels showing VEGF expression between the euglycaemic and hyperglycaemic groups, one cannot discount the possibility that the ratio between the two splice variants is changing in response to glucose. This topic is explored in more detail in sections 4.5.3 and 4.5.8.

VEGFb is known to inhibit angiogenic processes such as vasodilation, endothelial proliferation and migration (Woolard *et al.*, 2004). VEGFb has been shown to be downregulated in both physiologic and pathologic models of angiogenesis, such as various cancers and diabetic retinopathy (Bates *et al.*, 2002; Pritchard-Jones *et al.*, 2007; Perrin *et al.*, 2005). As such, we would expect VEGFb to be decreased in our explant culture, as wound healing is undergoing. VEGFb was therefore included as a profile to measure in the next two chapters.

Most studies have observed an effect of glucose on VEGF and VE-cadherin expression at 24h and beyond, but to the best of our knowledge there are not any studies that evaluate the effects of glucose itself (as opposed to glycation derivatives such as AGEs, as in the study by Otero *et al.* (2001)), at an earlier timepoint, such as our 4h timepoint.

3.6 Summary

VE-cadherin was shown to be affected by glucose, both at 4h and 24h, affecting endothelial cell-cell adherence, and this merits further investigation. Whether this results in increased leakage in the placental organ is not known, and it is not possible to investigate this using the placental explants methodology. Any differences in vascular leakage were therefore investigated using a dual-independent perfusion method in Chapter 5.

At both 4h and 24h, glucose was not found to affect VEGF production. However, the antibody we used was a pan-VEGF antibody, and as such did not differentiate between the two splice variants, VEGFa and VEGFb. This question is investigated in Chapter 4.

We used mannitol as an osmolarity control; however, results showed that it had effects independent of osmolarity, making it unusable as a control, and therefore from this point forward, its use was discontinued.

Analysing different locations gave more precise results for VE-cadherin, as location 1 represented wholly cytoplasmic VE-cadherin whilst location 3 represented wholly junctional VE-cadherin, a difference that could not be discerned by simply counting total number of vessels expressing VE-cadherin. However, counting intensity levels in VEGF immunoreactivity does not have the same benefits, as it is a pseudo-quantitative analysis, whilst total number of vessels immunopositive provides a more objective observation. Therefore, in the next chapters only total number of vessels immunopositive to VEGF are counted, disregarding intensity levels.

Chapter 4

The effect of 15mM glucose on the explant culture: analysis of VEGF splice variants in placentae from normal and diabetic pregnancies

4.1 Introduction

4.1.1 The diabetic placenta

As diabetes is a collective term for several types of diabetes with different aetiologies, it is unsurprising that different types (Type 1, Type 2, gestational) of diabetes affect pregnancies differently. We chose to study the placentae of Type1 diabetic pregnancies only. Here we discuss how the placental phenotype of Type 1 made it suitable for this study, and why placentae from gestational and Type 2 diabetic pregnancies were not used.

4.1.1.1 Pre-gestational and Type 1 placenta and pregnancy

There are two types of pre-gestational diabetes: Type 1 and Type 2. Here, we study the tissue response of Type 1 placentae to glucose.

The first reason behind this choice is the well-studied phenotype of the Type 1 placenta, specifically in our laboratory, where it has been studied for many years. Because of this in-depth knowledge of the Type 1 placenta, we can study the effects of glucose on VEGF isoforms. Type 1 diabetes displays a pathological increase in endothelial VEGF production, and junctional adhesion molecules are decreased, so that the overall phenotype is one that is leaky to macromolecules slightly larger than albumin (67kDa). Therefore, there is an established phenotype to compare both the euglycaemic and hyperglycaemic normal placental explants, and to observe how the diabetic tissue exposed to high glucose compares to diabetic tissue exposed to euglycaemia, i.e. whether the diabetic phenotype of high VEGF is reversible under euglycaemia. No such comparison can be made for Type 2 diabetes, as its pathological phenotype has not been as widely studied.

Secondly, Type 1 diabetes is controlled primarily with insulin, and insulin resistance increases during pregnancy. In order to maintain euglycaemia, the insulin requirement for non-obese,

singleton pregnancies increases from 0.7units/kg per day in the first trimester, to 0.8units/kg/day in gestational week 18, to 0.9units/kg/day in gestational week 26, and finally to 1.0units/kg/day in gestational week 36 (Jovanovic *et al.*, 1981). The authors also noted that towards the end of the pregnancy, the standard deviation of insulin required increased, so more individual monitoring is required at the end of the pregnancy to maintain euglycaemia. Obese subjects required very variable amounts of insulin, and therefore the authors did not include their values in their figures (see Figure 4.1). Herein lies another problem with Type 2 diabetes: it is a much less homogeneous disease, with some patients requiring insulin and some being treated by diet alone, and obesity complicates a great percentage of Type 2 diabetics. As a result, Type 2 diabetic patients have greater exposure to much higher glucose concentrations, with 30mM+ levels being reported. Therefore, the variation between subjects makes it difficult to make wide-reaching conclusions.

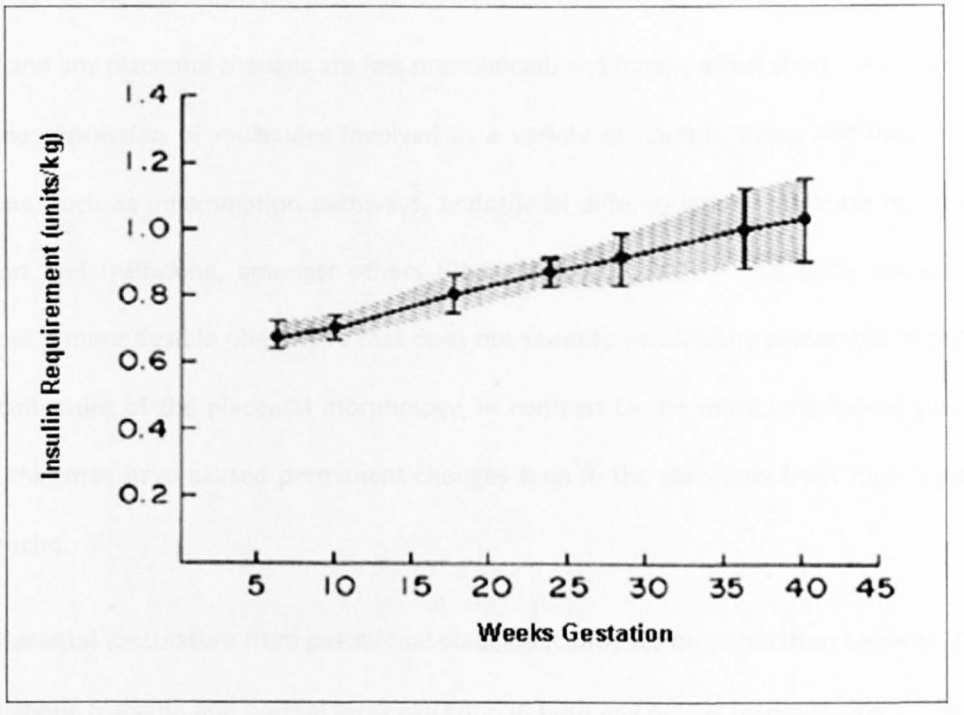


Figure 4.1: Insulin requirements of Type 1 diabetic women during pregnancy, with standard deviation (shaded area). The data points were taken at gestational weeks 7, 14, 21, 28, 32, 39, 41. Taken from Jovanovic *et al.* (1981) with permission from Elsevier.

Placentae from Type 1 diabetic women without any history of microvascular complications were therefore chosen for the reliability of results and less variation as compared to placentae from Type 2 diabetic women.

4.1.1.2 Gestational diabetes (GDM) placenta

Type 1 and Type 2 are pre-gestational diabetes, and therefore the placenta in these diabetic women is subjected to hyperglycaemia throughout the entirety of the pregnancy. Diabetes present at the start of gestation may alter formation of the placenta for the rest of the pregnancy; as the placenta may adopt different mechanisms to counteract the diabetic environment, these alterations may be long-lasting. In contrast, in gestational diabetes (GDM), which presents in the second or third trimester of the pregnancy (after the formation and maturation of the placenta), the placenta is subjected to a shorter duration of the pathological milieu, and any placental changes are less pronounced, and mainly affect short-term changes in the gene expression of molecules involved in a variety of chronic stress and inflammatory functions, such as inflammation pathways, endothelial differentiation, substrate metabolism, transport and trafficking, amongst others (Radaelli *et al.*, 2003). The GDM phenotype is therefore a more flexible phenotype that does not seem to involve any permanent changes to the architecture of the placental morphology, in contrast to the more long-lasting glycaemic insults that may have caused permanent changes seen in the placentae from Type 1 diabetic pregnancies.

Term placental vasculature from gestational diabetes (GDM) lies on a spectrum between Type 1 term diabetic placenta and normal term placenta, in both angiogenic response and evidence of junctional perturbation (Babawale *et al.*, 2000).

Placentae from GDM and Type 2 diabetic pregnancies were therefore not used for these studies.

4.1.1.3 Effects on the fetus of alterations in term diabetic placentae

As the main barrier between the maternal and fetal circulations, the placenta is a crucial discriminatory interface for nutrient uptake for the fetus. Both the syncytiotrophoblast and the fetal endothelium act as resistance in series in regulating materno-fetal transport (Leach and Firth, 1992). The leaky endothelium in diabetic placentae may therefore represent a reduced barrier leading to the "overnutrition" seen in diabetic pregnancies. Glucose readily crosses the placenta, both with simple diffusion and utilising glucose transporters 1 and 3, and, to a lesser extent, glucose transporter 4. GLUT1 was shown to significantly increase in diabetic placentae, but only in the basal membrane and not in the microvillous membrane (Gaither *et al.*, 1999), whilst expression of GLUT3 was shown to be significantly lower in diabetic pregnancies, perhaps as an adaptation to the diabetic milieu (Sciullo *et al.*, 1997). Once the syncytiotrophoblast has been traversed, as a hydrophilic molecule glucose can be transported through the paracellular clefts of the placental fetal endothelium. Disruptions of junctions here would influence rate of transfer. To counteract the resultant fetal hyperglycaemia, there is an increased insulin production in the fetus (Pedersen, 1954). According to the Pedersen hypothesis this may be behind the increased macrosomia seen in infants born to Type 1 diabetic mothers. Clinically, there are immediate neonatal consequences, whereby after parturition, newborns may experience hypoglycaemia and need to be given glucose.

4.1.2 VEGFb in pregnancy

Henceforth, we will be referring to the pro-angiogenic and pro-permeability VEGF splice variant as VEGFa, the anti-angiogenic and anti-permeability splice variant as VEGFb, and total VEGF as being the sum of VEGFa and VEGFb. Total VEGF in previous chapters was referred to simply as VEGF. In the literature, what we are calling VEGFa is sometimes referred to as $VEGF_{xxx}$, and what we are calling VEGFb is sometimes referred to as $VEGF_{xxx}b$.

VEGFb has been principally studied in the kidney (Qiu *et al.*, 2010) and the eye (Hua *et al.*, 2010) where overexpression studies have shown it to be both an anti-angiogenic and an anti-permeability isoform.

In the only study (to the best of our knowledge) looking at VEGFb in the human placenta, both VEGFb mRNA and protein were found (Bates *et al.*, 2006). These authors found about 22% of placental samples (including amnion, chorionic villi and stratum basale) containing VEGFb only, about 37% of samples to contain both VEGFa and VEGFb, about 17% of samples to contain VEGFa only, and about 22% to contain neither (exact figures not given; figures estimated by observing figure 1D) (Bates *et al.*, 2006). In particular, the authors found strong VEGFb staining in endothelial cells of blood vessels and syncytiotrophoblast in the chorionic villi but less staining in other tissues in the chorionic villi (Bates *et al.*, 2006). Western blotting showed that the majority of VEGF splice variants found in these samples was VEGFa. In pre-eclamptic placentae, the authors found significant up-regulation of VEGFa and significant down-regulation of the VEGFb splice variants, compared to normal placentae (Bates *et al.*, 2006).

Whether this splicing pattern also occurs in the diabetic placenta is not known. Glucose, one of the two likely candidates (the other being insulin) for causing any such disturbances in the ratio of VEGFa to VEGFb also remains to be shown and is the focus of this chapter.

4.1.3 Postprandial glucose levels in normal and diabetic pregnancies

Both in normal pregnancies and pregnancies of diabetic women, glucose homeostasis changes throughout gestation, the woman becoming progressively more insulin resistant as gestation progresses. A normal woman can adjust by producing more insulin, however, a Type 1 diabetic pregnant woman is reliant on external insulin injections, and therefore an increase in insulin requirement needs to be detected by the patient before glucose levels can be stabilised.

A study with 66 non-diabetic women found that, in the third trimester (34 weeks), a normal woman's preprandial glucose levels are around 3.5mM glucose, rising to 5.5mM postprandially. There is not much variation in these values, and the postprandial peak takes about 3 hours to return to preprandial values (Parretti *et al.*, 2001). The results of this study are summarised in Figure 4.2, which is adapted from data in the paper by Parretti *et al.* (2001).

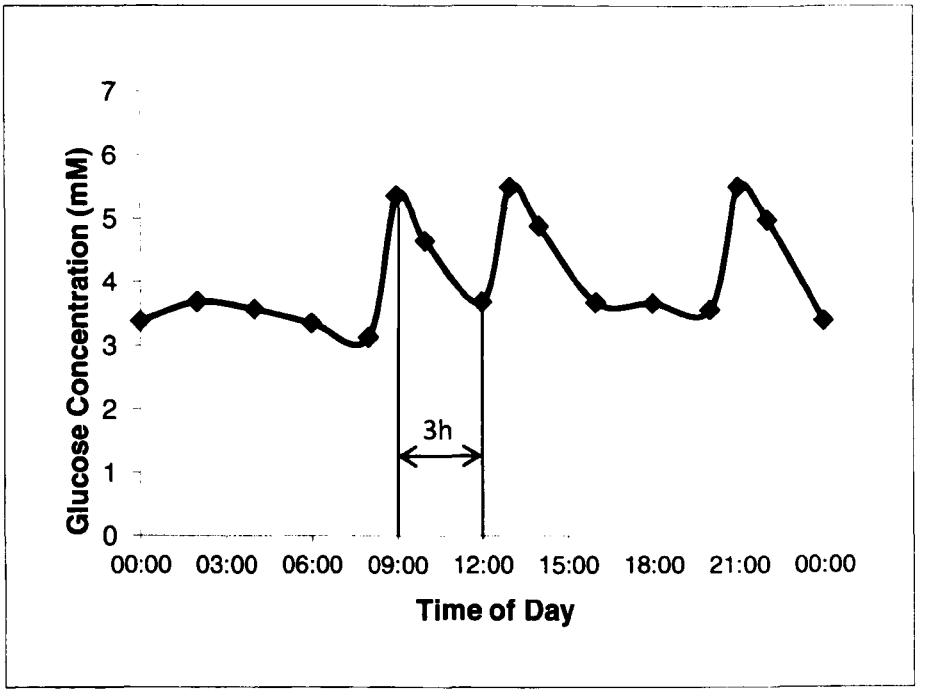


Figure 4.2: Typical daily glucose profile for a non-diabetic pregnant woman. Time for peak postprandial glucose concentration to return to fasted glucose levels is shown. Plotted from data from Parretti *et al.* (2001).

In a study including 111 pre-gestational diabetic (White class B-RF) pregnancies, at 33-36 weeks gestation the mean postprandial glucose concentration was found to be 7.4mM glucose (Combs *et al.*, 1992). However, all these women were extremely closely monitored and given individual diet plans. Even with this tight monitoring, 3.2±3.8% of readings were hyperglycaemic (>11.2mM glucose). This might seem low, but when considering there were at least 4 readings a day, in this four week gestational period there were on average 3-4 periods of hyperglycaemia per woman. The variation in these hyperglycaemic periods was also very high so some women might have had many more hyperglycaemic periods.

Other papers also comment on the high variability between Type 1 women in their hyperglycaemic excursions. Kerssen *et al.* (2004) show that there is a high degree of variability in two consecutive days of continuous glucose monitoring, with values as high as 14mM (Figure 4.3). In this example of a pregnant Type 1 diabetic patient, we can also see that the time it takes for the postprandial peak to return to preprandial levels is around 4h.

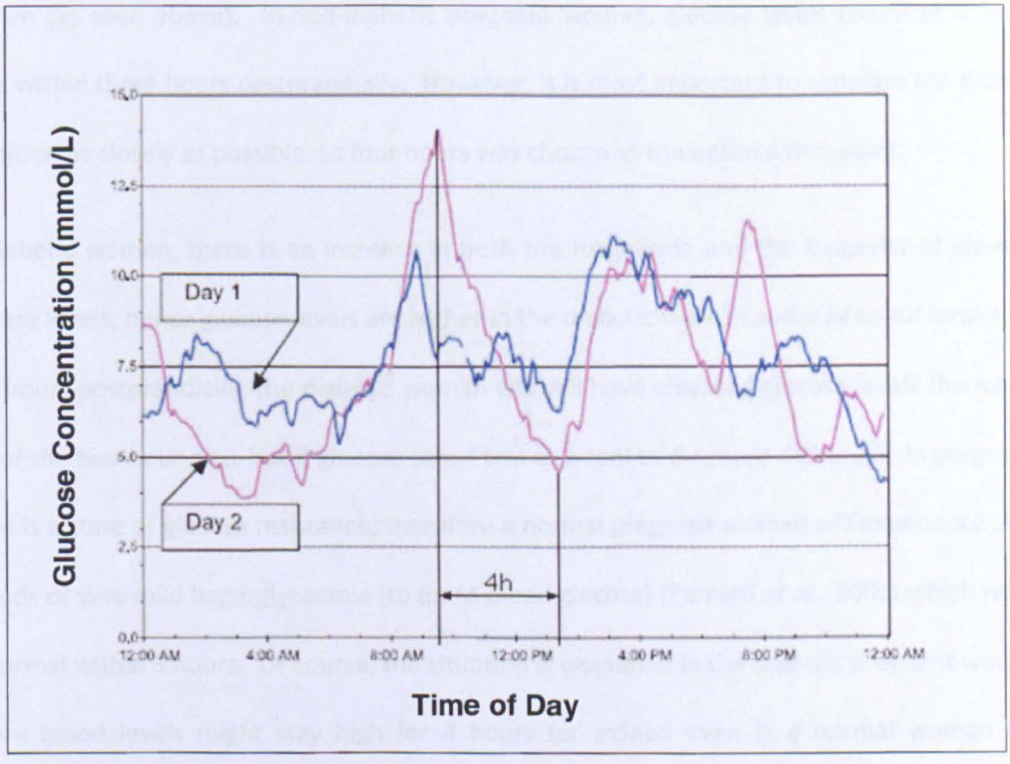


Figure 4.3: Two day continuous monitoring profile for a Type 1 diabetic pregnant woman. The daily variation and time for peak postprandial glucose concentration to return to fasted glucose levels are shown. Taken from Kerssen *et al.* (2004) with permission from John Wiley and Sons.

In their case report, Kyne-Grzebalski *et al.* (1999) also comment that postprandial levels in a Type 1 diabetic ranged from 1.5-16.9mM glucose for a woman who was thought to have good glucose control, due to a HbA_{1c} level of 41mmol/mol.

From these studies, one can conclude that even though mean postprandial glucose concentrations do not seem very high, in reality the variation of these values is great, and can easily reach 15mM glucose.

4.1.4 Our normal and diabetic explant cultures

As seen above, 15mM glucose concentration is often reached postprandially in Type 1 diabetic pregnancies, and therefore it is an appropriate concentration to use for our experiments to simulate hyperglycaemic insults as they happen *in vivo*. A timepoint of 4 hours was chosen for our experiments, as it correlates well with the postprandial glucose profile in diabetic pregnant women (as seen above). In non-diabetic pregnant women, glucose levels return to a fasted state within three hours postprandially. However, it is most important to simulate the diabetic condition as closely as possible, so four hours was chosen as the optimal timepoint.

In diabetic women, there is an increase in both the magnitude and the longevity of elevated glucose levels; hence glucose levels are higher in the diabetic woman and stay so for longer. By two hours postprandially, the diabetic woman will still have elevated glucose levels (hence the use of the two-hour post-insult glucose blood test as a tool to diagnose diabetes). In pregnancy, there is a state of glucose resistance; therefore a normal pregnant woman will experience short periods of very mild hyperglycaemia (to 6mM blood glucose) (Parretti *et al.*, 2001) which return to normal within 3 hours. Of course, the situation is worsened in the diabetic pregnant woman, where blood levels might stay high for 4 hours (or indeed even in a normal woman who develops gestational diabetes). The 4 hours timepoint is therefore very appropriate for our experiments.

4.2 Aims and objectives

The first aim was to observe whether glucose alters the VEGF splice variants in normal and Type 1 diabetic term placentae, using chorionic villous explants as our model. The experimental design again utilises the 4 hr incubation with 15mM glucose given that this correlates well with the postprandial glucose profile in diabetic pregnant women (see section 4.1.3). This will reveal

whether there is a diabetic phenotype described by the expression of VEGF splice variants that can be replicated with glucose insult.

The second aim was to observe whether there was a reversal in the Type 1 diabetic phenotype of high VEGF levels, as observed in perfused placentae from diabetic pregnancies by Leach *et al.* (2004) when such placentae are exposed to euglycaemic conditions. This was studied by comparing Type 1 diabetic chorionic villous explants incubated for 4h in euglycaemia to Type 1 diabetic chorionic villous explants incubated for 4h with hyperglycaemia (15mM glucose).

4.3 Methods

4.3.1 Materials

The source and catalogue number for all materials mentioned in this chapter are listed in Appendix 1.

4.3.2 Explant culture for 4h

Normal chorionic villous explants (n=4, 3 explants per placenta, with the same donor demographic requirements as those outlined in section 2.3.2) and Type 1 diabetic chorionic villous explants (n=4 placentae, 3 explants per placenta, see Appendix 2 for donor information) were incubated in oxygenated M199 medium which contained 5% (v/v) Fetal Calf Serum, 100U/ml penicillin, 100mg/ml streptomycin, 2µg/ml Fungizome, on net supports (pore size 700µm) in a twelve-well plate. The support ensured that the explants did not adhere to the bottom of the well. After 4h of incubation, in hyperglycaemic media (15mM D-glucose) or euglycaemic media (5mM D-glucose), the explants were removed and fixed in 1% (w/v) PFA (pH 7.2) for 30 minutes, before being washed in PBS, inserted in a drop of OCT and snap frozen with liquid nitrogen cooled isopentane.

4.3.3 Immunofluorescence protocol

Both diabetic and normal explants, incubated ± high glucose, were subjected to the immunofluorescence protocol as detailed in Chapter 2. The antibodies used were VEGF (5µg/ml, MAB293, R&D Systems) and $VEGF_{165}b$ (5µg/ml, MAB3045, R&D Systems and gift from D. Bates, University of Bristol) monoclonal antibodies. Negative controls did not contain the primary antibody. Given that both primary antibodies were monoclonal mouse anti-human IgG (no other VEGFb antibodies available), a single labelling technique had to be used on consecutive frozen sections.

4.3.4 Analysis

Using the selected random sampling method (adapted from systematic random sampling as described by Mayhew (2008)) (see Figure 2.2), the percentage of total immunopositive vessels were counted for both total VEGF and VEGFb using fluorescence microscopy (Nikon LaboPhot-2 fluorescence microscope, Nikon Instruments) and images were acquired (Nikon Coolpix 995 digital camera) in ADOBE Photoshop 6.0 Software. All 9 blocks were sectioned for each experimental condition and study group.

Analysis of data was performed using GraphPad Prism version 5.01 statistical software. To discern whether the various groups (normal euglycaemia, normal hyperglycaemia, diabetic euglycaemia and diabetic hyperglycaemia) were significantly different from each other, non-parametric Krustal-Wallis test was performed when comparing all groups, with Mann-Whitney U test performed on relevant groups if significant differences were found (see reasoning in section 2.3.9.1). Unless otherwise specified, all the statistics shown throughout Chapter 4 were performed with the Mann-Whitney U test (n=4 for both normal explants and diabetic explants). Where statistical significance is present, this indicates that a previous Kruskal-Wallis test (n=4

for both normal explants and diabetic explants) had shown a significant difference between the wider groups.

In order to ascertain whether the ratio of VEGF splice variants could be a predictor of vascular dysfunction in diabetes, the average percentage of immunopositive vessels for total VEGF for each placenta was plotted on the x-axis, with the corresponding average percentage of immunopositive vessels for VEGFb for the same placenta on the y-axis, giving a single point for each placenta analysed. Using GraphPad Prism version 5.01 statistical software, Spearman correlation analysis was then performed.

4.4 Results

For ease of understanding, the following abbreviations are used hereafter:

N5: normal chorionic villous explants incubated for 4h in 5mM glucose.

N15: normal chorionic villous explants incubated for 4h in 15mM glucose.

D5: Type 1 diabetic chorionic villous explants incubated for 4h in 5mM glucose.

D15: Type 1 diabetic chorionic villous explants incubated in 15mM glucose.

4.4.1 Analyses of total VEGF

There was a higher number of vessels showing immunoreactivity for total VEGF in the explants obtained from diabetic pregnancies compared to those from normal pregnancies. The percentage of total number of vessels immunopositive for total VEGF was 43% for N5, 51% for N15, 70% for D5 and 74% for D15 (Figure 4.4).

Mann-Whitney U statistical analysis showed that the percentage of total VEGF immunopositive vessels in N5 was significantly lower than D5 ($p<0.05$) and D15 ($p<0.05$), but not N15 ($p>0.05$). The percentage of vessels immunopositive to total VEGF in N15 was significantly different from D5 ($p<0.05$) and D15 ($p<0.05$). There was no significant difference in VEGFa immunoreactivity between D5 and D15 ($p>0.05$, Figure 4.4).

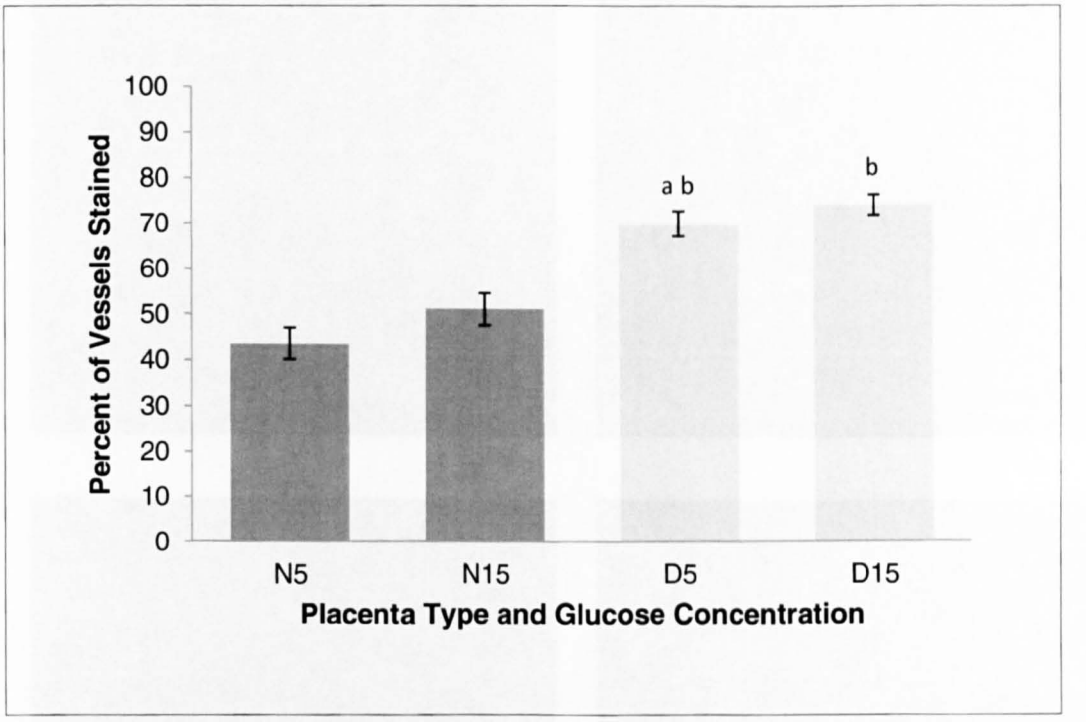


Figure 4.4: Percentage of vessels showing total VEGF immunoreactivity in normal and diabetic placentae. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=4 for normal groups, n=4 (*donors A-D*) for diabetic groups), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups. See Appendix 2 for diabetic donor details. a: $p<0.05$ relative to N5, b: $p<0.05$ relative to N15.

VEGF was immunolocalised mostly to the fetal vessels in the chorionic villi of normal explants. Some staining was observed in the trophoblast but none in the perivascular layer in normal explants, and glucose did not alter this localisation pattern (Figure 4.5A-B). In diabetic explants, total VEGF was seen predominantly in fetal vessels; there was some trophoblast staining and minimal perivascular localisation (Figure 4.5C-D). Again, glucose did not disturb this pattern.

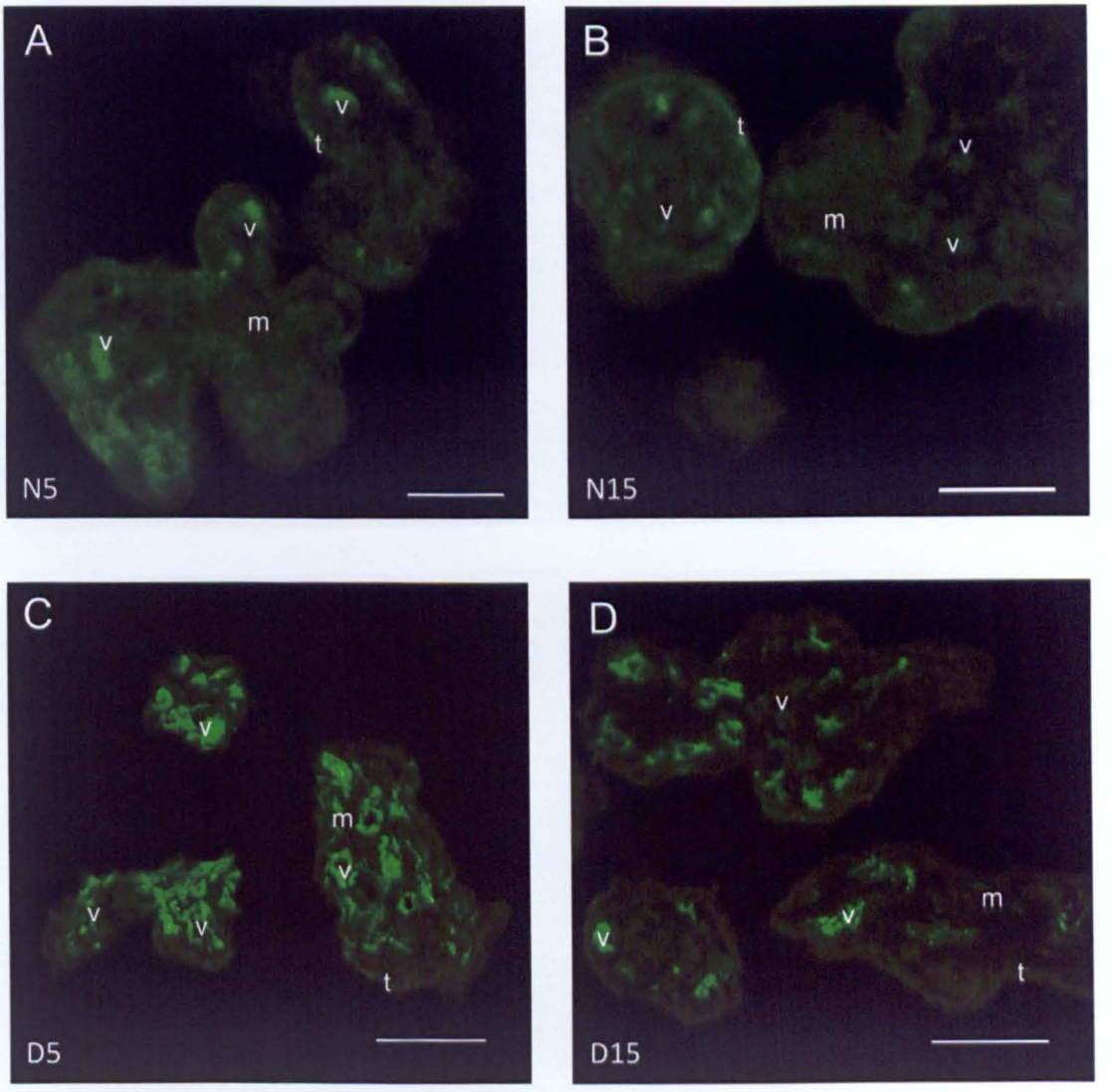


Figure 4.5: Representative micrographs showing total VEGF immunoreactivity in normal and diabetic explants. A: N5 group showing limited vascular VEGF immunoreactivity, and some trophoblast VEGF immunoreactivity B: N15 group showing a similar total VEGF profile to N5 (A), C: D5 group (*from donor B*) showing increased vascular VEGF immunoreactivity compared to the normal placental explants (A and B); similarly to A and B, it displayed some trophoblast immunoreactivity, but in addition also showed minimal mesenchymal immunoreactivity, D: D15 group (*from donor D*) showing a similar profile to D5 (C), of increased vascular VEGF immunoreactivity relative to the normal groups (A and B), some trophoblast immunoreactivity, and minimal mesenchymal immunoreactivity. Thus, the diabetic groups showed more vessels immunopositive for total VEGF; however, the glycaemic status (euglycaemic or hyperglycaemic media) did not affect vascular VEGF immunoreactivity. All groups showed some trophoblast immunoreactivity, but the diabetic groups also showed minimal mesenchymal immunoreactivity. N5, N15: normal explants incubated with 5mM and 15mM glucose, respectively, D5, D15: diabetic explants incubated with 5mM and 15mM glucose, v: vessel, t: trophoblast, m: mesenchyme. See Appendix 2 for diabetic donor details. Magnification: x200. Scale bar: 100um.

4.4.2 Analyses of VEGFb

There were significantly fewer vessels immunopositive for VEGFb in the N15 and diabetic groups than in N5 (Figure 4.6). The percentage of total number of vessels immunopositive for VEGFb was 57% for N5, 42% for N15, 44% for D5 and 39% for D15 (Figure 4.6).

Mann-Whitney U statistical analysis showed that the percentages of VEGFb immunopositive vessels were significantly different between N5 and D5 ($p<0.05$) and between N5 and N15 ($p<0.05$), but there were no significant differences between neither D5 and D15 nor N15 and the two diabetic groups (D5 and D15) (Figure 4.6).

Unfortunately, t=0h diabetic tissue was not obtained, so the VEGFb profile at that timepoint and comparison of t=0h with the 4h tissue could not be explored.

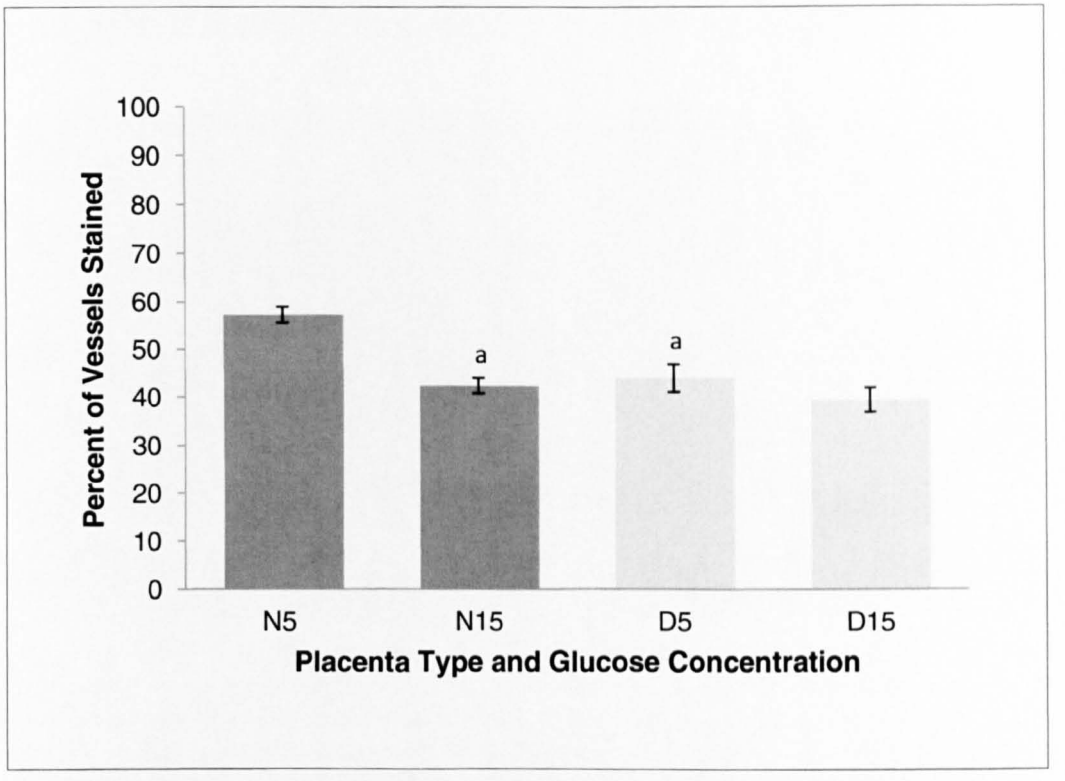


Figure 4.6: Percentage of vessels showing VEGFb immunoreactivity in normal and diabetic placentae. The statistical testing shown here refers to the Mann-Whitney U test between relevant groups in the data (n=4 for normal groups, n=4 (*donors A-D*) for diabetic groups), which were only analysed if the Kruskal-Wallis test had detected a significant difference in the wider groups. See Appendix 2 for diabetic donor details. a: $p<0.05$ relative to N5.

VEGFb was immunolocalised to the fetal vessels in the chorionic villi. Some staining was observed in the trophoblast in normal explants, but none in the perivascular area. Glucose did not alter this localisation pattern (Figure 4.7A-B). In diabetic explants, VEGFb was seen predominantly in fetal vessels. Similarly to normal tissue, there was trophoblast staining but no perivascular VEGFb localisation. Again, glucose did not disturb this pattern (Figure 4.7C-D).

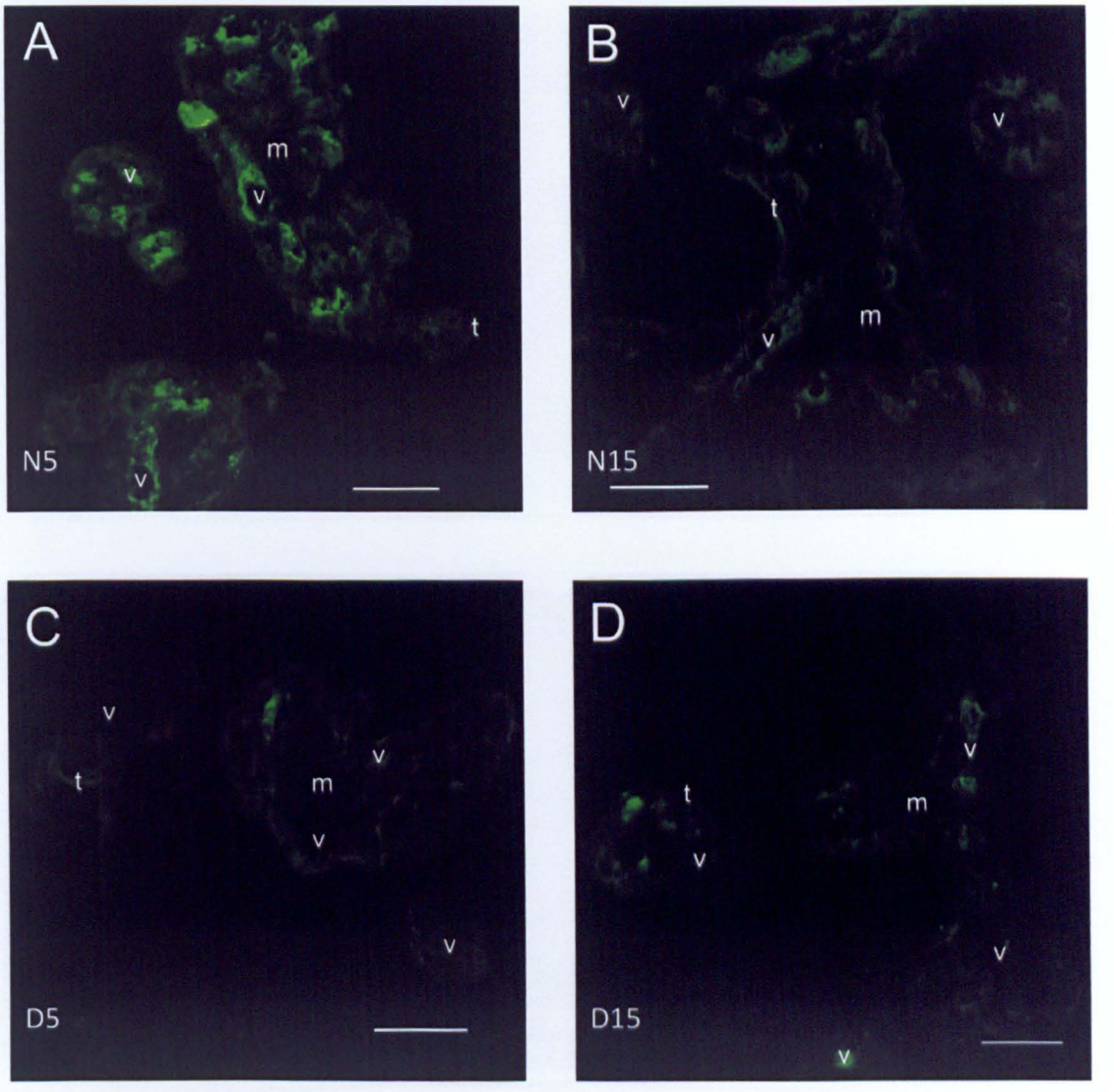


Figure 4.7: Representative micrographs showing VEGFb immunoreactivity in normal and diabetic explants. A: N5 group showing a majority of vessels VEGFb immunopositive, some trophoblast immunoreactivity and no mesenchymal immunoreactivity. B: N15 group showing decreased vascular VEGFb immunoreactivity relative to N5 (A), some trophoblast VEGFb immunoreactivity and no mesenchymal VEGFb immunoreactivity. C: D5 group (from donor C), showing similar VEGFb immureactivity to the N15 group (B), and decreased vascular VEGFb immunoreactivity relative to the N5 group (A). D: D15 group (from donor A) showing similar VEGFb immunoreactivity to N15 (B) and D5 (C). In short, the N5 group (A) shows more VEGFb immunopositive vessels than all other groups (B, C, D). All groups showed some trophoblast immunoreactivity but no mesenchymal immunoreactivity. N5, N15: normal explants incubated with 5mM and 15mM glucose, respectively, D5, D15: diabetic explants incubated with 5mM and 15mM glucose, v: vessel, t: trophoblast, m: mesenchyme. See Appendix 2 for diabetic donor details. Magnification: x200. Scale bar: 100um.

4.4.3 Total VEGF/VEGFb correlation and ratio

Analysis of data revealed a negative correlation between the percentage of vessels expressing VEGFb and total VEGF (Figure 4.8). The graph has a Spearman r value of -0.7412 (R^2=0.5494) and an alpha value <0.01, indicating a statistically significant negative correlation between total VEGF and VEGFb.

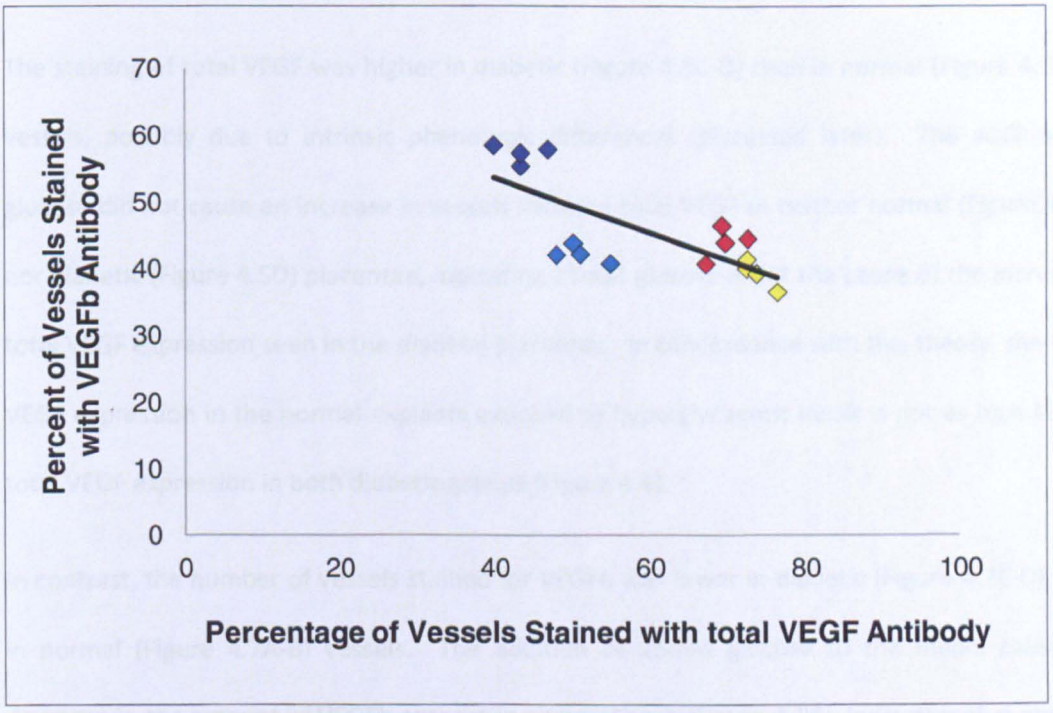


Figure 4.8: Significant (α<0.01) negative (r=-0.7412) correlation between total VEGF and VEGFb. Dark blue data points: N5 placentae, cyan data points: N15 placentae, red data points: D5 placentae, yellow data points: D15 placentae.

This significant negative correlation between total VEGF and VEGFb implies that the ratio of VEGFa/VEGFb is changing in the diabetic groups compared to N5, since

total VEGF = VEGFa + VEGFb.

Therefore, because of the significant correlation, VEGFa must be increasing when total VEGF is increasing and VEGFb is decreasing. This is indeed the situation in our diabetic explants compared to N5 (dark blue data points in Figure 4.8, and Figures 4.4 and 4.6). As the diabetic data (red and yellow data points in Figure 4.8) cluster further down the line of best fit than the

normal data (dark blue and cyan data points in Figure 4.8), the VEGFa:VEGFb ratio must be higher in the diabetic groups compared to the normal groups.

4.5 Discussion

4.5.1 Summary of results

The staining of total VEGF was higher in diabetic (Figure 4.5C-D) than in normal (Figure 4.5A-B) vessels, possibly due to intrinsic phenotypic differences (discussed later). The addition of glucose did not cause an increase in vessels showing total VEGF in neither normal (Figure 4.5B) nor diabetic (Figure 4.5D) placentae, indicating 15mM glucose is not the cause of the increased total VEGF expression seen in the diabetic placentae. In concordance with this theory, the total VEGF expression in the normal explants exposed to hyperglycaemic insult is not as high as the total VEGF expression in both diabetic groups (Figure 4.4).

In contrast, the number of vessels stained for VEGFb was lower in diabetic (Figure 4.7C-D) than in normal (Figure 4.7A-B) vessels. The addition of 15mM glucose to the media caused a decrease in the amount of VEGFb present in normal tissue (Figure 4.7B), indicating that glucose may be able to downregulate VEGFb production and dampen its anti-angiogenic and anti-permeability actions, allowing VEGFa to predominate and alter the vascular phenotype of the placenta. However, the same was not seen in diabetic tissue (Figure 4.7D), indicating a possible adaptation of the diabetic placenta to high glucose. VEGFb expression was not significantly different between the high glucose normal placentae and both diabetic groups (Figure 4.6). This further indicates that high glucose can induce the diabetic phenotype in normal placenta via down-regulation of VEGFb.

These results indicate that glucose affects the two splice variants differently, with down-regulation of the anti-angiogenic splice variant (VEGFb) being a predominant feature. There was also a significant negative correlation between total VEGF and VEGFb (Figure 4.8),

indicating a change in the ratio of VEGFa to VEGFb between the groups. This ratio of two VEGF splice variants may therefore be an important predictor of vascular dysfunction in diabetes.

4.5.2 Glucose effects on VEGF splice variants in normal and diabetic explants

Our results show that under euglycaemic conditions, explants from diabetic pregnancies have a lower percentage of vessels expressing the anti-angiogenic splice variant VEGFb (Figure 4.6) and a higher number of vessels expressing total VEGF (Figure 4.4), indicating an increase in the pro-angiogenic VEGFa when compared to the normal explants. After 4h hyperglycaemic insult, we observed that exposure to high glucose has very different effects on the normal explants as compared to the diabetic explants. Normal explants showed a significant down-regulation of VEGFb expression when exposed to 15mM glucose (Figure 4.7B), making N15 comparable to that seen in the diabetic explants (either D5 or D15, Figure 4.7C-D). Levels of total VEGF were not affected here. Plotting the correlation of total VEGF to VEGFb for the different experimental groups also proved enlightening, as this showed a significant negative correlation (when total VEGF increased, VEGFb decreased, Figure 4.8), and therefore the proportion of VEGFa to VEGFb must be changing, ie. there is a changing VEGFa/VEGFb ratio. This correlation is maintained with the inclusion of the diabetic explants in the analysis, and therefore study of the VEGFa/VEGFb ratio may be important in the prediction of vascular dysfunction in diabetes.

4.5.3 Pan-VEGF antibody

The alteration of the VEGFa/VEGFb ratio has been referred to as a potential therapeutic strategy for the amelioration of vascular angiogenenic pathologies (Nowak *et al.*, 2010; Zhao *et al.*, 2011). Ideally, it would be important in these experiments to try to calculate the ratio of the two molecules. However, currently there is no commercially available antibody which detects exclusively VEGFa and not VEGFb (Varey *et al.*, 2008), and the studies on the ratio of the

two splice variants is currently being calculated by techniques such as PCR (Bates *et al.*, 2002; Diaz *et al.*, 2008; Pritchard-Jones *et al.*, 2007; Qiu *et al.*, 2010; Schumacher *et al.*, 2007; Woolard *et al.*, 2004), which allows the differences in the amino acid composition to be exploited to discern the difference between VEGFa and VEGFb. Thus, we sought to calculate the proportion of VEGFb to total VEGF. However, when comparing the two, we observed that in N5 (see figure 4.4 and 4.6), the percentage of vessels immunopositive to total VEGF was lower than the percentage of vessels immunopositive to VEGFb, which is theoretically not possible if the two values were comparable.

Personal communications with R&D Systems (the supplier of the VEGF antibody used in this thesis, MAB293), revealed that the epitope of VEGF used to create MAB293 included alanine 29 to arginine 191, and therefore includes most of the VEGF molecule. However, there was a very minor possibility that the last 6 amino acids were critical for detection of VEGF. Further personal communications with R&D however confirmed that that MAB293 cross reacts with VEGFb 100%. This was tested with both direct ELISA and Western blotting (reducing and non-reducing conditions). The latter indicates that, even if the last 6 amino acids conferred VEGFa and VEGFb different secondary structures, they would both be detected by the antibody. Therefore, VEGFb would be detected by this antibody in our immunofluorescence protocol.

Personal communications with D. Bates (University of Bristol), who has experience of using MAB293 and other pan-VEGF antibodies, revealed that a possible reason for the discrepancy of immunopositive vessels using MAB293 and MAB3045 (the VEGFb antibody used) is the affinity of the antibodies to VEGF. Indeed, further communications with R&D confirmed this, as MAB293 has a detection limit of 5ng/well in ELISA and 50ng/lane in Western blotting whilst MAB3045 has a detection limit of 3ng/well in direct ELISA and 25ng/lane in Western blotting. Therefore, given the same amount of protein being present, MAB3045 will show more immunoreactivity than MAB293. This can account for the higher VEGFb levels seen in some samples compared to the total VEGF.

Due to this, we have been unable to perform a ratio analysis of the data, i.e. the proportion of VEGFb to total VEGF. In order to do this, we would have to use antibodies which have the same detection limits. In order to perform the ratio analysis, we would need an antibody which is specific to VEGFa and does not detect VEGFb. D. Bates is currently working on producing a polyclonal antibody which detects VEGFa exclusively, and therefore this analysis will be possible in the near future. In this chapter, we have instead performed a correlation analysis rather than a more direct VEGFb/VEGF (total) proportion analysis.

4.5.4 Reversal of diabetic phenotype in euglycaemic conditions

One of our aims was to observe whether a 4h incubation with 5mM glucose reversed the diabetic phenotype. If this had been the case, there would have been a significant difference in the number of vessels immunopositive to the VEGF splice variants between D5 and D15. This was not seen in our explants.

It is known that Type 1 diabetic placental vessels are more prone to excessive angiogenesis (Mayhew, 2002). It has also been shown that there is a high variation between Type 1 diabetic pregnant women's hyperglycaemic excursions (Kerssen *et al.*, 2004), and therefore a hyperglycaemic episode might be followed by euglycaemic periods. Had the Type 1 diabetic phenotype been reversible upon the cessation of glycaemic insult, it would have discarded glucose as an important mediator in the pathology of the diabetic placenta, as any changes would have been reversible upon better glycaemic control.

However, there was a significant negative correlation between total VEGF and VEGFb ($r=-0.7412$, Figure 4.8) when considering all data (normal and diabetic explants), indicating that the ratio of the splice variants is changing in the four groups (N5, N15, D5, D15). Furthermore, the diabetic groups (D5 and D15) all lie further down the correlation line than the normal groups

(Figure 4.8), indicating a higher VEGFa/VEGFb ratio in diabetic explants compared to normal explants.

If it is the ratio of VEGFa/VEGFb, rather than the total expression of the two splice variants, that is an important predictor of vascular dysfunction in the diabetic placenta, then better glycaemic control after 15mM glucose exposures might indeed ameliorate the outcome of the pregnancy. An analysis of the VEGFa/VEGFb ratio itself is however important to propose possible mechanisms: if the ratio of the VEGF splice variants in D5 is significantly different from N5 for example, it would suggest that persistent epigenetic changes have occurred, which are known to occur in diabetic pregnancies (reviewed by Leach (2011)). This is an important experiment to consider once a VEGFa antibody becomes available.

To test further whether the effects of 15mM glucose are reversible or not, a future experiment with the reversal of N15 to N5 again, followed by one or more cycles of glucose insult followed by reversals, would be informative. Polhill *et al.* (2004) in their study on human cortical fibroblasts have shown that 2h repeated short-term hits with hyperglycaemia (15mM) 3 times a day separated by 3h euglycaemia, followed by a 12h euglycaemic period (replicating postprandial hyperglycaemia) had a larger effect on most of the parameters they studied than a constant 15mM glucose insult. If episodic glucose insults (such as those seen by Kyne-Grzebalski *et al.* (1999)) have the same effect on the placental vasculature, we hypothesise that the future experiment suggested above would show more vascular effects (in terms of total VEGF and VEGFb levels) than a continuous glucose insult of equivalent total duration.

In this chapter, the closest we have come to a similar kind of reversal experiment is by comparing N15 and D5, where N15 represents a single hyperglycaemic insult to normal tissue and where D5 represents a tissue previously exposed to hyperglycaemia but subsequently experiencing a period of euglycaemia. When looking at total VEGF, our results show that N5 is significantly different from D5 but not from N15, and the latter two are also significantly different from each other (Figure 4.4); this would indicate that a single episode of

hyperglycaemia for 4h in normal tissue does not cause the diabetic total VEGF phenotype and that euglycaemia for 4h in diabetic tissue is not capable of restoring normal total VEGF levels. When looking at VEGFb, our results show that both N15 and D5 are significantly different from N5 but not from each other (Figure 4.6), suggesting that in normal tissue 4h of hyperglycaemia is causing the decrease in VEGFb, but 4h of euglycaemia in diabetic tissue is not sufficient to reverse the diabetic VEGFb phenotype.

4.5.5 Location of VEGFa and VEGFb

The splice variant VEGFb was only discovered in 2002 (Bates *et al.*, 2002). Therefore most studies into VEGF biology do not discriminate the actions of the two splice variants. The pan-VEGF antibody used in most studies is directed against both variants (Varey *et al.*, 2008). In adult tissue, global VEGF is normally found at basal levels acting as a survival factor; it is expressed in higher amounts only during inflammation and tissue damage, both to aid wound healing and as an early step in pathological angiogenesis. In the developing human placenta, there is physiological vasculogenesis and angiogenesis in the first trimester and a second wave of angiogenesis in the terminal villi of the third trimester placenta (Demir *et al.*, 1989). VEGF was found in the trophoblast and endothelium of all villi in the first trimester, whilst it was restricted only to the trophoblast of terminal villi in the last trimester and of course basal levels were expressed in the placental endothelium (Clark *et al.*, 1996; Leach *et al.*, 2002). In this study, diabetic explants exhibited a ubiquitous distribution of total VEGF in the endothelium of blood vessels, the trophoblast and (minimally) the mesenchyme, whilst VEGFb was found in both the endothelium of blood vessels and in the trophoblast, but not in the mesenchyme (see Figures 4.5C-D and 4.7C-D). This indicates that VEGFa is more ubiquitous than VEGFb. The number of vessels showing total VEGF in the diabetic explants was higher than that seen at 0h in normal tissue and agrees with previous studies which show intrinsic differences between diabetic and normal placentae (Leach *et al.*, 2004).

4.5.6 VEGFa in the diabetic explants

VEGFa in our diabetic explants is increasing with respect to normal explants, as total VEGF is increasing and VEGFb is decreasing (see section 4.4.3).

Data in this chapter was acquired from excised chorionic villi. Frank *et al.* (1995) has shown that dampened VEGFa is behind impaired wound healing in diabetes. If our explants were being affected by wounding, we would expect a decrease in the VEGFa levels in the diabetic explants. This was not seen. In fact, there is clear evidence in the literature that diabetes increases angiogenic processes and VEGFa production both in the human placenta (Leach *et al.*, 2004) and in other tissues, such as retinopathy in the eye, (reviewed by Crawford *et al.* (2009)). It is therefore interesting that when comparing normal and diabetic tissue (N5 and D5), the diabetic tissue displayed significantly more vessels expressing VEGFa after 4h in culture (D5 had more total VEGF (Figure 4.4) and fewer VEGFb (Figure 4.6) immunopositive vessels than N5, indicating more VEGFa immunopositive vessels). This finding is consistent with the diabetic phenotype seen in the term placenta, rather than the decrease one would expect in diabetic wound healing. This is good evidence that excision artefacts are not affecting our results.

Our data suggests that the increase in VEGFa in the diabetic placental explants is not modulated by glucose, as exposure to 15mM glucose for 4h did not change total VEGF and VEGFb expression (Figures 4.4 and 4.6). This insensitivity may be due to repeated exposures *in vivo* to this level of glucose – as stated earlier, postprandial levels of glucose takes several hours to return to normal in diabetic pregnant women (Ben-Haroush *et al.*, 2004), and as such the 15mM glucose used may not be perceived as 'hyperglycaemia' by the diabetic placenta.

4.5.7 VEGFb in the diabetic explants

In pre-eclampsia, VEGFb levels in the placenta have been shown to be decreased (Bills *et al.*, 2009). We have now found this to be true for another pathology of the placenta, as we found

that levels of the anti-angiogenic splice variant VEGFb were significantly higher in the normal placenta than in the diabetic placenta. VEGFb has a different role to VEGFa, and is thought to be involved in the stability of vessels, inhibiting the actions of VEGFa by binding to its receptor (VEGFR-2), and therefore being a competitive inhibitor (Cebe Suarez *et al.*, 2006). Our data supports this hypothesis. On addition of glucose, VEGFb levels significantly decreased and became similar to that seen in the diabetic placental explants (Figure 4.6) providing evidence that glucose is able to regulate this splice variant in normal tissue. By decreasing the levels of VEGFb, more VEGFa can bind to its receptors, and therefore activate pathways such as angiogenesis, which are seen in diabetes. Therefore our data allows us to hypothesise that VEGFb may be a key molecule in the development of placental pathologies and in diabetes high glucose itself may affect the placental vasculature by altering VEGFb, which consequently alters the ratio of VEGFa/VEGFb, allowing the activation of pathways that require VEGFa without actually altering VEGFa and/or total VEGF levels.

4.5.8 VEGFa/VEGFb ratio

We saw in section 3.1.1.1 that several studies show that glucose increases expression of total VEGF (Nakagawa *et al.*, 2006; Chen *et al.*, 2004; Vidro *et al.*, 2008; Feliers and Kasinath, 2010); these studies would have to be revisited to show which VEGF splice variant is up-regulated or down-regulated, and to see whether there is a change in the ratio of the two splice variants following glucose insult in these *in vitro* systems. Our study suggests that as total VEGF increases, VEGFb decreases, thereby altering the ratio of the two splice variants.

Harper and Bates (2008) review the possible implications for therapeutic approaches in tissues where the ratio differs, such as in cancerous tissue (Peiris-Pages *et al.*, 2010), non-cancerous tissue (Varet *et al.*, 2010), and pre-eclampsia (Bates *et al.*, 2006). We were not able to compare the ratio directly due to the different detection limits of the antibodies used (MAB293 and

MAB3045), but the significant negative correlation found suggests that in our explants there is a changing VEGFa:VEGFb ratio, as when total VEGF increases and VEGFb decreases, VEGFa must be increasing.

As the diabetic explants cluster further down the correlation line than the normal explants, this must mean that the diabetic explants have a different ratio than the normal explants.

If the changes in these VEGF splice variants correspond to a different placental phenotype and the functionality of the placental exchange is disturbed, glucose would not be the only nutrient that would be affected, as many other parts of the maternal-fetal exchange would show changes. The importance of the correlation between total VEGF and VEGFb and its effects on the functionality of placental exchange (as measured through changes in leakage to a 76Mr dextran tracer) can be seen in Chapter 5.

4.5.9 15mM glucose insult for 4h in Type 1 diabetic pregnancies

The 4h hyperglycaemic insult was shown to affect the vasculature of the placenta. A study (Kyne-Grzebalski *et al.*, 1999) showed that even well-controlled Type 1 diabetic pregnant women, who had normal or slightly elevated HbA_{1c} levels, experienced hyperglycaemic excursions to the levels we have studied (15mM blood glucose) postprandially. In their study, Kyne-Grzebalski *et al.* (1999) hypothesised that these brief hyperglycaemic excursions caused hyperglycaemia and hyperinsulinaemia in the fetus, thus resulting in macrosomic newborns. We have however shown that these hyperglycaemic insults affect the placenta as well as the fetus, and as such, not only is the fetus receiving an increased glucose load, but if there is increased leakage of the placental vasculature, perhaps also non-glucose nutrients might be able to traverse the placental barrier and enter the fetal circulation, thus contributing further to the macrosomia observed. We investigate the leakiness of the placental barrier in Chapter 5.

4.5.10 Consequences of postprandial hyperglycaemia

Several studies have correlated high postprandial glucose to macrosomia in the newborn, and some large population studies have been performed that confirm this. Combs *et al.* (1992) analysed data from 111 diabetic pregnancies, and found that macrosomia was associated with postprandial glucose levels up to 32 weeks gestation. That the association does not continue after that period is irrelevant, as the author states himself that women who tend to be hyperglycaemic at any one point in the pregnancy continue to do so at other points in the pregnancy; also, the study only went up to 36 weeks and did not include glycaemic levels at term. The authors recommended a postprandial glucose level of 7.6mM, as lower levels were seen to associate with small-for-gestational age offspring (Combs *et al.*, 1992). In their study of 98 Type 1 diabetic women, Mello *et al.* (2000) found that women with blood glucose levels of >5.3mM in the third trimester had a higher incidence of large-for-gestational age infants compared to women having >5.3mM blood glucose in the second trimester but not in the third. Infants from mothers with third trimester >5.3mM blood glucose blood also had a significantly higher incidence of hypocalcaemia and clavicular fracture than infants from mothers with blood glucose higher than 5.3mM only in the second trimester.

However, most papers seem to attribute the observed macrosomia to increased glucose concentration in fetal blood predicted by the Pedersen hypothesis, without considering that there might be deeper, more pronounced changes in the vasculature of the placenta. Indeed, Combs *et al.* (1992) do hypothesise that there might be other non-glucose nutrients that might partly contribute to macrosomia, but instead of looking at the vascular profile permeability status, they looked at food intake, without finding any differences. Whilst undoubtedly high glucose in the mother does result in fetal hyperglycaemia, as the GLUT1 and GLUT3 glucose transporters are independent of insulin, we have seen in this chapter that there are changes in the expression of angiogenic and permeability growth factors VEGFa and VEGFb directly attributable to glucose insult.

4.5.11 How VEGFb correlates to other tissues

VEGFb has been shown to be important in the reproductive system, with the overexpression of VEGFb inhibiting angiogenesis in these tissues (Qiu *et al.*, 2008), whilst inhibition of this splice variant reproduces the phenotype of overexpression of VEGFa (Artac *et al.*, 2009). We have seen that, when subjected to glucose insult, our normal explants (N5 and N15) showed decreased VEGFb. Based on the literature, a decreased expression of the anti-angiogenic splice variant should result in a phenotype similar to that caused by an increase in the pro-angiogenic splice variant, i.e. a more angiogenesis-prone tissue. This is indeed the phenotype we see in diabetic placentae, as increased angiogenesis of fetoplacental vessels is a key feature in these tissues (Mayhew, 2002).

As well as having been shown to be anti-angiogenic in various tissues, in the kidneys VEGFb has been found to have anti-permeability effects (Ferguson, 2007). This fits well with our results, as the diabetic placenta has been shown to be leakier than the normal placenta (Leach *et al.*, 2004), and we have shown that diabetic explants have decreased levels of VEGFb expression in comparison to normal explants. Therefore this anti-permeability effect might be a global effect not restricted to renal tissue (Qiu *et al.*, 2010). Whether the presence of decreased VEGFb levels correspond to higher permeability in the placenta will be explored in Chapter 5.

4.6 Summary

Compared to explants from normal pregnancies (N5), placental explants from diabetic pregnancies (D5 and D15) have a higher number of total VEGF immunopositive vessels and a lower number of VEGFb immunopositive vessels, indicating an increase in VEGFa.

On addition of glucose, both normal tissue and diabetic tissue do not produce more total VEGF; however, the expression of VEGFb in normal tissue, but not diabetic tissue, decreases

in response to a 4h incubation with 15mM glucose, indicating different regulatory mechanisms between VEGFa and VEGFb.

The significant negative correlation in total VEGF/VEGFb indicates a different VEGFa/VEGFb ratio between normal and diabetic explants, and therefore targeting this change in ratio might in future ameliorate the clinical outlook for pregnant diabetic patients.

However, whether this ratio accurately predicts the functional role of the placenta cannot be ascertained with explants. Therefore, the functional effect of 15mM glucose exposure is investigated with a dual-perfusion methodology in Chapter 5.

Chapter 5
Functional studies on term placental microvascular beds perfused with 15mM glucose

5.1 Introduction

5.1.1 Glucose effects on the vasculature

Hyperglycaemia in diabetes is known to increase the permeability of the endothelium regardless of what type (Type 1, Type 2 or gestational) of diabetes is investigated. Albumin retention, a measure of capillary permeability, has been associated with patients with Type 1 diabetes (Valensi *et al.*, 1997). In Type 2 diabetes, transvascular permeability seems to be increased, as the fractional escape rate of low-density lipoprotein is increased in Type 2 diabetic patients compared to healthy individuals (Kornerup *et al.*, 2002), and this has been attributed to chronic high glucose, at least in monkeys (Litwak *et al.*, 1998). Even the presence of elevated glucose alone (in the absence of overt diabetes) can have an effect on permeability, as in a study of hypertensive patients with or without metabolic syndrome (and hence elevated glucose, but no overt diabetes) but all free from vascular disease, the patients with metabolic syndrome showed increased capillary leakage of albumin (Dell'Omo *et al.*, 2004). The common link between these studies seems to be high glucose, thus suggesting that it is this variable causing the leakage seen. In diabetes-induced animals, where high glucose is the main characteristic, blood constituents have been shown to have leaked from blood vessels in several tissues, such as the heart (Yamaji *et al.*, 1993), skeletal muscle (Beals *et al.*, 1993) and brain (Huber *et al.*, 2006), as well as in the retina and kidney (as discussed in section 1.3.4).

Most of the studies above looked at diabetic animals, not hyperglycaemia in itself, and were studies conducted for several days. However, the mechanisms of the increase in permeability due to high glucose was also studied, and were found to be fast acting (start of response within minutes, peak of permeability at around 30 minutes) and mediated by the PKC pathway in porcine aortic endothelial cells (Hempel *et al.*, 1997), and in rat studies diabetes-induced leakage could be reversed by PKC inhibitors (Yuan *et al.*, 2000).

5.1.2 The Pedersen hypothesis and our experiments

The Pedersen hypothesis (1954) is the most widely followed theory about how hyperglycaemia as seen in diabetic pregnancies affects the fetus, which was thought to solely account for the macrosomia seen in the fetus at term. This hypothesis states that whilst glucose freely crosses the placental barrier by facilitated diffusion, insulin is unable to cross this barrier (Keller and Krohmer, 1968). As the fetal pancreas starts producing insulin from 11 weeks gestation (Piper *et al.*, 2004), the *in utero* fetus produces insulin to counteract the hyperglycaemic environment derived from the diabetic mother. Thus, even though the fetus' insulin response is normal, the fetus is subjected to high glucose and insulin levels, replicating the diabetic phenotype in the womb and allowing macrosomia to occur.

However, we have seen in the last chapter that even a 15mM glucose exposure for 4h causes vascular changes, in the form of lower VEGFb expression in the endothelium. This change in the vasculature may affect vascular permeability in the placenta, and if the integrity of the placental barrier is disrupted through increased leakage of nutrients, the fetus would be subjected to a higher amount of nutrients, encouraging excessive growth as seen in macrosomia. If there is increased leakage in the placental vasculature, this would indicate that the Pedersen hypothesis does not solely account for the macrosomia seen in diabetes. Indeed, the HbA_{1c} levels, a measure of overall glycaemic control (but which does not give information about short hyperglycaemic excursions) cannot always predict macrosomia (Schwartz *et al.*, 1994). Combs *et al.* (1992) also hypothesised that macrosomia might be partly caused by nutrients other than glucose, but looked at the effect of diet on the infants' weight, and did not find any significant differences. However, to the best of our knowledge, there is no literature looking at this phenomenon from a perspective of altered vascular leakage, which may be causing the macrosomic phenotype. In our explant methodology, we were unable to study vascular leakage, as this requires the separation of the fetal circulation from the maternal circulation. Therefore,

in this chapter we are looking at the effect of glucose on vascular leakage using a dual-independent perfusion model.

5.1.3 Glucose transport in the normal and diabetic placenta

The main glucose transporter found in the trophoblast and endothelial layer of the placenta is GLUT1 (Takata *et al.*, 1992; Hahn *et al.*, 1995; Takata *et al.*, 1997). Glucose crosses the placenta mostly through facilitated diffusion in the maternal-facing microvillous and fetal-facing basal membrane of the trophoblast. Evidence shows that the endothelium of microvessels does not contribute greatly to the flux resistance to glucose (and other small hydrophilic molecules) (Takata and Hirano, 1997; Eaton *et al.*, 1993; Leach *et al.*, 1995), and that glucose passes directly through the paracellular clefts of the endothelium (Leach and Firth, 1997) to the fetal circulation. Glucose transporters in the endothelial cells are thought to play a part in the uptake of glucose from the fetal circulation into the endothelial cells for their own metabolism (Hahn *et al.*, 1995; Hauguel-de Mouzon *et al.*, 1997).

Expression of glucose transporters in the trophoblast layer is asymmetrical, with a three-fold higher expression of GLUT1 in the microvillous (maternal-facing) than on the basal membrane (fetal-facing) (Takata *et al.*, 1992; Jansson *et al.*, 1993). This, in addition to a six-fold increased surface area of the microvilli compared to the basal membrane (Teasdale and Jean-Jacques, 1988), leads to greater capacity of uptake by the syncytiotrophoblast compared to the basal membrane, which acts as a rate-limiting step in glucose transport (Jansson *et al.*, 1993). The glucose gradient between the maternal circulation and the fetal circulation also positively correlates with glucose uptake, as shown in an elegant placental perfusion study by Schneider *et al.* (2003). This study showed that at equal glucose concentration in both circulations, the maternal circulation contributed 70% of placental glucose uptake, and the fetal circulation only contributed 30%. This maternal contribution was increased to 95% of total placental uptake

when the maternal circulation was 1.1mM glucose higher than the fetal, but only dropped to 50% when the fetal circulation was higher by the same amount (Schneider *et al.*, 2003). The rate limiting step in glucose transport on the fetal-facing surface affects glucose release to the fetal circulation (maternal to fetal circulations), but only affects placental uptake in the other direction (fetal to maternal circuits).

The density of GLUT1 is also differentially regulated throughout pregnancy, with a doubling in GLUT1 expression on the basal side in the third trimester compared to the second trimester, whilst expression on the microvillous membrane remains constant throughout pregnancy (Jansson *et al.*, 1993). By lowering the amount of glucose present in the fetal circulation by uptake in the placenta (but not increase in transplacental flux), the gradient between maternal and fetal circulations increases, and this is thought to lead to a bigger maternal to fetal transplacental glucose flux.

In the diabetic placenta, there is an increase in glucose flux from mother to fetus. Hyperglycaemia (>20mM glucose) has been shown to downregulate the expression of GLUT1 (Hahn *et al.*, 1998); however, the increased glucose gradient across the placenta overcompensates for this decrease. Also, insulin upregulates the expression of GLUT1 on the basal membrane, which is the rate-limiting step, and therefore the outcome of these changes is increased glucose flux to the fetus. Hence, the placental barrier might become compromised and its efficiency decreased, as seen in rat studies (Thomas and Lowy, 1995; Thomas and Lowy, 1992) which showed increased leakage of glucose, perhaps as a result of the increased vascular permeability seen in placentae from diabetic pregnancies (Teasdale, 1981). The result from these changes is that the density of GLUT1 doubles in the rate-limiting basal membrane whilst there are no changes in the microvillous membrane, leading to a 40% increase in basal membrane glucose uptake.

In this chapter, we sought to analyse the rate of transfer of glucose across the placental barrier.

5.1.4 Role of VE-cadherin and PECAM-1 in permeability

In previous chapters we have looked at VE-cadherin as an important adhesion and anti-proliferation molecule. Here, VE-cadherin is studied in the context of increased permeability.

VE-cadherin is an important regulator of permeability. As seen in Section 1.2.2.1.1 and shown in Figure 1.9A, VE-cadherin is present in the junctional space when the endothelium is stable. It is anchored to the cytoskeleton by its complex with β-catenin, plakoglobin, and actin. When there is an increase in permeability, the molecules disassociate from the actin and VE-cadherin is internalised (Figure 1.9B).

Whilst platelet-endothelial cell adhesion molecule-1 (PECAM-1) is localised to the junctional space, it is not involved in the junctional integrity of the paracellular cleft, and instead is involved in leucocyte binding and inflammatory responses (Albelda *et al.*, 1991). Indeed, in placental vasculature, PECAM-1 is present in term placental endothelium, and its expression increases when subjected to pro-inflammatory cytokine TNFα (Dye *et al.*, 2001); however, PECAM-1 remains at cell-cell contacts in junctional instability processes, such as increases in angiogenesis (Wright *et al.*, 2002), and is therefore an excellent endothelial marker for our purposes.

5.1.5 The placental dual-independent perfusion model

Placental perfusions have been used for many years as a methodology to study placental function. Panigel was the head of one of the first laboratories utilising perfusions, and he wrote a review in 1962 on these methodological experiments, documenting the various variations in placental perfusion techniques (Panigel, 1962). He demonstrated that different cotyledons can be perfused independently of one another, and therefore, one cotyledon could be perfused being isolated from the rest of the placenta.

The dual-independent placental perfusion seeks to recreate in one cotyledon both the maternal and the fetal circulation. The two circulations in this model are completely independent of each other, hence the name. The flow, maternal and fetal pressure, and temperature in the perfusion chamber are constantly monitored and maintained at a physiological level seen *in utero*. Thus, the flow is maintained at 20ml/minute for the maternal circulation and 5ml/minute for the fetal circulation, the pressure is maintained between 20-80mmHg for the maternal circulation and below 20mmHg for the fetal circulation, and the temperature is maintained at 37°C. Other than these key figures, there are some variations in the protocol (see section 5.1.6, below).

The main purpose of placental perfusions is to carry out studies into transport of drugs, nutrients, etc. between the two circulations.

5.1.6 Placental perfusion models in the literature

As the placental perfusion has been used for such a long time, it is natural that it would evolve with the times. Before Henning Schneider's contribution to the field, perfusing a placenta would mean delicately finding and cannulating the remnants of the spiral arteries, which was a very delicate and difficult process. This was made much easier by Schneider's discovery that by piercing the basal membrane and inserting the cannulae in the intervillous space, the same degree of perfusion could be achieved (Schneider *et al.*, 1972).

There are several variations to the placental perfusion protocols that have been used, to suit different experiments. One of the most important variations is the choice of which perfusate to use. Blood is the best perfusate for this methodology, as non-blood perfusate was seen to cause mitochondrial damage (Contractor *et al.*, 1984) and increased leakage from vessels (Panigel, 1962). However, use of human blood, especially in long duration experiments, could be problematic: firstly for long duration perfusion, a large quantity of blood would be needed,

as otherwise haemolysis occurs (Panigel, 1962), secondly the blood needs to be heparinised to keep it from coagulating, thirdly it could be difficult to sterilise the equipment between perfusions, and fourthly and very importantly, a 3h perfusion with blood media showed microvillous damage and oedema (Contractor *et al.*, 1984). For all these reasons, some experimenters use media instead of blood.

A variety of media can be used as perfusate. The main problem with using media is the low osmolarity of the solution, which increases vascular leakage. In order to counteract this leakage, high molecular weight proteins, such as dextran, albumin, or Subtosan (a synthetic substitute for plasma), could be used to increase the osmolarity of the perfusate, reducing, but not completely eradicating, the leakage (Panigel, 1962; Leach and Firth, 1992). Therefore, it is important to monitor any eventual leaking from the vessels in control experiments.

A feature of the *ex utero* perfused placental vasculature is that it can experience vasoconstriction, and therefore in some experiments, adenosine was used to ensure vasodilation. However, adenosine has been shown to disrupt the increased permeability in the perfusion model in response to histamine (Leach *et al.*, 1995), and therefore there is a concern that adenosine might affect other molecules capable of similar effects, such as VEGF, and is therefore not used in our set-up. Furthermore, as the current methodology monitors fetal and maternal pressure, adenosine is no longer strictly needed, as pressure can be monitored and perfusions discontinued if vasoconstriction occurs.

Another consideration in the perfusion methodology is which tracer to use. Cyanocobalamin (B12) is of a useful size to study leakage in the placenta, and is easy to label, whether radioactively or with a fluorescent molecule (Leach *et al.*, 1995). However, B12 is readily taken up by cells, and therefore to use it as a tracer, experimenters have used an excess of unlabelled cyanocobalamin, which is used to saturate its carrier-mediated uptake mechanisms prior to the perfusion of the labelled cyanocobalamin. Albumin is another tracer that has been used in the literature but has the problem that its permeability varies depending on which labelling method

is used (Rumbaut *et al.*, 1999), and also that albumin can interact with other proteins in the media, affecting its permeability (Eaton *et al.*, 1993). Dextran can also be used, as it is not absorbed by the tissue, although it is toxic if used for a long period of time. However, large molecular weight dextran is not metabolically active, and thus it is used in our studies.

The sourcing of the placenta is another variable: in early studies, placentae were obtained both from vaginal births and Caesarean sections. However, today we know that in the process of detaching from the uterus and passing through the birth canal, the placenta is subjected to various hormones and signalling mechanisms which might cause vascular collapse. Therefore, our laboratory uses exclusively placentae obtained through Caesarean sections.

5.1.7 Effect of glucose on vascular beds

Perfused glucose has been shown in mainly animal studies to affect the vascular bed of several tissues.

In a non-diabetic rat model, the vascular bed of skeletal muscle was investigated under high insulin (without high glucose) or 1.5g/kg high glucose, and it has been shown that under high insulin alone, there was vasodilation of vessels but no change in vasomotion. With high glucose (and with endogenous insulin being produced by the rat), this effect was lost, and instead there was vasoconstriction and increased vasomotion (Renaudin *et al.*, 1998).

In glomerular (kidney) rabbit tissue perfused with 30mM glucose, angiotensin sensitivity was increased compared to 5.5mM glucose perfusion. When a NO inhibitor was added to the 5.5mM glucose perfusions, the sensitivity was similar between the two groups, indicating that glucose in this model causes vasoconstriction via impairment of the NO signalling pathway (Arima *et al.*, 1995) (see section 1.3.3.1).

In *in situ* perfused diabetic rat brain tissue, high glucose resulted in the increase of protein transporter multidrug resistance protein-2 but not of organic anion transporter-3 at the blood-brain barrier (Hawkins *et al.*, 2007b). By using the same methodology (*in situ* brain perfusion), increased permeability of the blood-brain barrier to sucrose was observed in diabetic animals (streptozotocin-treated rats), and this was in conjunction with decreased occludin and ZO-1 (Hawkins *et al.*, 2007a).

Importantly for our experiment, perfused frog and mesenteric vascular beds show increased hydraulic conductivity (water permeability) but not reflection coefficient (macromolecular permeability) in response to 20mM glucose perfusion; this increased leakage was not mediated by free-radical production, the PKC pathway, albumin glycation or glucose metabolism (Perrin *et al.*, 2007).

All these studies were performed in animal studies, as there would be ethical and technical difficulties in performing them on human tissue. No such problems exist for the human placenta. In this chapter, we have perfused a cotyledon of the human placenta to observe what effect glucose has on the placental vascular bed.

5.1.8 Our dual-independent perfusion model

The dual-independent placental perfusion protocol set out in Leach *et al.* (2004) was used, with a few modifications. Firstly, the perfusion was lengthened to 3h (from the short duration in the Leach *et al.* (2004) paper), as we wanted to represent the postprandial period accurately. A trial run was made with the intention to carry on for 4h, to match the explant studies more closely; however, we observed that we had a higher rate of failure, mostly from increased leakage, after that period of perfusion, and it was therefore extremely difficult to work with. Therefore, we decided to use a 3h perfusion instead to minimise eventual damage to the placental vascular bed; this duration has also been used by other groups in placental perfusions

when researching diabetic phenotypes concerning glucose (King *et al.*, 2003; Kraemer *et al.*, 2006; Osmond *et al.*, 2000).

Media was used instead of blood, as even though blood would have given a more physiological simulation of the *in utero* environment, the duration of perfusion would probably lead to haemolysis and the volume of blood required for this experiment did not make the use of blood ethically possible.

Both circuits were closed, instead of the open maternal circuit used by Leach *et al.* (2004). We closed both circuits because the duration of the perfusion meant it would have been uneconomical to have an open perfusion; however, to limit the build up of toxic substances such as lactic acid, we replaced both the maternal and fetal media every 1h.

Other than these two exceptions, our perfusion followed the principles of the ideal perfusion set out above. A dextran tracer of known molecular weight (76Mr; greater than 66Mr albumin) was used to measure leakage in the last 10 minutes of our perfusion model. Figure 5.1 illustrates the dual-independent perfusion model.

5.1.9 Advantages and disadvantages of using a perfusion system vs. explant culture

The perfusion methodology represents a vast improvement over both explants methodologies and cell culture studies, as it gives a functional read-out by allowing measurements of permeability and transport between the two circulations. An important factor is that the entire placental infrastructure is in place in perfusions, as opposed to having isolated tissue in the explants culture, leading to a more physiological model. Another improvement is that as the whole perfused vascular tree remains intact, there are no excision artefacts.

One of the disadvantages of using a perfusion methodology is the short duration of the perfusion, as perfused placental cells remain viable for a maximum of 48h, compared to 11 days

for explants. However, this is not of concern to us, as our perfusions were of much shorter duration.

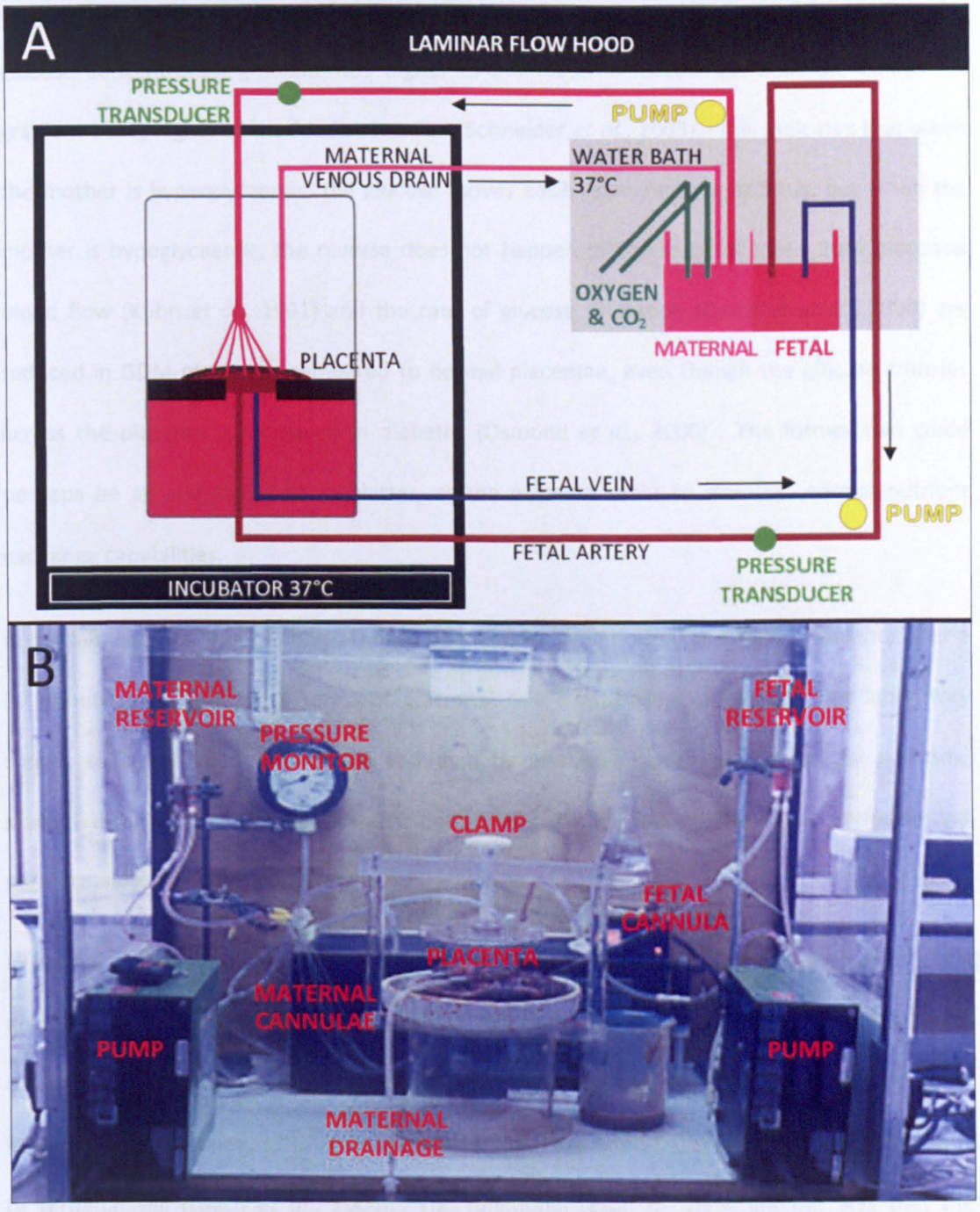


Figure 5.1: Diagram (A) and photo (B) illustrating the dual-independent perfusion model. The media flows from the maternal reservoir through five maternal cannulae to the intervillous space in the placenta, and from the fetal reservoir through one fetal cannulae to a fetoplacental vein supplying the perfused cotyledon of the placenta. The maternal circulation is drained from the placenta through a drainage tube in the clamp back to the maternal reservoir, whilst the fetal circulation is drained from the placenta through the corresponding artery to the vein supplying the fetal circulation, back into the fetal reservoir.

5.1.10 Perfusion of normal placentae and placentae from diabetic pregnancies

Placental perfusions have been used on both normal and diabetic placentae, with very illuminating results. In normal placentae, both glucose uptake and flux were higher when the glucose concentration gradient was higher in the maternal circulation than the equivalent gradient being higher in the fetal circulation (Schneider *et al.*, 2003). This indicates that when the mother is hyperglycaemic, the glucose moves easily from mother to fetus, but when the mother is hypoglycaemic, the reverse does not happen to the same degree. Both placental blood flow (Kuhn *et al.*, 1991) and the rate of glucose utilisation (Osmond *et al.*, 2000) are reduced in GDM placentae compared to normal placentae, even though the glucose transfer across the placenta is increased in diabetes (Osmond *et al.*, 2000). The former two could perhaps be an adaptation to the latter, as the placenta seeks to maintain normal nutrient exchange capabilities.

In our lab, Leach *et al.* (2000, 2004) have characterised the normal and Type 1 placenta after a 30 minute perfusion. In a series of placental dual-independent perfusions, our laboratory sought to define both the normal and diabetic placental barrier phenotype, by perfusing placentae for a 20 minute equilibration period, immediately followed by tracer perfusion and paraformaldehyde perfusion fixation.

In normal placentae, 92.9% of vessels exhibited VE-cadherin immunopositive (Leach *et al.*, 2004) and 98% showed occludin immunopositive junctions (Leach *et al.*, 2000), combined with negative to very weak immunoreactivity to anti-phosphotyrosine in 19.2% of vessels, a marker for junctional instability. VEGF immunoreactivity was low and localised to the trophoblast layer of terminal villi (Leach *et al.*, 2004). The functional result of these findings was that the placental vasculature was minimally leaky to 76Mr dextran tracer.

In contrast, perfused Type 1 placentae exhibited only 49.4% VE-cadherin immunopositive vessels, whilst 74.3% of vessels showed strong immunoreactivity to anti-phosphotyrosine.

VEGF immunoreactivity in Type 1 placentae was higher and more ubiquitously expressed (in the trophoblast and endothelium of stem, intermediate and terminal villi). The functional result of these findings was numerous vascular profiles exhibiting 76Mr dextran tracer leakage. Of the molecules studied in Leach *et al.* (2004) only PECAM-1 remained at similar levels in placentae from diabetic pregnancies compared to normal placentae (96.3% vs. 96.6%).

The phenotype of placentae from gestational diabetes is somewhat in between the two extremes (normal and Type 1 diabetes): 79.6% of vessels showed VE-cadherin immunoreactivity and 96.0% occludin immunoreactivity, whilst anti-phosphotyrosine immunoreactivity was observed in 56.4% of vessels (Babawale *et al.*, 2000). No information on VEGF levels or localisation, or on tracer leakage, for GDM was given in these or any other papers.

Interestingly, these placental vascular disturbances were found in placentae of women who had otherwise normal pregnancies (except for the presence of diabetes), and no further microvascular complications (such as retinopathy or nephropathy).

5.2 Aims and objectives

There were three aims in this chapter.

The first aim was to evaluate whether 3h 15mM glucose insult in an *ex vivo* model altered expression of:

- VE-cadherin
- Total VEGF
- VEGFb

The second aim was to observe whether the changes in expression of these molecules resulted in a change in the functionality of the human placenta in response to a 3h 15mM glucose

exposure to the placenta from the maternal circulation, as measured by 76Mr dextran tracer leakage.

The third aim was to see whether the expression of total VEGF and/or VEGFb could be good predictors of vascular leakage in the human placenta exposed to 15mM glucose.

5.3 Methods

5.3.1 Materials

The source and catalogue number for all Materials mentioned in this chapter are listed in Appendix 1.

5.3.2 Dual-independent perfusion model

Placentae (n=12) were taken from normal pregnancies undergoing term elective Caesarean sections in Queen's Medical Centre, Nottingham (and with the same donor demographic requirements as those outlined in section 2.3.2), with informed patient consent and ethical approval.

Immediately after delivery, the placenta was transferred to a 37°C chamber with the umbilical cord kept clamped until cannulation, to prevent the collapse of the fetoplacental vessels. A vein and an artery supplying one randomly chosen cotyledon were each cannulated with a nasogastric (5mm) tube to establish the fetal circulation within 20 minutes of arrival of the placenta, and the cotyledon was then clamped and sealed in a Perspex chamber to allow for a closed circuit. Five 5mm nasogastric tubes (which branched from one higher-calibre line) were inserted in the intervillous space through the basal plate of the cotyledon, and drained through a drain point in the Perspex chamber, to establish the maternal circulation. The cannulation

process was completed within 30 minutes of delivery to minimize hypoxic damage to the placenta.

Both circulations were connected to peristaltic pumps providing a 20ml/min flow to the maternal circulation and a 5ml/min flow to the fetal circulation, replicating the physiologic flow rates seen *in utero* (Panigel, 1962). A 20 minute open circuit equilibration period was started, to reverse any eventual hypoxic and ischaemic changes due to the delivery of the placenta (Leach and Firth, 1992), and to wash away any blood in the finest branches and capillaries. During this period, both maternal and fetal circulations were open circuits. After this 20 minute equilibration period with oxygenated perfusate (M199 with added albumin (5g/l), heparin, dextran (20,000Mr; 8g/l) and sodium bicarbonate (2.2g/l), 10mM glucose was added (to give a total of 15mM glucose) to the maternal circuit of the experimental group (n=6), but not to those in control perfusions (n=6). Both the maternal and fetal circulations were closed at this stage. The maternal and fetal flows were maintained at 20 and 5ml/min, respectively, and maternal and fetal perfusion pressures were 20-80mmHg and below 20mmHg, respectively.

Both the maternal and fetal circulations were checked at this point and throughout the experiment for leakage; if at any point there was any significant leakage (defined as a loss of more than 10% of perfusion media from the respective circulations), the experiment was defined as unsuccessful and terminated. Due to this, 6 perfusions had to be terminated early and discarded, and the remaining 6 were successful (n=3 for both euglycaemic and hyperglycaemic perfusions).

Microvascular beds were perfused for 3h, changing the media every hour. In the last 10 minutes, a 76Mr dextran tracer (76Mr; 0.5mg/ml; conjugated with Tetramethyl Rhodamine Isothiocyanate (TRITC)) was introduced to the fetal circuit of all perfusions. Following fetal perfusion fixation (1% w/v PFA, 30 minutes) tissue biopsies were immersion fixed for 45 minutes, rinsed in PBS, and then snap frozen in liquid nitrogen cooled isopentane.

5.3.3 Sampling of perfusion media and glucose measurements

During the dual-independent placental perfusion, 1ml samples of perfusion media were taken from both maternal and fetal circulations hourly, immediately before replacement of the media. This media was then frozen for subsequent use for glucose measurements.

Measurement of glucose concentration at each timepoint was performed first using Medi-test Glucose test strips for urine (RPC), using perfusion media with 5mM and 15mM glucose as measurement controls. The test-strips used colour values corresponding to 'negative' (0), 'normal' (0.8mM), 50mg/dl (2.8mM), 150mg/dl (8.3mM), 500mg/dl (27.8mM), and >1000mg/dl (>55.6mM) glucose.

Secondly, we measured glucose concentration in the samples using a HemoCue Glucose 201+ (HemoCue AB) blood glucose analyser. Each sample was tested in triplicate. Perfusion media with 5mM and 15mM glucose (known concentrations) were also analysed to assess the reliability of the machine to measure perfusion media samples.

Lastly, glucose concentration measurements were performed with a YSI Model 2300 STAT PLUS Glucose and Lactate Analyser. Perfusion media with 0mM, 5mM, 10mM, 15mM and 20mM glucose (known concentrations) were also measured in triplicate to assess the reliability of the machine to measure perfusion media samples.

5.3.4 Immunofluorescence (with VE-cadherin, total VEGF, VEGFb, PECAM-1 and tracer)

Immunofluorescence (using the methodology from Chapter 2) was performed on slices of samples using antibodies against VE-cadherin (555661, BD Biosciences; 5μg/ml), total VEGF (MAB293, R & D Systems; 5μg/ml), VEGFb (MAB3045, R&D Systems; 5μg/ml), and the endothelial marker PECAM-1 (BBA7, R&D systems; 5μg/ml). Goat anti-mouse FITC-conjugate

(F-0257, Sigma-Aldrich, 20μg/ml) was used as a secondary antibody for all primary antibodies (VE-cadherin, total VEGF, VEGFb and PECAM-1 antibodies).

5.3.5 H&E staining

Haematoxylin and Eosin (H&E) staining was performed to allow structural detail to be evaluated. Slides were rehydrated in tap water, submerged for 5 minutes in Harris Haematoxylin, and washed in a bath of running tap water until the water cleared. They were then dipped in acid alcohol for 10 seconds, washed until there were no streaks on the slides, and dipped in Lithium Carbonate/Scott's Tap Water. Slides were then submerged in 1% Eosin for 5 minutes, and washed again in tap water. A series of dehydration steps were then performed, by dipping the slides in 50%, 70%, 90% and 100% alcohol for 10 seconds each. Finally, slides were dipped in fresh and finishing xylene for 2 minutes each, and mounted with DPX.

5.3.6 Analysis of data

The percentage of immunopositive vessels was analysed using selective random sampling and unbiased counting (as previously described in section 2.3.9 and Figure 2.2). The vessels which showed staining were sub-divided according to their location, using a 1-3 scale (location 1 being cytoplasmic localisation; location 3 being junctional localisation, see Figure 2.3A-D) for VE-cadherin only. The percentage of vessels showing leakage was detected using the PECAM-1 staining as a guide to where vascular profiles were in the FITC channel, and switching to the TRITC channel of the same area to see whether there were any hotspots around the vascular profiles (signifying leakage of 76Mr dextran tracer from the fetal circulation).

GraphPad Prism version 5.01 statistical software was used for statistical analysis. The Mann-Whitney U test was used to evaluate statistical significance for most groups. When analysing

the tracer leakage (functionality of the perfused placenta), our euglycaemic perfusion was also compared to tracer leakage from a 1h euglycaemic perfusion, with all conditions being the same except for duration, to make sure our longer 3h perfusion did not cause a functional change in the placenta. For correlations between tracer leakage and total VEGF or VEGFb Spearman correlation was used. For the glucose concentration analysis, the euglycaemic perfusion samples were compared to each other and to the hyperglycaemic samples with a Mann-Whitney U test (5mM maternal vs. 5mM fetal and 5mM fetal vs. 15mM fetal).

5.4 Results

5.4.1 Functional characteristics of 3h perfusion

The 3h perfusion exhibited normal pressure readings, which stayed constant for the duration of the perfusion at 20-80mmHg for the maternal circulation, and <20mmHg for the fetal circulation (Fig. 5.2).

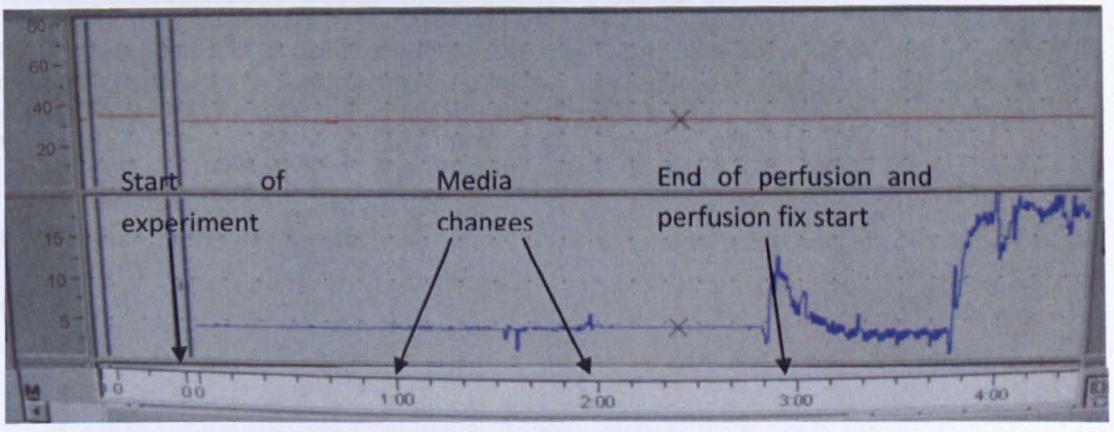


Figure 5.2: Representative trace of maternal (red trace) and fetal (blue trace) pressure throughout the 3h perfusion. There is no change in pressure in either circulation in the 3h perfusion. The change in pressure seen in the fetal trace at the end of the perfusion corresponds to the PFA fixation stage and the handling of the placenta following this.

Comparison with a previous set of 1h perfusions showed that there was no significant difference ($p>0.05$) in the number of vessels showing leakage, thus ensuring the integrity of the placental endothelial barrier during the 3h perfusion (Figure 5.3).

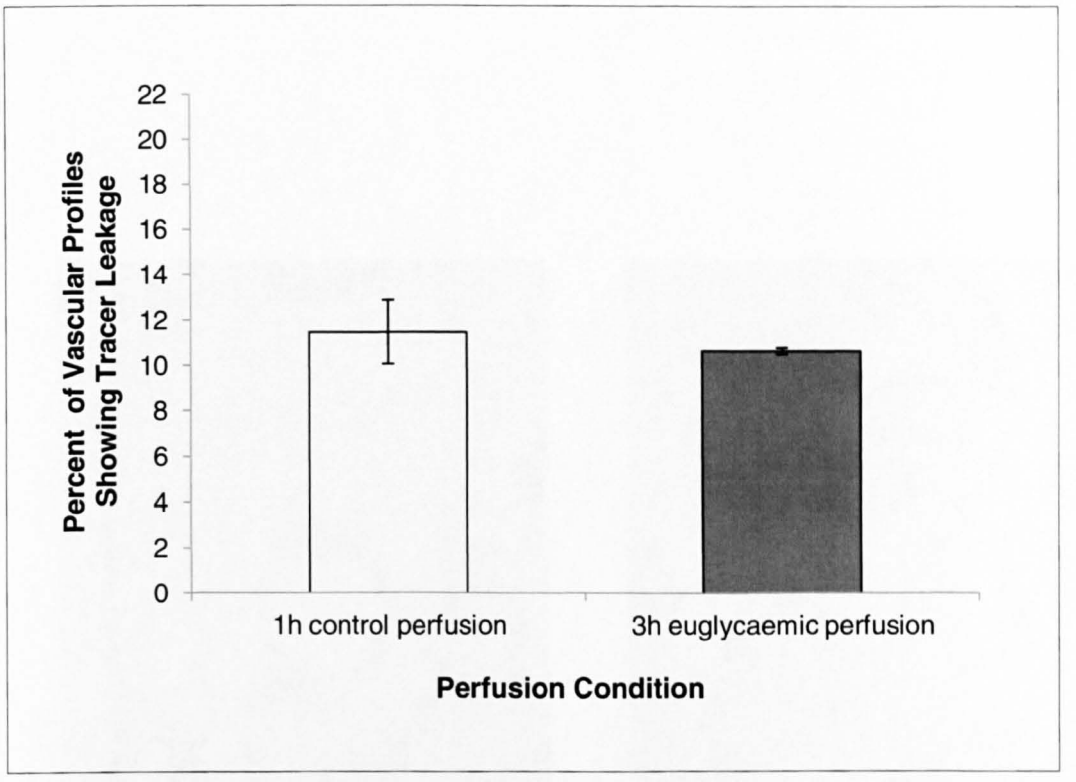


Figure 5.3: Evaluation of 3h perfusion on functionality of the placenta. No significant differences ($p>0.05$) were found between a 1h eugluycaemic perfusion and a 3h euglycaemic perfusion in the leakage of 76Mr dextran. Data (n=3 for both 1h control perfusion and for 3h euglycaemic perfusion) was analysed using Mann-Whitney U test.

Furthermore, H&E staining showed normal placental morphology and no damage to tissue (Figure 5.4).

5.4.2 Molecular phenotype of the perfused placenta

The expression of VE-cadherin, total VEGF and VEGFb in euglycaemic and hyperglycaemic perfusions is illustrated in Figure 5.5.

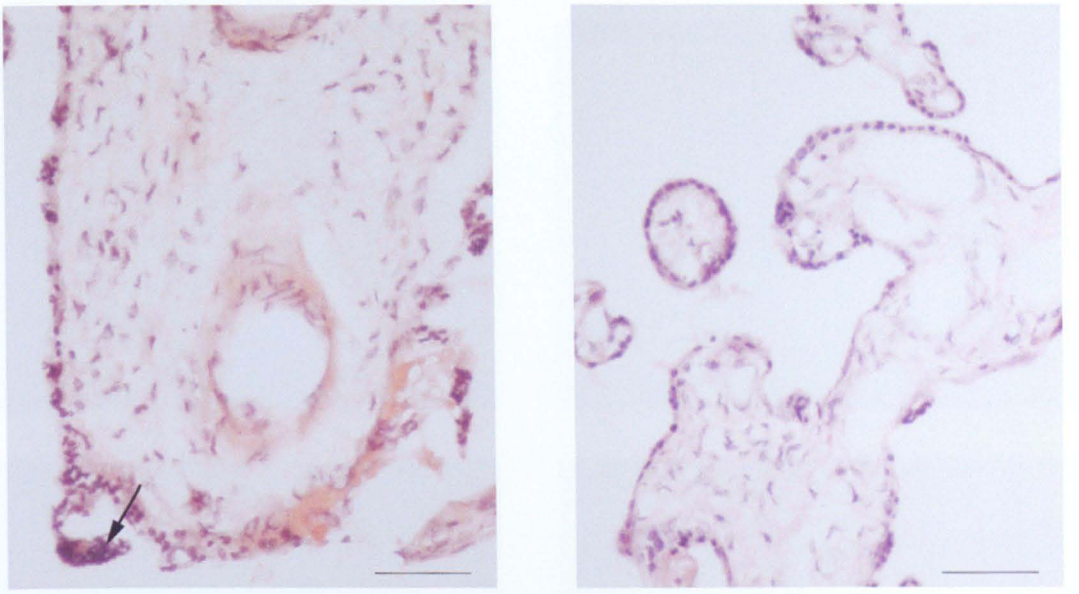


Figure 5.4: H&E staining of perfused placenta, showing normal morphology. A: Stem villous with a central vessel surrounded by fibrin (orange colour). A syncytial knot can be seen in the process of separating from the trophoblast (arrow), a normal process in term placentae. B: Intermediate and terminal villi. In both micrographs, the morphology of the tissue shown looks normal. Magnification: x200. Scale bar: 50um.

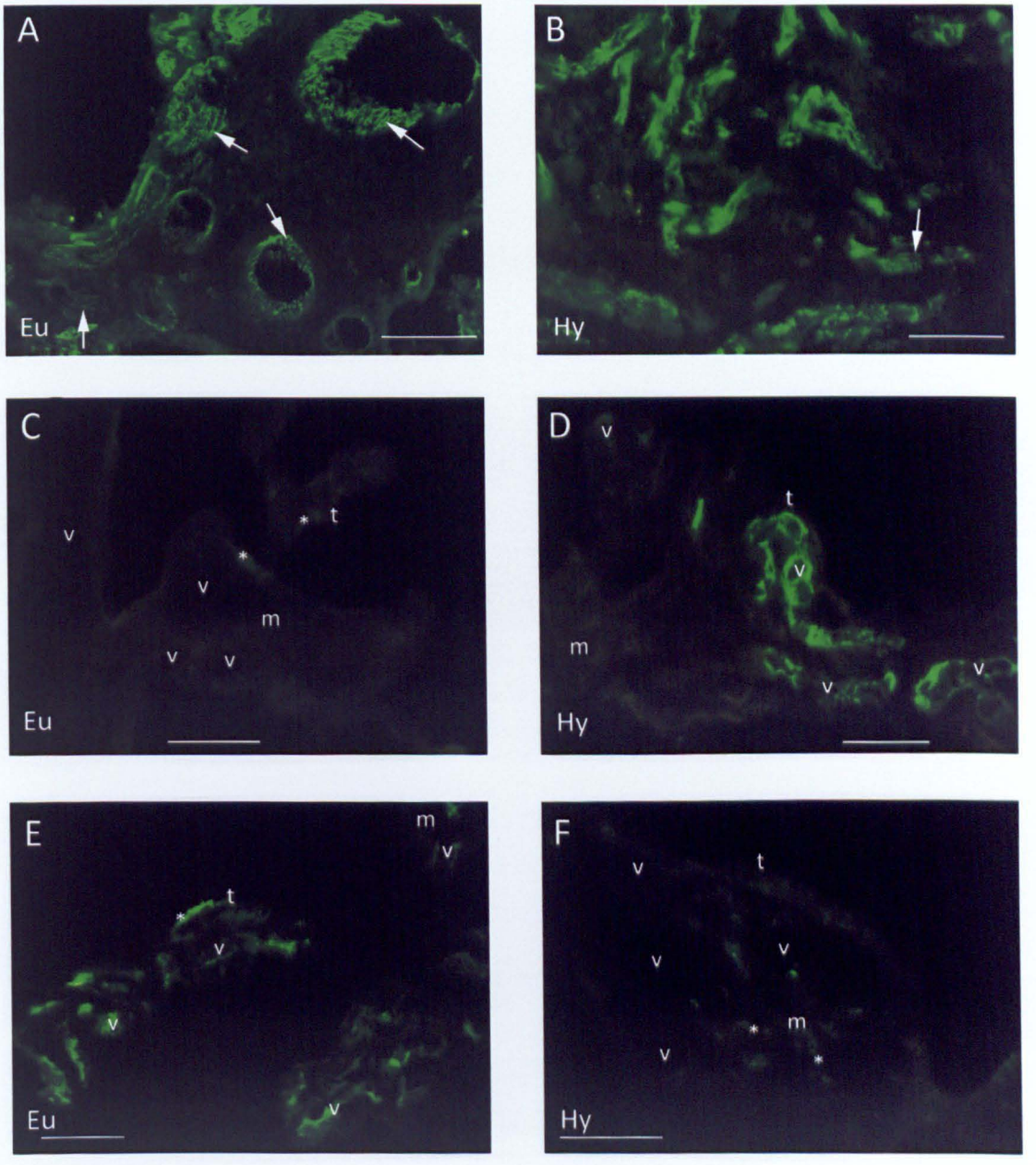


Figure 5.5: Molecular phenotype of euglycaemic and hyperglycaemic perfused placentae. A: VE-cadherin phenotype in euglycaemic perfused placentae, found mostly at junctional locations. B: VE-cadherin phenotype in hyperglycaemic perfused placentae, displaying more VE-cadherin in cytoplasmic location than the euglycaemic perfusions (A). C: Total VEGF phenotype in euglycaemic perfused placentae, showing few vessels immunopositive for VEGF, and some trophoblast immunoreactivity, but only minimal mesenchymal immunoreactivity. D: Total VEGF phenotype in hyperglycaemic perfused placentae, which display more vessels immunopositive for total VEGF than the euglycaemic perfusions (C). E: VEGFb phenotype in euglycaemic perfused placentae, showing vessels immunopositive to VEGFb, and some trophoblast (mesenchymal immunoreactivity shown in F). F: VEGFb phenotype in hyperglycaemic perfused placentae, showing fewer VEGFb immunopositive vessels than the euglycaemic perfusions (E). Glucose thus alters the perfused placenta molecular phenotype by reducing VE-cadherin junctional localisation, increasing total VEGF, and decreasing VEGFb immunoreactivity. Eu: euglycaemia, Hy: hyperglycaemia, arrow: VE-cadherin junctional location, v: vessel, t: trophoblast, m: mesenchyme, asterisks: non-vascular VEGF splice variants immunoreactivity. Magnification: x200. Scale bars: 100um.

5.4.2.1 VE-cadherin results

In both euglycaemic (Figure 5.5A) and hyperglycaemic (Figure 5.5B) perfusion, VE-cadherin was localised mostly in the paracellular junctions of the endothelial cells, giving beads-on-a-string pattern of staining, with little VE-cadherin having cytoplasmic localisation, giving diffuse staining. There was a statistically significant decrease ($p<0.05$) in VE-cadherin-immunopositive vessels on addition of 15mM glucose (Figure 5.6).

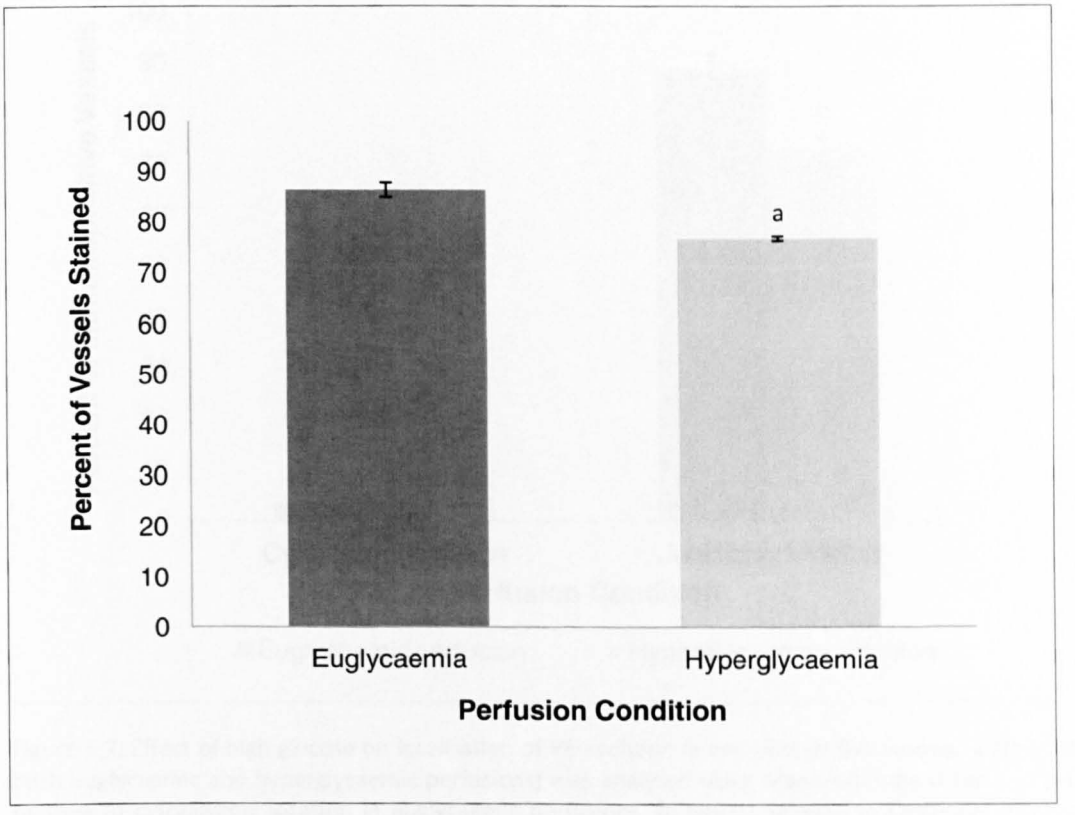


Figure 5.6: Effect of high glucose on percentage of vessels immunopositive to VE-cadherin. Data (n=3 for both euglycaemic and hyperglycaemic perfusions) was analysed using Mann-Whitney U test. a:$p<0.05$ relative to euglycaemia.

There was also a statistically significant decrease ($p<0.05$) in junctional VE-cadherin (location 3, as defined in Figure 2.3) in hyperglycaemic perfused placentae compared to euglycaemic-perfused placentae, and a statistically significant increase ($p<0.05$) in cytoplasmic localised VE-cadherin (location 1, see Figure 2.3) in the placentae perfused with hyperglycaemic media compared to the placentae perfused with euglycaemic media (Figure 5.7).

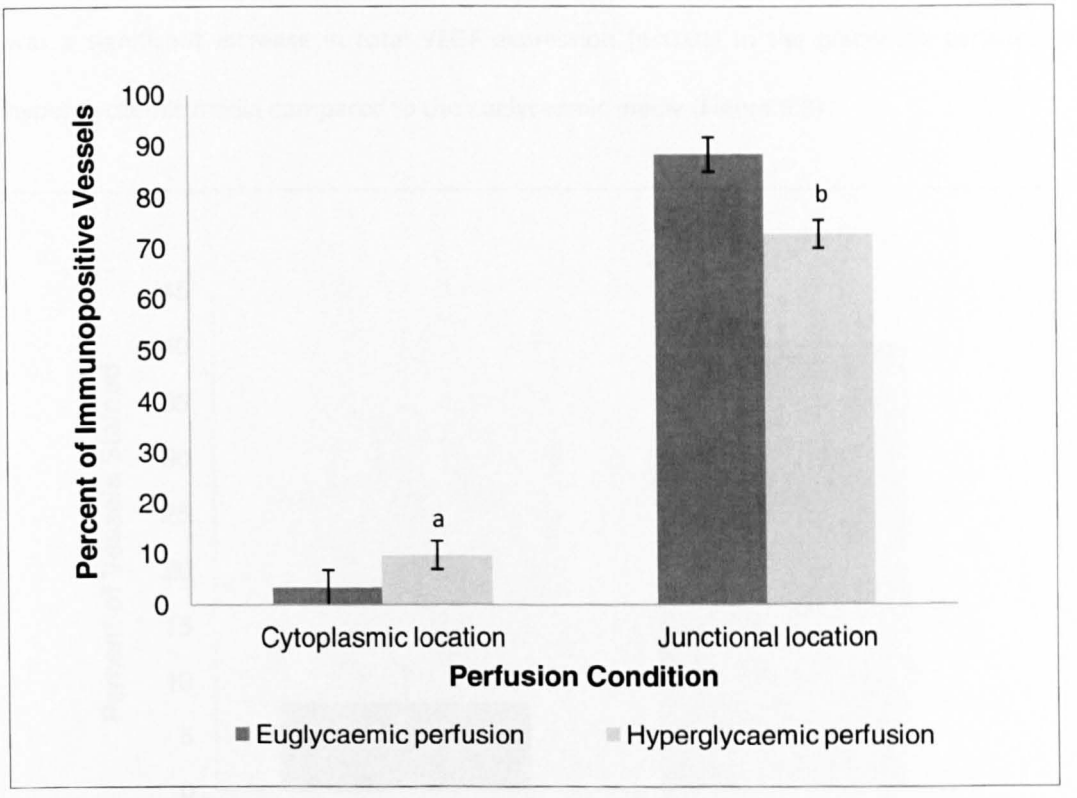


Figure 5.7: Effect of high glucose on localisation of VE-cadherin in immunopositive vessels. Data (n=3 for both euglycaemic and hyperglycaemic perfusions) was analysed using Mann-Whitney U test. a: $p<0.05$ relative to cytoplasmic location in euglycaemic perfusions, b: $p<0.01$ relative to junctional location in euglycaemic perfusions.

5.4.2.2 Total VEGF results

Total VEGF was immunolocalised to the fetal vessels in the chorionic villi, partly in the trophoblast and minimally in the mesenchyme in normal euglycaemic placentae; this pattern of total VEGF localisation was unchanged in the hyperglycaemic perfusions (Figures 5.5C-D).

Only the total VEGF in the placental vessels was counted, as per the aims of this chapter. There was a significant increase in total VEGF expression ($p<0.01$) in the placentae perfused with hyperglycaemic media compared to the euglycaemic media (Figure 5.8).

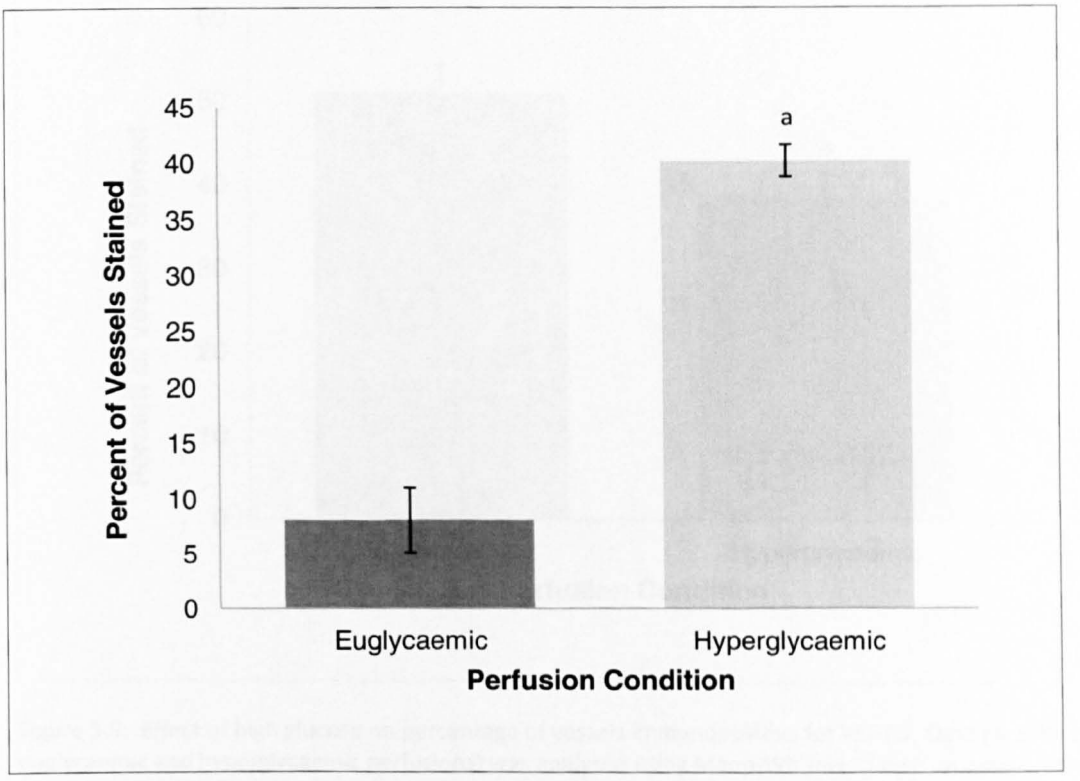


Figure 5.8: Effect of high glucose on percentage of vessels immunopositive for total VEGF. Data (n=3 for both euglycaemic and hyperglycaemic perfusions) was analysed using Mann-Whitney U test. a: $p<0.01$ relative to euglycaemic perfusion.

5.4.2.3 VEGFb results

Under both euglycaemic and hyperglycaemic perfusions, VEGFb immunolocated in fetal vessels' endothelium, perivascular layer and trophoblast (Figure 5.5E-F). The percentage of VEGFb immunolocalisation in the latter two tissues was not counted as it did not answer the aims of this chapter. There was a significant decrease in VEGFb immunopositive vessels in the hyperglycaemic perfusion group compared to the euglycaemic group ($p<0.05$) (Figure 5.9).

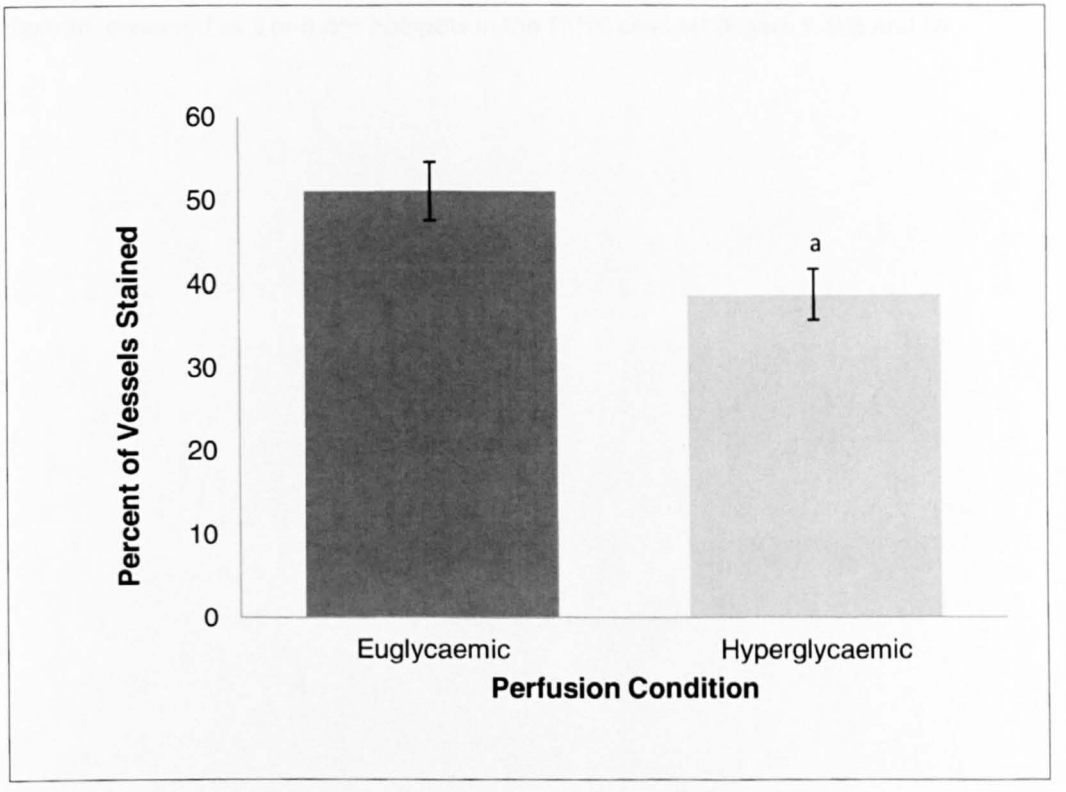


Figure 5.9: Effect of high glucose on percentage of vessels immunopositive for VEGFb. Data (n=3 for both euglycaemic and hyperglycaemic perfusions) was analysed using Mann-Whitney U test. a: $p<0.05$ relative to euglycaemic perfusion.

5.4.3 PECAM-1 immunoreactivity and tracer leakage

Immunopositive vessels were not counted, but on visual inspection all vessels were immunopositive to PECAM-1, except vessels with very severe tracer leakage. This was true for both euglycaemic and hyperglycaemic perfusions (Figure 5.10A and C).

Leakage was analysed by comparing PECAM-1 immunolocalisation, to find vascular profiles (as PECAM-1 is known not to be affected by glucose), on the FITC channel, and detection of 76Mr dextran, observed as 3 or more hotspots in the TRITC channel (Figure 5.10B and D).

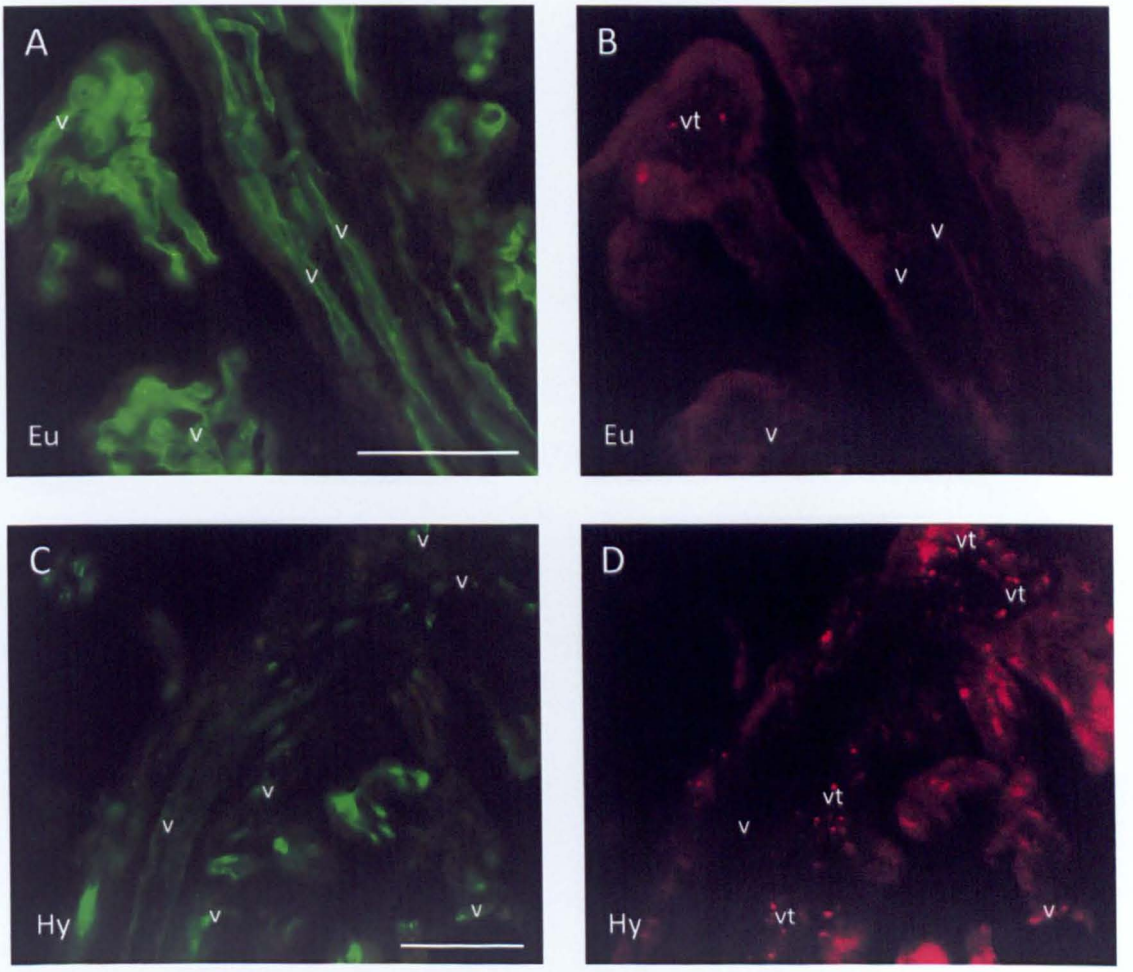


Figure 5.10: Functional phenotype of euglycaemic and hyperglycaemic perfused placentae. A: PECAM-1 immunoreactivity in euglycaemic perfused placenta (Eu), highlighting where vessels (v) are located, taken in the FITC microscope channel. B: Corresponding TRITC microscope channel illustrating the same field of view as A, showing there is tracer leakage (vt) in one vessel out of four highlighted. C: PECAM-1 immunoreactivity in hyperglycaemic perfused placenta (Hy), highlighting where vessels (v) are located, taken in the FITC microscope channel. D: Corresponding TRITC microscope channel illustrating the same field of view as C, showing there is more vessels showing tracer leakage (vt). Eu: euglycaemia, Hy: hyperglycaemia, v: vessels, vt: vessels with tracer hotspots. Magnification: x200. Scale bar: 100um.

By comparing the PECAM-1 immunoreactivity in the FITC channel with the tracer leakage hotspots in the TRITC channel, we found that there was a mild significant increase ($p<0.05$) in tracer leakage in the vessels of the hyperglycaemic perfusion compared to the euglycaemic perfusion (Figure 5.11).

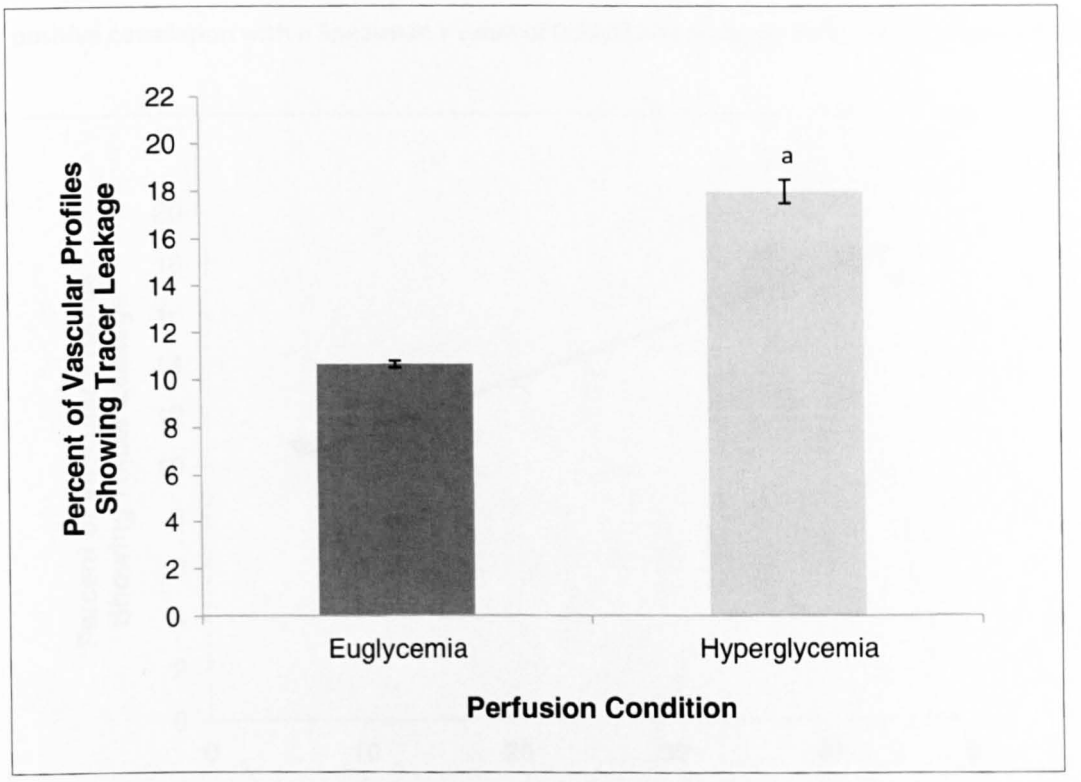


Figure 5.11: Effect of hyperglycaemia on tracer leakage. Data (n=3 for both euglycaemic and hyperglycaemic perfusions) was analysed using Mann-Whitney U test. a: $p<0.05$ relative to euglycaemia.

5.4.4 Correlation of tracer leakage to total VEGF and to VEGFb

Spearman correlation analyses were conducted between the percentage of vessels exhibiting tracer leakage and total VEGF and splice variant VEGFb.

Spearman correlation between tracer leakage and total VEGF revealed a non-significant ($p>0.05$) positive correlation with a Spearman r value of 0.5429 and an alpha value >0.05. (Figure 5.12).

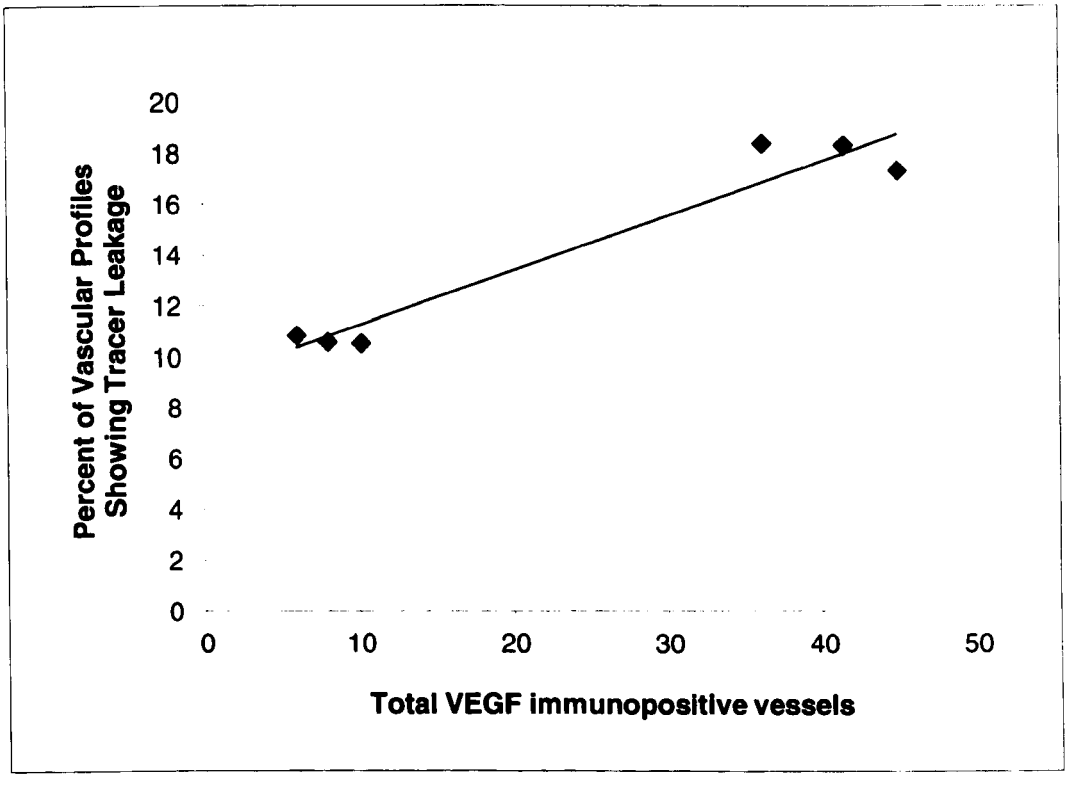


Figure 5.12: Non-significant ($\alpha>0.05$) positive ($r=0.5429$) correlation between total VEGF and tracer leakage. Data ($n=6$) was analysed with Spearman correlation.

A statistically significant ($p<0.05$) negative correlation was revealed between tracer leakage and VEGFb with a Spearman r value of -0.8857 and an alpha value <0.05 (Figure 5.13).

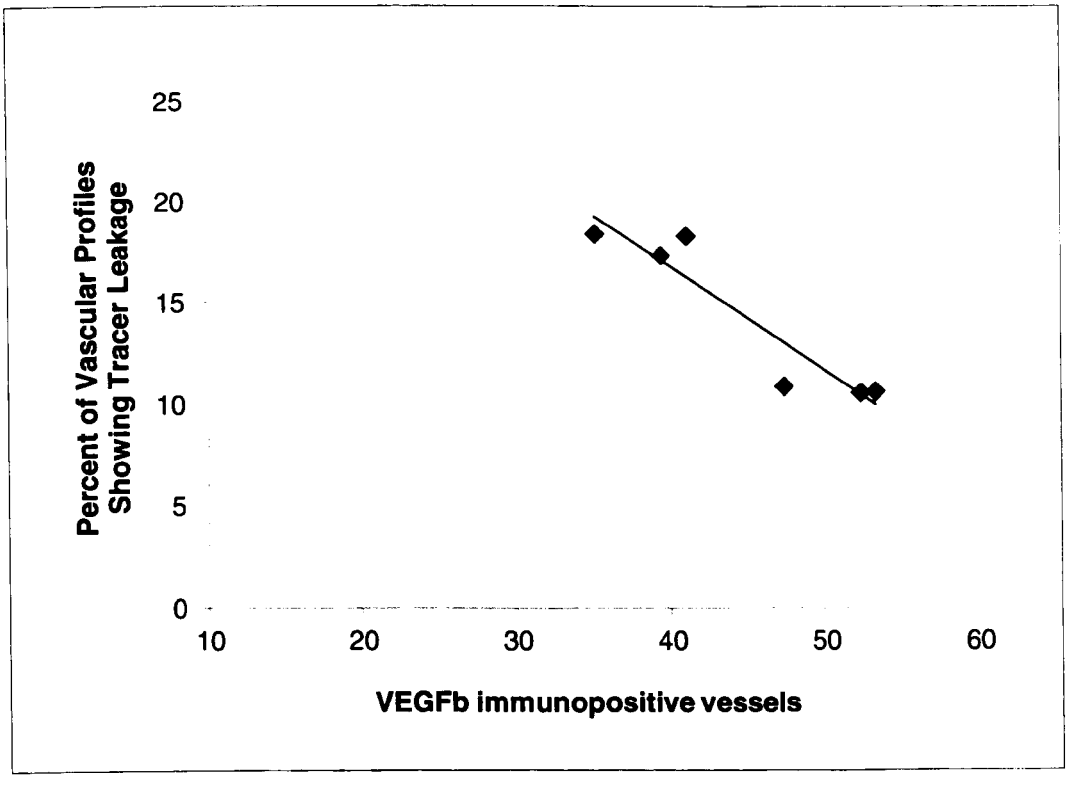


Figure 5.13: Significant ($\alpha<0.05$) negative ($r=-0.8857$) correlation, between VEGFb and tracer leakage. Data ($n=6$) was analysed with Spearman correlation.

5.4.5 Glucose measurements

The results shown is section 5.4.5.1 and 5.4.5.2 refer to measurements with the YSI Model 2300 STAT PLUS Glucose and Lactate Analyser. The results of the glucose measurements by HemoCue Glucose 201+ blood glucose analyser are shown in Appendix 3.

5.4.5.1 Measured glucose readings vs. actual glucose concentrations

The YSI Model 2300 STAT PLUS Glucose and Lactate Analyser gave the most precise results for the known concentration samples, although the 5mM and 15mM glucose concentration samples seemed to be consistently low (Table 5.1).

	Control readings:		
0mM glucose	0	0.02	0.05
5mM glucose	3.92	3.89	3.85
10mM glucose	10.5	10.3	10.3
15mM glucose	13.9	14.1	14.1
20mM glucose	20.5	20.4	20.8

Table 5.1: Glucose measurements (using YSI Model 2300 STAT PLUS Glucose and Lactate Analyser) of the known glucose concentration control samples.

5.4.5.2 Glucose measurement results

As a reminder, at the start of the experiment the fetal circulations of both groups were perfused with 5mM glucose. It was only the maternal circulations which were perfused with either 5mM glucose (for the so-called 'euglycaemic perfusions') or 15mM glucose ('hyperglycaemic perfusions').

In the fetal circulation media samples taken at 1h and 2h, there was a significant difference ($p<0.05$) in the glucose concentrations from the euglycaemic and hyperglycaemic perfusions (5mM and 15mM glucose in the maternal circulations, respectively), whilst the media sampled at the end of the perfusion protocol (3h) did not reveal any differences. There were also no significant differences ($p>0.05$) between the fetal and maternal circulation samples from the euglycaemic perfusions (Figure 5.14).

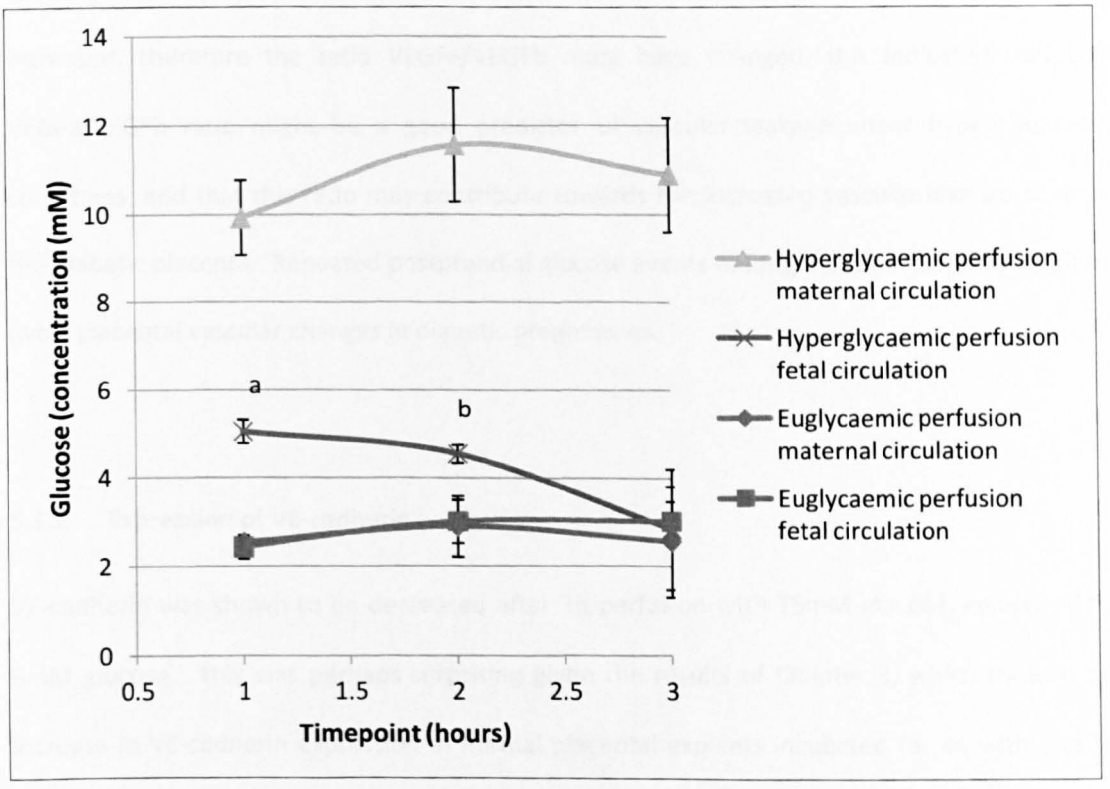


Figure 5.14: Glucose measurements in the maternal and fetal circulations of the euglycaemic and hyperglycaemic perfusions. The hyperglycaemic perfusion maternal circulation was not included in the analysis, given its known higher glucose concentration. Data (n=3 for all timepoints and circulations) was analysed using Mann-Whitney U test. a: $p<0.05$ relative to 1h euglycaemic perfusion fetal circulation, b:$p<0.05$ relative to 2h euglycaemic perfusion fetal circulation.

5.5 Discussion

5.5.1 Summary of results

The statistically significant decrease of both VE-cadherin junctional localisation (Figures 5.5A-B and 5.7) and VEGFb expression (Figures 5.5E-F and 5.9), and the statistically significant increase in total VEGF expression (Figures 5.5C-D and 5.8) seen in hyperglycaemic perfusions compared to euglycaemic perfusions indicate that there might be a phenotypic change in the vasculature of the perfused placenta after a 3h 15mM glucose insult. Indeed, we found that the functionality of the hyperglycaemic-perfused placenta had changed, and was mildly but statistically significantly more leaky to 76Mr dextran (Figures 5.10B and D and 5.11). There was a significant correlation ($\alpha<0.05$, $r=-0.8857$) between VEGFb and the percentage of vascular profiles showing leakage (Figure 5.13), but not between the latter and total VEGF (Figure 5.12). Similarly to Chapter 4, as total VEGF increased, and VEGFb decreased, VEGFa must have increased, therefore the ratio VEGFa/VEGFb must have changed; this indicated that the VEGFa/VEGFb ratio might be a good predictor of vascular leakage under hyperglycaemic conditions, and that this ratio may contribute towards the increased vascular leakage seen in the diabetic placenta. Repeated postprandial glucose events of long duration (3h) may result in overt placental vascular changes in diabetic pregnancies.

5.5.2 Expression of VE-cadherin

VE-cadherin was shown to be decreased after 3h perfusion with 15mM glucose, compared to 5mM glucose. This was perhaps surprising given the results of Chapter 3, which showed an increase in VE-cadherin expression in normal placental explants incubated for 4h with 15mM glucose. This highlights the importance of the 3D architecture and a more physiological method with flow present in the perfused vascular bed as opposed to the limited 3D architecture of the explants with no flow. The placental perfusion protocol uses intact placental tissue, without

any vascular injury except at the site of cannula insertion. The vasculature in explants instead displayed considerable injury, as they were severed from the main placenta organ in the excision process. The sample of placenta which was fixed before the excision process (Figure 2.6C) although unusable for counting due to the high number of red blood cells remaining in the tissue, did show a majority of vessels with intensity 3 VE-cadherin immunoreactivity, resembling the phenotype seen in normal perfused placenta. This could indicate that the tissue injury from the excision process in Chapter 3 caused the initial decrease in VE-cadherin immunopositive vessels, hence explaining the difference in our VE-cadherin results between Chapter 3 and Chapter 5.

Leach and colleagues have shown that in short normal placental perfusions, VE-cadherin expression ranges between 92.9-99.3% of all vascular profiles (Babawale *et al.*, 2000; Leach *et al.*, 2004). In our 3h euglycaemic perfusion (Figure 5.5A), we are seeing 86.3% immunopositive vessels (Figure 5.6), a decrease of around 10 percentage points from previous studies. This could be due to the long duration of perfusion, as some vessels could be under stress from lactic acid produced by the stressed placenta. However, we have also observed that the functional role of the placenta was not disturbed, as tracer leakage after the 3h perfusion was statistically similar to that of a 1h perfusion (Figure 5.3). Furthermore, most (88.2%) of the VE-cadherin immunoreactivity was in a junctional location (Figure 5.7), and therefore is contributing to junctional integrity.

Hyperglycaemia (Figure 5.5B) caused a decrease in VE-cadherin immunoreactivity to 76.6% (Figure 5.6). Interestingly, this is similar to the 79.6% VE-cadherin immunopositive vessels seen in GDM (Babawale *et al.*, 2000). This makes logical sense, as GDM presents in the second or third trimester of pregnancy after vascular maturation, and is therefore a shorter duration of insult than Type 1 diabetes where the vasculogenesis, angiogenesis and maturity of the placenta occur in a hyperglycaemic environment.

However, if we look at the difference between euglycaemic and hyperglycaemic VE-cadherin immunoreactivity (hence taking into account the lower VE-cadherin seen in our euglycaemic perfusion), the decrease in VE-cadherin immunopositive vessels in GDM compared to normal placentae in Babawale *et al.* (2000) is around 19 percentage points, whilst in our 3h perfusion it is around 10 percentage points (Figure 5.6), and furthermore the VE-cadherin junctional location (location 3) decreases in our hyperglycaemic perfused placentae compared to the euglycaemic perfused placentae (72.5% vs. 88.2%, a difference of ≈15 percentage points) (Figure 5.7). In this analysis, the decrease in VE-cadherin immunoreactivity in hyperglycaemia compared to euglycaemia is about half of that seen in GDM. This would mean that one hyperglycaemic insult is not sufficient to create the VE-cadherin phenotype seen in GDM, and therefore multiple hyperglycaemic insults and/or other factors contribute to the GDM phenotype. This would give hope for treatment of GDM patients after diagnosis, as the damage to the placental vasculature could be limited by monitoring and counselling the patient.

VE-cadherin is a junctional protein, which regulates cell-cell adhesion through adherens-junctions. However, one must remember that these types of junctions are not the only regulators of endothelial leakiness, as tight junctions play an important and size-limiting role. Thus, whereas VE-cadherin is lost from 10% of vessels, concomitant loss of tight junctional integrity in large vessels is also needed for the altered phenotype of increased leakage. We shall discuss leakage in our perfusion model in section 5.5.5.

5.5.3 Expression of total VEGF

Total VEGF levels after the euglycaemic perfusion (Figure 5.5C) were 7.7% of all vessels (Figure 5.8). This is a very small percentage of vessels, and corresponds well with the findings of Leach *et al.* (2004), who saw only minimal VEGF immunoreactivity in normal perfused placentae.

Under 3h 15mM glucose insult, the level of total VEGF increases substantially (Figure 5.5D), being found in 38.8% of vessels (Figure 5.8). This is a very large increase, and we would have previously expected a large increase in permeability as a result. However, a couple of considerations need to be made.

VEGF increases permeability in part by VEGFR-2 phosphorylating VE-cadherin, which in turn will disrupt the junctional stability (Esser *et al.*, 1998). However, as discussed in section 5.5.2, there was only about a 15 percentage point difference in VE-cadherin junctional localisation (location 3) in response to hyperglycaemia compared to euglycaemia (Figure 5.7). This suggests that not all VEGFR-2 are being activated by VEGF.

We know that VEGFb acts as a competitive inhibitor of VEGFa (Cebe Suarez *et al.*, 2006). Therefore, more total VEGF being present does not necessarily mean more VEGFa bound to VEGFR-2, and therefore more total VEGF would not necessarily mean an increase in VEGF-induced leakage. This theory is in agreement with a perfusion study by Brownbill *et al.* (2007), where VEGF-containing perfusions for 30 minutes from the fetal circulation had no effect on the tracer leakage they observed.

Another consideration is that adherens junctions (and VE-cadherin) are not the only junctions which need to be disrupted to cause an increase in permeability. Tight junctions also need to be destabilised before increased leakage can occur. It has been shown in rat retinas (Antonetti *et al.*, 1999) that following VEGF injection there was an increase in occludin phosphorylation which was maximal 45 minutes following injection and had started to reverse by 90 minutes (i.e., phenotype not permanent). Another study showed that this occludin phosphorylation was caused via PKC activation by VEGF (Harhaj *et al.*, 2006). Importantly, this pathway is mediated by the VEGFR-2 receptor, so this is also potentially subject to VEGFb competitive inhibition. None of these studies test the effect of VEGFb on occludin. Therefore, analysis of VEGFb is essential to understand the mechanisms at play in a 3h hyperglycaemic perfusion.

5.5.4 Expression of VEGFb

VEGFb was present in 51.0% of vessels after a 3h euglycaemic perfusion (Figures 5.5E and 5.9). This was somewhat lower than what Bates *et al.* (2006) found in normal placental samples, but their samples included amnion, chorionic villi and stratum basale, whilst we were looking at VEGFb expression in placental vessels only, so a difference in expression between our results and Bates' is not of concern.

After a 3h hyperglycaemic perfusion (Figure 5.5F), 38.6% of vessels were immunopositive for VEGFb (Figure 5.9), representing a decrease of 12 percentage points compared to euglycaemic perfusion (Figures 5.5E and 5.9). Decreases in VEGFb expression have been found in diabetic vascular complications such as diabetic retinopathy (Perrin *et al.*, 2005), as well as in common diabetic pregnancy complications, such as pre-eclampsia (Bills *et al.*, 2009; Bates *et al.*, 2006). However, to the best of our knowledge, this is the first time that VEGFb decrease has been shown following such an acute, short and mild (only one 3h 15mM glucose hit) insult.

In an abstract by Reeve *et al.* (2006), VEGFb levels in vessels from GDM placentae were not seen to be significantly different from normal placentae, whilst the levels in placentae from Type 1 diabetics were significantly lower than normal and GDM placentae. However, there was a progressive decrease in VEGFb from normal>GDM>Type 1. The standard deviation was higher than in our experiments, possibly because the glycaemic levels present in the placentae immediately before perfusion could have been different (i.e., some could have been in a hyperglycaemic environment immediately before delivery, some in an euglycaemic environment). However, the non-significant decrease between VEGFb levels in GDM compared to the normal placentae was of 10 percentage points (normal 50%, GDM 40%) (Reeve *et al.*, 2006), which is similar to our data. Therefore, VEGFb may play a role in GDM pathological phenotypic changes.

The decrease in VEGFb represents an uneven decrease compared to the increase in total VEGF (VEGFb 12 percentage point decrease, total VEGF >30 percentage point increase, Figures 5.9 and 5.8, respectively). If VEGFb were acting as a competitive inhibitor of VEGFa, as described by Cebe-Suarez *et al.* (2006), VEGFb would be the determinant splice variant that regulates VEGF-induced leakage, as an increase in VEGFa would not be sufficient if the VEGFR-2 receptor responsible for mediating its effects were already being bound by the inhibiting splice variant VEGFb.

5.5.5 Tracer leakage

After the 3h euglycaemic perfusion (Figure 5.10B), 10.6% of vascular profiles showed tracer leakage to 76Mr dextran tracer (Figure 5.11). This compares well with the study of Leach *et al.* (2004) which shows 'minimal' tracer glucose in normal placentae perfused only for a 20 minute equilibration period followed by a ten minute tracer perfusion. Furthermore, in their abstract, Reeve *et al.* (2006) reveal that there were 10.75% of normal vascular profiles leaky to 76Mr dextran tracer, which is similar to our findings.

In our hyperglycaemic perfused placentae (Figure 5.10D), we observed 17.9% of vascular profiles leaky to 76Mr dextran tracer (Figure 5.11). This relates well with studies on diabetic patients which have shown increases in permeability in other tissues (Yamaji *et al.*, 1993; Beals *et al.*, 1993; Huber *et al.*, 2006). We can add the placenta to the list of tissues that become leakier in response to high glucose. Hempel *et al.* (1997) have shown that, at least in porcine aortic endothelial cells, the increased permeability is due to activation of the PKC pathway; this pathway is therefore a possible cause of the increased leakage we observed in our perfused placentae. In the same paper, the authors discuss the fast response of this increase in permeability (peak permeability after 20mM glucose insult at 40 minutes), and this also fits in

well with our experiments, as we have seen an effect after 3h acute, as opposed to chronic, hyperglycaemic insult.

The difference in leakage between euglycaemic and hyperglycaemic perfused placentae is 7 percentage points (Figure 5.11), which relates well with the 10 percentage point decrease in VE-cadherin immunoreactivity (Figure 5.6) and the 12 percentage point decrease in VEGFb expression (Figure 5.9), but less so to the 31 percentage point increase in total VEGF expression (Figure 5.8). This could indicate that VEGFb decreases are more predictive of vascular leakage than total VEGF increases in those tissues that express the b splice variant.

Our 17.9% vascular leakage also relates well with the findings of Reeve *et al.* (2006) that show 76Mr dextran tracer leakage in 20.12% of vascular profiles in GDM placentae. These very similar results indicate that the phenotype seen in GDM can be caused by a single postprandial glucose excursion, and that subsequent hyperglycaemic insults may add very little to the final phenotype. Of course though, the phenotype we observed in this chapter may be reversible, and may need further hyperglycaemic insults to become permanent.

In their paper of perfused Type 1 placentae, Leach *et al.* (2004) do not give the percentage of vascular profiles showing leakage. The leakiness of the Type 1 placenta is, however, likely to be more than in either the functional phenotype we observed in section 5.4.3 or the GDM phenotype, as in Leach *et al.* (2004) the Type 1 placenta has a much more pronounced phenotype in terms of increases of total VEGF and decreases of VEGFb.

In the hyperglycaemic perfusion, the functional effect of the increased total VEGF and decreased VEGFb was a mild but significant increase in leakage (from 10.6% to 17.9%, Figure 5.11). We would not expect to see too much change in the functional phenotype of the placenta, as we must remember that even poorly controlled diabetic patients, although with complications such as macrosomia, are capable of successfully carrying a pregnancy, and

therefore any functional changes in the vasculature of their placentae must be subtle in nature, otherwise their offspring would not survive.

VEGFb has been shown to be important in vascular permeability. However, whether the VEGFb decrease shown here is a cause or consequence of the increased leakage, has not been investigated. However, as VEGFb has been shown in human glomerular endothelial cells to cause decreased permeability (Bevan *et al.*, 2008), it is likely that this is the case in our placental perfusion model. The causality of the VEGFb decrease could be investigated by performing a timecourse experiment, where the tracer leakage is monitored and the beginning of leakage observed. If a decrease in VEGFb is detected shortly before the occurrence of tracer leakage, then VEGFb is a possible cause of the increased permeability, whilst if the tracer leakage precedes the decrease in VEGFb, then the decreased splice variant is an effect of increased permeability. Such an experiment might require several preliminary experiments to ascertain the timepoint of increased leakage, and further experiments to identify the timing of VEGFb decrease. An *in vitro* cell culture experiment investigating the causality of VEGFb decrease could also be used, simplifying the setup but losing the 3D placental architecture present in the perfusion model, hence leading to a less accurate physiological model.

Whether the amount of VEGFa present is also important, and hence whether the ratio of VEGFa/VEGFb is critical in the vascular dysfunction exhibited by leakage, will be discussed next.

5.5.6 Correlation between VEGF splice variants and tracer leakage

There was a positive significant correlation ($p<0.05$) between VEGFb and the percentage of vascular profiles exhibiting leakage (Figure 5.13), but not between total VEGF and tracer leakage ($p>0.05$, Figure 5.12). As VEGFa is a pro-permeability and VEGFb is an anti-permeability splice variant, this result indicates that it is the proportion of the variants (ratio) rather than absolute quantities of the two molecules (total VEGF) that is important in the formation of leaks

in the otherwise normal vasculature in response to 15mM glucose, with alteration of the VEGFb splice variant being a predominant feature.

This is a clinically powerful correlation, as if this is shown to hold true not only in normal placentae subjected to one hyperglycaemic insult but also in diabetic placentae with multiple hyperglycaemic and other insults, it could pave a way for further treatment. We already know that HbA_{1c} levels cannot fully account for the macrosomia observed in diabetic pregnancies (Schwartz *et al.*, 1994), and that postprandial high glucose more accurately reflects the status of the placental-fetal unit (Jovanovic-Peterson *et al.*, 1991; Kyne-Grzebalski *et al.*, 1999). However, if the VEGFb to leakage correlation holds true for the conditions mentioned above and if the VEGFa/VEGFb ratio could be accurately measured, we could have an accurate predictor of vascular dysfunction in the placenta, and a predictor of macrosomia in the infant.

5.5.7 Our 3h perfusion model

It was mentioned in section 5.1.6 that a possible complication of using media instead of blood as a perfusate, is that media might cause leakage of the vasculature regardless of glycaemic levels. This was a concern to us, especially since we had a longer duration of perfusion than our laboratory usually employs (1h). However, we can see in Figures 5.3 and 5.11 that only 10.6% of vessels in the euglycaemic perfusion showed leakage, which is an acceptable level for the analysis of the effect of hyperglycaemia on the leakage of dextran. In addition, when compared to a 1h normal placental perfusion, which are normally performed in our laboratory, there is no statistically significant difference (10.6% vs. 11.5%, respectively, Figure 5.3). Furthermore, the pressure readings from both maternal and fetal circulations remained constant throughout the perfusion (Figure 5.2); therefore, no blockages and/or significant leaks occurred (Panigel, 1962), as these would have been shown in the pressure trace.

VE-cadherin immunolocalised to the junctional space in around 88% of vessels in euglycaemia, dropping to around 73% in hyperglycaemia (Figure 5.7). Although these values are higher than in explants (perhaps due to the excision artefacts in the latter), even the euglycaemic immunopositive vessels were fewer than seen in previous perfusions, where 92.9-99.3% of vessels were immunopositive for VE-cadherin (Babawale *et al.*, 2000; Leach *et al.*, 2004).

5.5.8 PECAM-1 immunoreactivity

PECAM-1 immunoreactivity did not change in the hyperglycaemic perfusion compared to the euglycaemic perfusion (Figure 5.10A and C), and was lost from the endothelium only when there was severe 76Mr dextran tracer leakage. In fact, in this experiment it was used primarily to visualise more clearly the endothelium around blood vessels in order to count tracer leakage. It has been shown that 24h exposure to high glucose (30mM) does not affect PECAM-1 location at the junctional space in HUVEC, and only after 13 days was PECAM-1 affected by the hyperglycaemic environment (Baumgartner-Parzer *et al.*, 1995). Furthermore, in short duration perfusions comparing placentae from Type 1 diabetic pregnancies to normal placentae, PECAM-1 expression remained similar (96.3% vs. 96.6%, respectively) (Leach *et al.*, 2004), demonstrating that in placental vessels long term exposure to high glucose, as seen in Type 1 diabetic pregnancies, has no effect on PECAM-1 immunoreactivity. Therefore, PECAM-1 was a useful marker to highlight the vasculature.

5.5.9 Glucose measurement methods

We detailed in Section 5.3.2 how we sampled the media to calculate the glucose concentration in it. To summarise, we attempted to calculate the glucose concentrations in this media by three methods: urine test-strips, HemoCue 201+ blood glucose analyser and YSI Model 2300 STAT PLUS Glucose and Lactate Analyser. The most precise results were obtained with the YSI

Model STAT PLUS Glucose and Lactate Analyser (Table 5.1), and are discussed in sections 5.5.9.2 and 5.5.10. The results from the HemoCue Glucose 201+ blood glucose analyser are shown in Appendix 3, and both this and the urine strips are discussed in section 5.5.9.1.

5.5.9.1 Problems arising from glucose test strips and HemoCue Glucose 201+ measurements

When using the glucose test strips, normally used for urine glucose testing, according to the manufacturer's instructions, the resulting colour values for the 5mM, 15mM and 25mM glucose standards all corresponded to the colour values at around 27.8mM glucose. As this did not give any valuable information, this test was discontinued. Human urine does not have the same osmolarity as human blood or our perfusion media, and this could have been the cause of the failure of the test-strips to accurately record the glucose concentrations in our media.

Thus, we then attempted the measurement of glucose concentration with the HemoCue Glucose 201+ glucose analyser, normally used for measuring blood glucose concentrations (according to the manufacturer). The control 5mM glucose perfusion media tested gave values of 4.5mM, 7mM and 8.6mM glucose (average 6.7mM glucose) and the control 15mM glucose tested gave values of 14.5mM, 20.5mM, and 20.6mM glucose (average 18.6mM glucose). These were very inaccurate values with great variation between them, and therefore the data from the perfused media samples would have been inaccurate, and no conclusions could be discerned from it (for results, see Appendix 3).

A search of the literature revealed that the HemoCue Glucose 201+ analyser is not ideal for measuring glucose in our perfusion media. Bellini *et al.* (2007) showed that this system tended to overestimate glucose values by around 1mM glucose. Furthermore, a study by Deshpande *et al.* (1996) reveals that the HemoCue Glucose 201+ analyser is more accurate in plasma than in whole blood, and identifies haematocrit as being a confounding factor in blood. Our perfusion

media contains albumin and dextran to replicate the osmolarity of blood, and therefore may be confounding our results.

Most research groups which measure glucose transport across the placenta use radiolabelled glucose, for example Schneider's group (2003). This may indeed be the most accurate and precise method of measuring glucose flux in the placenta. Also, the flux is measured in μmol/min/g, and therefore even an accurate measurement would have been meaningless, as we did not measure the weight of the perfused cotyledon. However, as measuring glucose flux across the placenta was not one of our main aims for these experiments, these additional methods were not included in our experimental design.

5.5.9.2 YSI Model 2300 STAT PLUS Glucose and Lactate Analyser measurements

Following the methods discussed in section 5.5.9.1, we then sought a more accurate and precise method of measuring glucose, to allow the identification of the small differences that we expect from our data. We therefore performed measurements again using the YSI Model 2300 STAT PLUS Glucose and Lactate Analyser. This latter method gave the most precise results, although the known glucose concentrations of 5mM and 15mM glucose gave consistently low readings (see Table 5.1). The results obtained by using this method will be discussed in the next section (5.5.10).

5.5.10 Glucose concentrations in perfused media

The results show that in response to hyperglycaemia in the maternal circulation (compared to euglycaemia in the maternal circulation), there is increased glucose flux across the placenta (and hence increased glucose concentration in the fetal circulation) in the first and second hours of the perfusion. In contrast, in the third hour there is less glucose movement from the

maternal to the fetal circulation (Figure 5.14). This is an interesting result which has implications for glucose management in the diabetic pregnancy, as even short maternal hyperglycaemic periods (1h) result in peaks in fetal blood glucose. In a laboratory setting, this short period between onset of hyperglycaemia and increased glucose transfer to the fetal circulation implies that shorter perfusions might be appropriate for future perfusion studies into glucose effects.

The placenta seems to show an adaptation to the hyperglycaemic insult by the third hour, with glucose levels returning to levels similar to the euglycaemic perfusions. However, at 3h, the standard deviation increased with respect to earlier timepoints (Figure 5.14), indicating a variation in individual placentae response to hyperglycaemia. Decreased flux across the placenta may be due to a decrease in GLUT1 transporters in the trophoblast, as reported by Hahn *et al.* (1998), who observed a decrease in GLUT1 mRNA and protein after a 24h 25mM glucose cell culture experiment with human trophoblast cells. Individual differences in this adaptation may have caused the variation we observed in our results.

There was no significant difference between the maternal and fetal euglycaemic perfusions. According to Schneider (2003), where there are equal maternal and fetal glucose concentrations, the maternal circulation provides 70% to the glucose uptake of the placentae, whilst the fetal circulation only contributes to 30%. This should theoretically lead to a significant difference between the maternal and fetal glucose circulations. At the 3h sampling, the euglycaemic maternal sample showed a small (non-significant) decrease compared to the euglycaemic fetal circulation (see figure 5.14). The small sample size and the variation seen among our samples could be masking the difference.

5.5.11 Critique of experimental design

The perfusion model we used in this chapter is the best physiological method we could have used under our circumstances. However, even though it represents a vast improvement over the explants methodology used in Chapters 2-4, it does not completely simulate the *in* utero environment. One of the problems is that the placental perfusion is metabolically stable, which might be unlike the placenta in pregnancy. Another point of note is that perfusions are unable to observe parameters such as changes in blood flow (Bourget *et al.*, 1995). However, the placental perfusion model is still to date the closest model to the *in utero* environment.

It is noteworthy that there is no osmolarity control in this experiment. We saw in Chapter 3 that mannitol was not a good control for glucose experiments in our settings. We could have used L-glucose if necessary, but it proved unnecessary, as the normal range of blood osmolarity is 280-303mOsm/l, and our perfusion media was 292 mOsm/l for the euglycaemic perfusate and 302mOsm/l for the hyperglycaemic media, i.e., both were within the normal range. Therefore, we decided that the use of a control was unnecessary, as the difference in osmolarity in such perfusates was of non-significant physiological importance.

5.5.12 The emerging diabetic phenotype

In this chapter, we have seen how high glucose can simulate the diabetic phenotype of high VEGFa, lowered VEGFb, disturbed junctional stability, and most importantly, differences in the functionality of the placenta in respect of leakiness of the vascular endothelium. However, we must remember that high glucose represents only half of the diabetic phenotype. The Pedersen hypothesis (1954) as explained in section 5.1.2 states that the fetus is exposed to high insulin levels *in utero* in response to high maternal glucose. How hyperinsulinaemia affects the phenotype of the human perfused placenta will be explored in section 6.2.2.4.

5.6 Summary

Our results indicate that there is an altered phenotype in the normal placenta when exposed to a single 15mM glucose insult for 3h. This phenotype resembles the placental phenotype seen in GDM, but not Type 1 diabetes, in terms of VE-cadherin immunolocalisation, VEGFb expression, and importantly, the functional change in vascular leakage to 76Mr dextran tracer. We have therefore succeeded in re-creating a diabetic phenotype with a single 15mM glucose insult.

VEGFb was then seen to be significantly correlated ($\alpha<0.05$) to the tracer leakage in our perfusion model, indicating that the measurement of the proportion of this splice variant compared to total VEGF might be an important predictor of vascular leakage, at least in normal placenta subjected to short (3h) hyperglycaemic insult.

Whether the diabetic phenotype can be recreated, and whether VEGFb plays the same important role in the development of vascular leakage for the other main complication in the diabetic pregnancy, fetal hyperinsulinaemia, is a major avenue for further investigation.

Chapter 6
General Discussion

6.1 Summary of findings

There are three main findings that have arisen from this piece of research that contribute to the body of knowledge in the placental vasculature field.

Firstly, the observation that a single 15mM glucose insult provokes a change in the phenotype of the vasculature of a normal term placenta by increasing the total VEGF expression and decreasing VEGFb expression (hence VEGFa must be increasing and therefore the ratio of the two splice variants must be changing) in the endothelial layer, recreating the phenotype seen in the diabetic milieu.

Secondly, the fact that VEGFb is differentially regulated by glucose than its splice variant VEGFa in the placenta, as glycaemic insult decreases VEGFb expression whilst total VEGF expression does not change in explant culture. This pattern of downregulation was altered in placentae from Type 1 diabetic pregnancies, where no downregulation of VEGFb expression was seen on further glycaemic insult.

Thirdly, the leakage to macromolecules seen in the perfusion model as a result of a 3h perfusion with 15mM glucose is significantly correlated with VEGFb but not total VEGF, a functional read-out which indicates that VEGFb is important in this process. As VEGFb decreased and total VEGF increased in hyperglycaemic perfusions compared to euglycaemic perfusions, VEGFa must be increasing, thereby changing the VEGFa/VEGFb ratio. This ratio may act as a predictor of vascular dysfunction in the placenta.

6.2 General Discussion

6.2.1 Possible models for glucose effects on placental vascular leakage

Three different possibilities for how glucose could be affecting the expression of total VEGF, VEGFb, VE-cadherin and leakage of the placental vasculature seen after acute 15mM glucose insult are here proposed:

The first model (Figure 6.1A), valid only in the explants, is that glucose exerts its effects on the trophoblast, and the trophoblast releases signalling molecules and hormones into the media which will then travel to the endothelial layer, which will subsequently become leaky under the effects of these molecules. Signalling molecules may be transported via the media to the endothelium in the explants studies. This is not possible in the perfused placenta as the two circulations are separate, and therefore the endothelium would not be exposed to any secreted substance in the maternal circulation. This model could theoretically be a possibility in the explants, as there is only one reservoir for the media (the well of the plate) and therefore secreted substances could be transferred through the media to the endothelium; however, the distance the media would have to travel in possibly collapsed vessels to the capillaries in terminal villi means that it is unlikely that the media the trophoblast is exposed to would reach the endothelium of the smallest vessels.

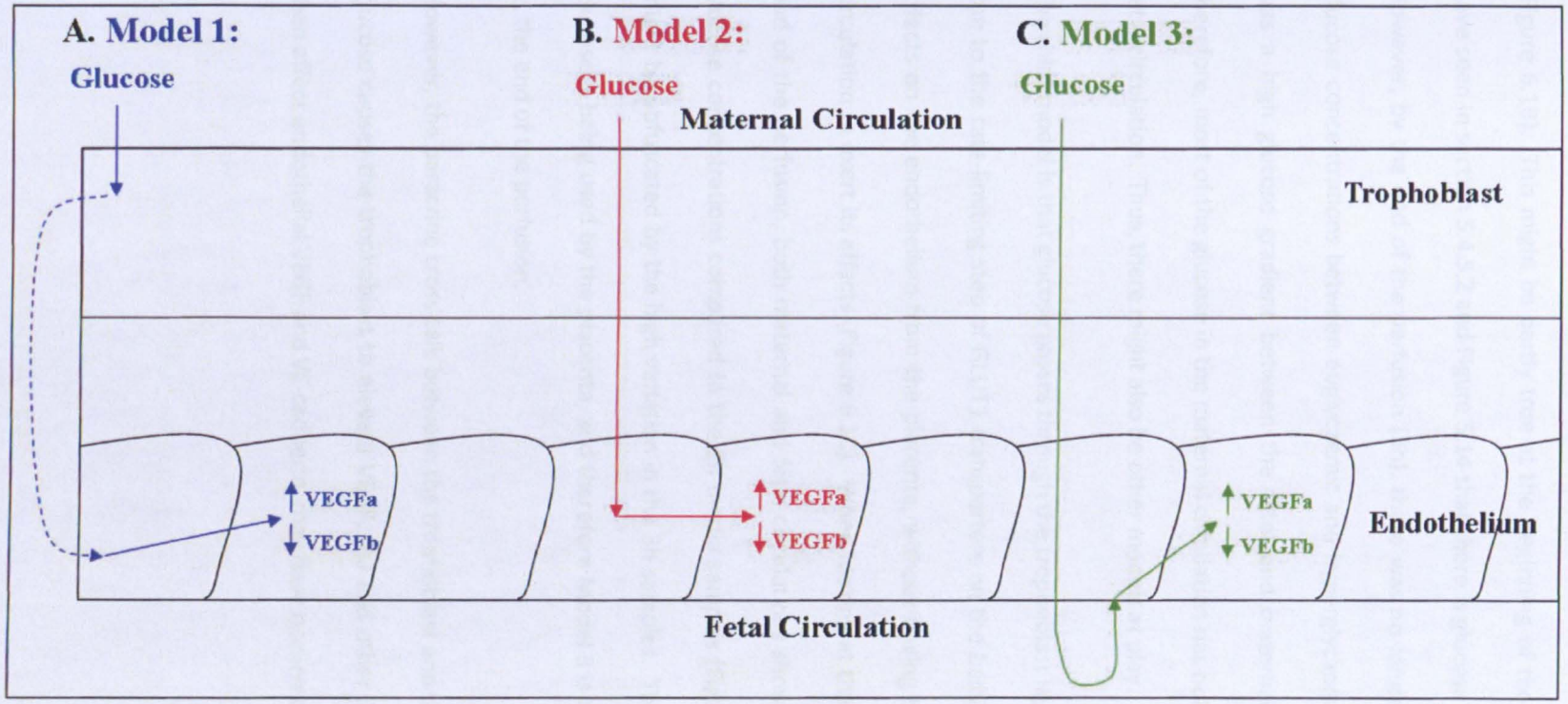


Figure 6.1A: Model 1 (in blue). Glucose exerts its effects on the trophoblast. It then releases pro-permeability molecules which diffuse through the trophoblast and mesenchymal layers. Here, they exert their effects on the endothelium, altering the expression of vascular VEGFa and VEGFb, resulting in a leakier phenotype. In explants only, the cytokines, hormones etc. could be secreted into the media, and may exert their pro-permeability effects on the endothelium by this route.

Figure 6.1B: Model 2 (in red). Glucose passes through both trophoblast and endothelium through facilitative diffusion to be released into the fetal circulation. Glucose then exerts its effects on the endothelium from here, altering the vascular expression of VEGFa and VEGFb, resulting in a leakier phenotype.

Figure 6.1C: Model 3 (in green). Glucose passes through the trophoblast by facilitative diffusion, but due to the rate-limiting step of GLUT-1 transport on the fetal-facing basal layer, it does not immediately cross the endothelial barrier. Glucose then exerts its pro-permeability effects from the placenta, increasing vascular VEGFa and decreasing vascular VEGFb and resulting in a leakier phenotype, without having to transport to the fetal circulation to have an effect.

The second model is that glucose passes through both the trophoblast layer and the endothelial layer by facilitative diffusion, and exerts its effects on the vasculature in the fetal circulation (Figure 6.1B). This might be partly true at the beginning of the perfusion (1h and 2h), as we have seen in section 5.4.5.2 and Figure 5.14 that there is glucose flux to the fetus at 1h and 2h. However, by the end of the perfusion (3h), there was no longer any significant difference in glucose concentrations between euglycaemic and hyperglycaemic fetal circulations and there was a high glucose gradient between the fetal and maternal hyperglycaemic circulations; therefore, most of the glucose in the maternal circulation has not traversed the placenta to the fetal circulation. Thus, there might also be other models at play.

The third model is that glucose passes through the trophoblast layer by facilitative diffusion, but due to the rate-limiting step of GLUT1 transporters on the basal (fetal-facing) layer, exerts its effects on the endothelium from the placenta, without having to diffuse through to the fetal circulation to exert its effects (Figure 6.1C). When looking at the 3h media samples, i.e., at the end of the perfusion, both maternal and fetal circulations show a non-significant decrease in glucose concentrations compared to the 2h media samples (Figure 5.14), although the results might be obfuscated by the high variation in the 3h samples. These results indicate that some glucose is being used by the placenta, and therefore Model 3 is the most likely to be occurring at the end of the perfusion.

However, the paracrine cross-talk between the trophoblast and the endothelium, whereby high glucose causes the trophoblast to elevate VEGF, NO and other inflammatory mediators which then affect endothelial VEGF and VE-cadherin, may have occurred but was not tested.

6.2.2 Clinical implications of this study

6.2.2.1 The Pedersen hypothesis: the whole story?

The Pedersen hypothesis predicts that complications in diabetic pregnancies are due to the fetal hyperinsulinaemia caused by the maternal hyperglycaemia, as glucose readily crosses the placenta (Pedersen, 1977). Indeed, even in non-diabetic pregnant women, altered glucose production partially explained fetal weight variations and thus macrosomia (Rohl *et al.*, 2001; Ahlsson *et al.*, 2010). In diabetic pregnancies, the outcome of the combination of hyperglycaemia (which is a major anabolic fuel) with hyperinsulinaemia (a major anabolic hormone) is an increase in fat (Fee, 1960) and protein stores. As fetal hyperinsulinaemia only occurs after 20 weeks gestation (when the fetal pancreas starts producing insulin), this weight gain occurs mostly in the third trimester. However, it has also been discovered that maternal HBA_{1c} levels (representing average glycaemic levels in the prior few months) do not fully account for the increased macrosomic rates in the offspring of diabetic women, and that the postprandial glycaemic levels of the mother were more relevant in predicting macrosomia (Kyne-Grzebalski *et al.*, 1999).

However, the Pedersen hypothesis does not take into account any eventual changes that high glucose might have on the placental vasculature. We have shown in this thesis that even single short duration insults (3 and 4h) are sufficient to cause increased leakage of the feto-placental endothelium, changing in the process the pattern of pro- and anti-permeability molecules. This vascular dysfunction may therefore play a part in the aetiology of the diabetic pathology in the fetus. Therefore, the Pedersen hypothesis, whilst still very relevant to this topic, is not the only factor in the fetal milieu of a diabetic pregnancy, as had been assumed until now, but rather working in conjunction with increased leakage of the human placenta.

6.2.2.2 Our study and metabolic syndrome

A current topic in the literature is neonatal macrosomia as a risk factor for developing metabolic syndrome as an adult. It is thought that the *in utero* environment of a diabetic pregnancy predisposes the fetus to subsequent weight gain and insulin resistance.

The effects of GDM to the fetus were observed in Pima Indians, a population known to be genetically predisposed to Type 2 diabetes (Dabelea *et al.*, 2000). In that study, pairs of siblings were studied, the gestation of the younger of the pair having been complicated by GDM, the older sibling's gestation having been normal. Siblings who had been exposed to a diabetic environment *in utero* were found to be significantly more obese (50% increase in BMI) at 21 years of age compared to the older sibling.

Exposure to Type 1 diabetes *in utero* also shows an increased predisposition to develop metabolic syndrome, as a small study of the offspring of 15 pregnant Type 1 diabetic women found more insulin-resistant individuals than in the control group (16 controls with Type 1 diabetic fathers) (Sobngwi *et al.*, 2003). Another study showed a doubling in the risk of becoming overweight and developing metabolic syndrome in offspring with gestations complicated by GDM and Type 1 diabetes (Clausen *et al.*, 2009). Interestingly, the risk of metabolic syndrome was higher in GDM than in Type 1, a possible repercussion of glucose slowing down fetal growth in the first trimester when hyperinsulinaemia is not a feature (Creasy and Resnik, 1999).

It is interesting that both Type 1 and GDM pregnancies are affected by increased offspring predisposition to metabolic syndrome, as they are different diseases with different aetiologies. However, maternal hyperglycaemia links them both, and therefore may be an important factor in predicting metabolic syndrome occurrence later on in life. In these experiments, not only have we shown that maternal hyperglycaemia has further effects than the Pedersen hypothesis predicts, but the increased leakage of the placental barrier may have further effects than just

the immediate *in utero* environment and may contribute to the later development of metabolic syndrome seen in the studies above. Being able to predict when hyperglycaemic insult is increasing the leakage of feto-placental vessels is thus very important.

6.2.2.3 Prediction of leakage in the placental vasculature: use of chorionic villous sampling?

As we saw in Chapter 5, VEGFb expression significantly correlates with vascular leakage. When an antibody specific to VEGFa becomes available, it will be possible to accurately calculate the ratio of VEGFa/VEGFb, which could prove important. With further research, both VEGFb levels alone and the VEGFa/VEGFb ratio could translate clinically as a tool to predict when the feto-placental vessels are leaky in the pregnancy. However, in order to do this, one would need to sample the placenta in the third trimester.

Chorionic villous sampling (CVS) is a technique used for diagnostic purposes for chromosomal and genetic conditions in the fetus, and would be an ideal procedure to obtain samples for further analysis of VEGFb or the VEGFa/VEGFb ratio. However, it is used primarily in the late first trimester (12-14 weeks gestation), and this timing is too early to directly use the results from our term placenta perfusions, as it is known that the dual-independent model cannot simulate the first trimester (Bourget *et al.*, 1995; Vahakangas and Myllynen, 2006).

Fortunately, a study has been performed regarding CVS in second and third trimester pregnancies (Smidt-Jensen *et al.*, 1993). The indications for CVS in women undergoing this procedure in the third trimester were 'abnormal fetus or oligohydramnios' (gestational weeks 15-38) or 'intrauterine growth restriction' (IUGR) (gestational weeks 27-35). Among the 58 women of the former indication, there was a total of 12 unintended losses of the fetus (7 spontaneous abortions and 5 stillbirths) -- not an uncommon outcome for abnormal fetuses -- but there were no losses among those who had normal test results and who intended for the pregnancy to continue. There were also no unexpected losses among the women with the

IUGR indication. Furthermore, after their late CVS there were no immediate complications (except for lower back pain and contractions for less than 30 minutes), and there was no correlation between increased feto-maternal bleeding and gestational age when CVS was carried out. Thus, CVS has been shown to be as safe in third trimester pregnancies as in first trimester pregnancies, and would be an ideal procedure to clinically measure VEGFb or the VEGFa/VEGFb ratio in placental vessels.

However, a fully quantitative protocol would be needed to quantitate VEGFb or VEGFa/VEGFb levels if a clinical application is eventually reached, rather than our pseudo-quantitative work with immunofluorescence. Vessels could in this case be carefully separated from the other placental tissues, and Western blotting performed to evaluate protein levels.

If in further experiments VEGFa and VEGFb were shown to be secreted into the fetal circulation and if their levels (on their own or with a calculated ratio) were predictive of vascular leakage, it could be useful to sample cord blood and test for VEGFa and VEGFb levels. In this case, ELISA would be an appropriate technique to quantitatively analyse protein levels.

6.2.2.4 Fetal hyperinsulinaemia perfusion studies

In this thesis, we investigated the effects of glucose on the placental vasculature. However, maternal hyperglycaemia is only half of the diabetic *in utero* environment, the other half being fetal hyperinsulinaemia. Thus, it is important to study the effect of fetal hyperinsulinaemia to see whether it contributes to the diabetic phenotype observed in Type 1 (Leach *et al.*, 2004) and GDM (Babawale *et al.*, 2000) placentae.

One of the consequences of maternal hyperglycaemia, as seen in diabetic pregnancies, is that glucose enters the fetal circulation by facilitated diffusion. The rate-limiting step in this transfer of glucose is the capacity of the insulin independent glucose transporter GLUT1 (Takata and Hirano, 1997), levels of which have been shown to be increased in the syncytiotrophoblast of

placentae of women with Type 1 diabetes (Jansson *et al.*, 1999) but not GDM (Jansson *et al.*, 2001); this may contribute to the increased glucose transport in diabetic pregnancies.

The fetal pancreas starts to develop between 4 and 6 weeks gestation, the β-cells appear at 10.5 weeks, and by 20 weeks insulin production starts and the fetus is now responsible for its glucose homeostasis (Jaffe, 1991). Thus, although insulin cannot traverse the placental barrier in appreciable amounts, as the fetus is subjected to hyperglycaemia, the fetal pancreas will produce increased insulin to compensate and to return to a euglycaemic environment. This forms the basis for the Pedersen hypothesis (1954) of fetal hyperinsulinaemia.

During pregnancy, the control of insulin action in the placenta passes from the mother, as insulin receptors are localised on the syncytiotrophoblast in the first trimester, to the fetus, as insulin receptors are present in the endothelium of the fetal vessels at term (Desoye *et al.*, 1994; Jones *et al.*, 1993). As the second and third trimester (after insulin production starts in the fetus) of pregnancy is characterised by longitudinal expansion of chorionic villous trees, the presence of high insulin in the fetal circulation might affect the vasculature in the placental villi, affecting the exchange function of the placenta. The effect of high insulin on the vasculature should be the focus of further work.

Our laboratory has performed an as-yet unpublished study with insulin perfusion from the fetal circulation, with the intention to study the immunolocalisation of several key angiogenic and permeability molecules. This author studied VEGFb expression in these experiments (see Appendix 4); however, the other results are equally important and a summary of these results, performed by colleagues, is given here.

The molecules being studied were the important adherens junction molecules VE-cadherin and β-catenin, the important tight junctional molecule occludin, and total VEGF. The leakage of the vasculature to 76Mr dextran tracer was also observed under fetal circulation hyperinsulinaemia and control perfusions. Colleagues (M. Barclay, J. Lucas, R. Lewis, A. Ikram, P. Rodgers, S.

Walker) performed the analysis of the above molecules, whilst the author of this thesis was responsible for the VEGFb immunoreactivity (see Results in Appendix 4).

The junctional profile of the insulin perfused placentae was significantly different from that of the normal placentae. There were significantly fewer vascular profiles immunoreactive for β-catenin and occludin (in stem villi), indicating that both adherens and tight junctions respectively were being destabilised. β-catenin is usually bound to a complex including VE-cadherin, holding the latter at the junctional space. When β-catenin is displaced to the nucleus, VE-cadherin is free to diffuse out of the junctional space. The only abnormality from this altered profile was VE-cadherin, which did not show any significant differences in immunopositive vessels. However, the author of that analysis (M. Barclay) admits that she did not differentiate between cytoplasmic and junctional locations of immunoreactivity, as the author of this thesis did herself in previous chapters. Therefore, it is not possible to know without further analysis if there was more VE-cadherin localisation in the cytoplasm, which would indicate adherens junctional instability. However, the results for β-catenin, a molecule intrinsically linked to VE-cadherin junctional localisation, and occludin (analysed by J. Lucas, R. Lewis and A. Ikram for the former and P. Rodgers for the latter) certainly suggest VE-cadherin junctional instability to have occurred.

The junctional instability seen above suggests that endothelial activation might be ongoing. Immunofluorescence with an anti-VEGF antibody indeed showed that there was more total VEGF present in the insulin perfused placentae than in the normal placentae, indicating that there may be increased vascular permeability in the insulin perfused placentae.

The authors finally looked at the integrity of the barrier function of the placenta by looking at the leakage to 76Mr dextran tracer. It was shown in these experiments that the insulin perfused placentae showed significantly more leakage than normal perfused placentae.

My part within this project revealed that there was a significant decrease in the number of large vessels (found in stem villi) immunopositive for VEGFb in response to insulin perfusion compared to normal perfusion ($p<0.05$, Figure A4.3). This was matched with loss of the tight junctional molecule occludin from the same area. Microvessels (in intermediate and terminal villi) did not show any differences in VEGFb expression. The mesenchymal layer surrounding the large vessels of the stem villi and the maternally-facing trophoblast layer did not seem to be affected in terms of VEGFb expression by hyperinsulinaemia. The trophoblast is the tissue furthest away from the fetal circulation, and thus in contact with the maternal circulation, and therefore any changes affecting the trophoblast may affect the maternal environment. These studies indicate that fetal hyperinsulinaemia affects the feto-placental vasculature but as insulin does not cross the placenta it does not affect the maternal circulation.

6.2.3 Critique of experimental design

6.2.3.1 Explants vs. perfusion

The chorionic villous explant methodology has the potential to be a very useful technique, allowing the easy *in vitro* manipulation of placental tissue to answer several research questions regarding the placenta. It is a relatively easy technique which can elucidate the effects of long-duration insult with glucose on junctional and permeability molecules. However, one difficulty is that it is not a universal methodology; it must be adapted to suit each experimental aim. We wished to use this technique to study expression of VEGF splice variants and VE-cadherin on the endothelium of placental blood vessels. To the best of our knowledge we were the first group to do so, as this technique has to date only been used to study trophoblast function.

During the course of our validation experiments, however, we discovered a flaw in the explant methodology for this use: high number of profiles immunopositive for VEGF and low VE-cadherin localisation to the junctional space (Figures 2.8 and 2.9). We hypothesised that this

was due to a wound healing effect that was initiated by the excision procedure. However, we have only observed the effect of this possible wound healing effect on VE-cadherin and VEGF; other cytokines expressed in wound healing might be produced and be affecting the tissue. This point is worthy of note, and any experimenters who are considering the use of explants in their set-up should include a validation experiment to see how their chosen target is being affected by the wound healing effect we have observed. Strangely, this topic has not been seen before in previous explant studies. Although wound healing is a process that normally only affects the endothelium of blood vessels and not the trophoblast layer, the latter is bathed in the same media as the vessels, and paracrine molecules that would normally be secreted by the endothelium in wound healing could be affecting the trophoblast. Perhaps this is the cause of the unexplained trophoblast degeneration seen in some explant methodologies.

In the context of this thesis, if a wound healing process caused by excision artefacts was occurring, it would mean that our experiments would only show a physiological effect of glucose if the total number of vessels showing immunostaining increased; if the total number of immunopositive vessels stayed the same but the intensity of staining increased, only the vessels already affected by the excision artefacts would be affected by the hyperglycaemic condition.

In contrast, the perfusion methodology has been extensively used to study the transfer of molecules across the placental barrier, which involves the endothelium, and thus the lack of any negative effects of the perfusion model on the blood vessels' lining has been extensively documented.

One of the important findings in this thesis was that VEGFb could be an indicator of vascular dysfunction in diabetes. We have inferred that a single dose of 15mM glucose, a level seen in diabetic pregnancies, can change the VEGF splice variants ratio in both the 4h explants model in Chapter 4, and the 3h perfusion model in Chapter 5 (see section 4.4.3 for reasoning on ratio). From these experiments, we have concluded that glucose might be regulating the leakage of macromolecules from the vasculature through altering levels of VEGFb (Figure 5.13), this being

regulated by glucose, whilst total VEGF was not correlated with tracer leakage (Figure 5.12). However, the timings in the two models differ: in the explants a more physiologically relevant 4h timepoint was used, whilst in the perfusion a shorter 3h duration of perfusion was employed. The perfusion technique has a high failure rate: other groups have found that the failure rate for perfusions is around 75%. Our group has found that the chance of leakage (and hence perfusion failure) of media from either circulation increases with time, and therefore we chose the more reliable 3h perfusion rather than the less reliable 4h perfusion.

Another limitation of the dual-independent placental perfusion model is that it is not representative of the first trimester placenta (Vahakangas and Myllynen, 2006; Bourget *et al.*, 1995). Use of first trimester placental tissue with explants or other methods may provide valuable information for this earlier gestational period and for Type 1 diabetes, as in this pathology glucose insult starts in the first trimester.

6.2.3.2 Antibodies used

The two VEGF antibodies used in this thesis were MAB293 and MAB3045 for total VEGF and VEGFb respectively. MAB293 was used because there are currently no commercial antibodies specifically for VEGFa. Once one is developed, the VEGFa/VEGFb ratio can be calculated directly, rather than having to deduce that VEGFa must be increasing when VEGFb expression is decreasing and total VEGF expression is increasing (see section 4.4.3).

Currently, the only method to quantitatively calculate the VEGFa/VEGFb ratio is using RT-PCR; amplifying mRNA using different primers yielding a 130bp product (corresponding to VEGFa) and a 64bp product (corresponding to VEGFb) (Nowak *et al.*, 2008). Protein levels, however, are not always reflected by mRNA levels, and therefore an antibody for VEGFa protein would prove useful.

Another criticism of the two antibodies used is that they have different detection limits, whereby MAB3045 detects more protein than MAB293, leading to an inconsistency in the results. If the detection limits for the two antibodies had been similar, the proportion of VEGFb/total VEGF could have been calculated, which would have been truly representative of the VEGFa/VEGFb ratio. Antibodies with comparable detection limits would therefore be preferable in these types of experiments.

6.2.3.3 Alternative methods to the use of immunofluorescence for quantitation of results

The other main critique of this study is that the expression of VE-cadherin and the VEGF splice variants has been analysed entirely by immunofluorescence, which is a pseudo-quantitative technique, and therefore cannot give exact values to the expression of these molecules. This could have been performed through Western blotting. However, Western blotting would not indicate the location (junctional or cytoplasmic) of VE-cadherin, and therefore would not be physiologically relevant. For the VEGF splice variants, likewise, it would not indicate the location of the splice variants in the maternally-facing trophoblast or in the fetal-facing endothelium of the fetal blood vessels. In our experiments, we have shown that in response to high glucose in the maternal circulation, the expression of these VEGF splice variants was significantly different, and the splice variants were differentially regulated, in the fetal endothelium; therefore the effects of glucose have resulted in effects on the other side of the placental barrier. To quantitate levels of total VEGF and VEGFb in a relevant way, it would be necessary to isolate trophoblast from endothelium, and then analyse the resulting separated tissue with Western blotting. This analysis is an essential future step if further experiments indicate a potential clinical use for VEGF splice variants levels and/or ratio between the two.

We have observed in the placental perfusion that VEGFb (but not total VEGF, Figures 5.13 and 5.12, respectively) in the feto-placental vessels correlates with feto-placental vascular leakage.

To quantitate these amounts through Western blotting without first separating trophoblast from endothelium could obfuscate results, as *in vivo* the trophoblast is not in contact with the endothelium, and thus VEGFa/VEGFb levels here probably would not correlate with endothelial dysfunction, as the two circulations are exposed to different environments and stimuli.

Another criticism could be that ELISA was not performed. This could have given valuable information on the amount of VEGF splice variants secreted into the fetal circulation, and thus whether effects downstream of this release could be expected. ELISA was indeed attempted on several occasions, but unfortunately did not give interpretable results, and this avenue of research was thus abandoned, due to the limited samples available. An ELISA on the samples from the fetal circuit (rather than from the maternal circuit) of the perfusion experiment would be the most physiologically relevant, as it would indicate release of VEGF splice variants in the circulation which would be most likely to affect the placental vasculature and fetus.

6.2.4 Regulation of factors influencing VEGF splicing

Our studies show that glucose affects the alternative splicing of VEGFa and VEGFb, but we have not investigated the mechanisms behind this change. In order to fully understand the mechanisms behind this splicing switch, the SR proteins responsible for selection of the splice variant to be produced, ASF/SF2 and SRp40 for VEGFa and SRp55 for VEGFb, need to be studied under conditions of high glucose. To the best of our knowledge, this has not been performed in the literature at present. Being the prototypical SR protein, most studies have focused on ASF/SF2.

SR proteins are post-translationally modified via several mechanisms, including glycosylation (Soulard *et al.*, 1993), methylation (Rho *et al.*, 2007), and, most well studied, phosphorylation. This latter modification is controlled by SRPK1-2 and members of Cdc2-like nuclear kinases (Clk/Sty and Clk2-4). The regulation of SR proteins through post-translational modifications in

general, and phosphorylation in particular, allows alternative splicing to occur without new protein synthesis (Stamm, 2002), and thus a splice selection switch can be observed relatively quickly after the stimulus, with degradation of the existing splice variant and replacement with the new, a process that can take less than one hour after the initial stimulus. This fits well with our observations of changes in splicing (decrease in VEGFb, Figures 4.6 and 5.5) 3h and 4h after initial glucose insult.

SR protein phosphorylation status is thought to regulate two processes, the first of which is the localisation of the protein in the cell (cytosolic, nuclear speckles, or nuclear splicing site). This change in localisation has been well studied for ASF/SF2. The change in localisation from cytosol to nucleus is due to phosphorylation by SRPK1-2 of serine residues (Aubol *et al.*, 2003), which then results in transport to nuclear speckles (Lai *et al.*, 2000). In the nucleus, Clk/Sty and Clk2-4 hyperphosphorylate ASF/SF2, which causes the translocation from speckles to splice site, and protein:protein binding to U1 70kDa subunit occurs, allowing U1 snRNP to the 5' splice site (Eperon *et al.*, 2000; Kohtz *et al.*, 1994; Wu and Maniatis, 1993). When two sites are in competition, such as is the case for VEGF pre-mRNA splicing, ASF/SF2 seems to favour the proximal site (Ge and Manley, 1990; Krainer *et al.*, 1990); this is indeed the case for VEGF, as ASF/SF2 is required for VEGFa splicing (Nowak *et al.*, 2008). After splicing has occurred, protein phosphatase 1 (PP1) dephosphorylates SR proteins to signal for their return to the cytoplasm. The dephosphorylation of ASF/SF2 leads to an increase in binding to nuclear export factor TAP/NFX1, leading to an increase in cytosol localisation. PP1 has a different cellular localisation depending on where the protein they are regulating is found (Trinkle-Mulcahy *et al.*, 2003). Thus, insights into splicing regulation could be garnered by looking at the localisation of proteins involved in splicing and their regulators. This is an important avenue for future investigation to elucidate the exact mechanisms behind effects of glucose that we observed on total VEGF and VEGFb.

The second process regulated by phosphorylation of SR proteins is the binding to the splicing machinery snRNPs. Phosphorylation of ASF/SF2, for example, enables tighter binding to the U1 70kDa subunit (Xiao and Manley, 1997), and decreases binding to TAP/NXF1 (Huang *et al.*, 2004). The binding of RS domains to RNA also changes with phosphorylation, affecting splicing (Shen and Green, 2006; Xiao and Manley, 1997). Therefore, studies looking at whether SR proteins are in complexes with specific proteins (such as U1 snRNP and RNA or TAP/NXF1) can also shed light on the regulation of splicing.

Looking at SR phosphorylation can also be useful, but does not provide a definite answer as to whether the proteins are participating in splicing, as both hypophosphorylation and hyperphosphorylation have been shown to inhibit splicing (Cao *et al.*, 1997). It is thought that when the RS domain is hyperphosphorylated, the interaction between it and U1 snRNP becomes too strong, inhibiting U1 snRNP's disassociation from the splice site, therefore reducing splicing. Additionally, it has been shown that both hypophosphorylation (by PP1 addition) and hyperphosphorylation (by CLK/Sty and SRPK1 overexpression) cause a diffuse nuclear distribution of ASF/SF2 protein (Prasad *et al.*, 1999).

Although glucose studies have not been performed in the literature at the present time, other studies that are of potential importance to our study have been conducted. Insulin receptor activation by insulin has been shown to increase phosphorylation of SRp40 and SRp55, two SR proteins known to regulate VEGF splicing, and result in splice site selection change (Jiang *et al.*, 2009; Patel *et al.*, 2005). These changes were mediated by the PI3K pathway (important in VEGF's role as a survival factor and NO production (see section 1.2.1.3), and resulted in alternative splicing of PKCβII (PKC is an important pathway in hyperglycaemia (see section 1.3.3.1) and is capable of decreasing VEGFb production (Nowak *et al.*, 2010)), which leads to increased glucose uptake. $PKB^{-/-}$ tissue also showed a decrease in SRp55 phosphorylation. A TNFα (a pro-inflammatory cytokine) increase has been shown to cause the dephosphorylation of ASF/SF2 and PKB through an increase in ceramide (involved in apoptosis) and allosteric PP1

activation. This might be of relevance to our study if our wound healing assumption is correct. ASF/SF2 and SRp55 have been shown to be regulators of tissue factor in human monocytic cells (Tardos *et al.*, 2008). Alternative splicing of tissue factor has been shown to be increased in Type 2 diabetic patients due to the combined effect of hyperglycaemia and hyperinsulinaemia (Boden and Rao, 2007).

The investigation of the effects of glucose on the splice factors involved in VEGF splicing, namely ASF/SF2, SRp40, and SRp55, would thus be an important future step for the full understanding of mechanistic details of the findings reported in this thesis.

6.2.5 Future work

6.2.5.1 Reversal of phenotype studies

In this thesis, we have found that the diabetic phenotype could be replicated with a single 3h or 4h hyperglycaemic (15mM glucose) insult. However, in a pregnancy, the postprandial values eventually return to basal level until the next meal. Whether the effects of our hyperglycaemic insult are permanent or reversible, and whether further insults worsen the phenotype, could therefore change the clinical outcome of the pregnancy: if the changes are reversible, prompt treatment could limit damage to the placenta and hence the fetus. Thus, it could prove clinically useful to experiment on whether or not hyperglycaemia followed by a period of euglycaemia, followed by another glycaemic insult, reverses the phenotype observed. Although perfusions are the best physiological model of the *in utero* environment, the duration required and frequent change of media would make it impractical to use as a model in this instance, and explants have the possible added complication of excision artefacts. An experiment trying to research this question was therefore twice attempted by the author of this thesis in a HUVEC cell model of permeability (see Appendix 5 for materials and methods). However, these experiments revealed that changing the media, a necessary step to study

reversal of hyperglycaemic phenotype, was disrupting the HUVEC monolayer, leading to holes forming in the monolayer, and therefore the permeability measurements taken were worthless. Time and financial constraints meant that this experiment had to be abandoned.

In order to investigate whether the phenotype which results from glucose insult is reversible or not, the experiment detailed above (and in Appendix 5) requires a re-design, and careful consideration should be given to the methodology used.

6.2.5.2 Investigations on 'wound healing' in chorionic villous explants cultures

The chorionic villous explant methodology would be an ideal model to study the reversibility experiments described in section 6.2.5.1, as it allows longer duration experiments and lower failure rate than the perfusion model and is more robust than experiments with HUVEC cell cultures (see section 6.2.5.1 for experimental difficulties with HUVECs). However, it does not allow permeability studies, and has the confounding factor of a possible 'wounding' response caused by the excision artefacts observed.

However, our experiments were not designed to definitively show if there was a wound healing response or if the increase in VEGF and decrease in VE-cadherin seen in Chapter 2 were due to wound healing or other processes. The main evidence in favour of the wound healing theory is the recovery seen by 24h; this shows a decrease in VEGF and increase in VE-cadherin compared to the earlier timepoints. Furthermore, the tissue which was excised after washing and fixation showed low VEGF and high VE-cadherin immunoreactive vessels as expected of uninjured tissue. However, this latter evidence is only qualitative due to the high amount of blood trapped in the vessels which makes counts unreliable (Figure 2.6A-D). In addition, the fact that high VEGF and low VE-cadherin is only seen in explants but not in perfused placentae indicates that the artefacts are probably being caused by a disturbance in the 3D architecture, and a 'wound' could be just such a disturbance. In further experiments investigating wound healing, other

possibilities, such as the roles of VEGF as a survival factor and endothelial proliferation, should be also investigated.

To investigate the theory of wound healing response, a full spectrum of inflammatory cytokines, anti-inflammatory cytokines and other molecules that affect the wound healing response should be studied in explants. If our hypothesis on the wounding of explants is correct, the cytokines expressed should match the pattern seen in wound healing (for a review listing the molecules involved in wound healing, see Singer and Clark (1999)). If a wound healing process was proven to be occurring, other experiments investigating how this was being resolved would be necessary, to see if there is a plateau in wound healing after 24h, or if the process is ongoing.

6.2.5.3 VEGFa/VEGFb ratio in insulin perfusion studies

Section 6.2.2.4. and Appendix 4 detail our group's research into fetal hyperinsulinaemic perfused placentae in a currently unpublished study. Unfortunately, S. Walker and this author used different euglycaemic perfused placentae to calculate total VEGF and VEGFb expression. Furthermore, J. Lucas, R. Lewis and A. Ikram used another set of euglycaemic placentae for their tracer leakage studies. These discrepancies mean that it is not possible to perform a correlation analysis between total VEGF and tracer leakage and between VEGFb and tracer leakage (as performed in Chapter 5 for hyperglycaemic perfusions) without a complete re-count of these results with the same control placentae.

6.2.5.4 Diabetic and hyperglycaemic/hyperinsulinaemic perfusion models

In Chapter 5, we described our hyperglycaemic perfusions on normal placentae; further to these experiments, two more critical studies should be performed using perfusion models. The first of such studies should be to perfuse normal placentae for 3h with both hyperglycaemia in

the maternal circuit and hyperinsulinaemia in the fetal circuit, to see whether concurrent insult with these two pathological conditions affect the placenta any differently. Secondly, perfusions on diabetic placentae, both from Type 1 and GDM, should be performed with both maternal hyperglycaemia and fetal hyperinsulinaemia, to compare with normal placentae, and also perfused with only either hyperinsulinaemia or hyperglycaemia. In addition, radiolabelled glucose could be used if transplacental flux and/or glucose consumption is being studied. However, the diabetic perfusion studies are likely to take a significant amount of time to complete, due to the scarcity of such tissue combined with the high failure rate of the perfusion model due to cannulation and leakage problems. This is the reason why we perfused only normal placentae in our experiments.

The exploration of these topics could further knowledge that might lead to clinically predicting dysfunction in the human placenta, therefore aiding the treatment of diabetic pregnant women.

6.3 Concluding remarks

This thesis has implications in two areas of research: VEGF biology and materno-fetal health.

In this thesis, we have identified the anti-angiogenic and anti-permeability molecule VEGFb as an important player in the control of vascular integrity, and we have seen that VEGFb levels might even act as a predictor of vascular dysfunction. Our findings thus suggest that future studies investigating VEGF actions should certainly include this splice variant, to establish whether it is this splice variant or VEGFa or even their relative proportions, which is important in each of VEGF's effects and roles. This will have implications in many fields, as VEGF is seen as a key molecule in diverse pathologies, e.g., diabetes or cancer.

The Pedersen hypothesis states that it is the fetal hyperinsulinaemia seen in response to maternal hyperglycaemia that causes the fetal macrosomia seen in diabetic pregnancies. However, this prevailing theory does not take into account that maternal hyperglycaemia alone

(i.e., independently of high insulin) might be causing some changes, nor does it consider that the placenta itself might be affected, as any placental vascular changes have the potential to affect the fetus and its development.

In this thesis, we have shown that hyperglycaemia itself can affect the functional phenotype of the placenta. Furthermore, these effects were seen with a single acute glucose insult, and were independent of insulin. Therefore, the Pedersen hypothesis would appear to be an incomplete model of the pathology seen in diabetic pregnancies, and this should be considered in the care of the diabetic pregnant woman.

Appendix 1
Materials

Sigma-Aldrich

Agarose (A9539)

Albumin from Bovine Serum (A7906)

Albumin, tetramethylrhodamine isothiocyanate bovine (A2289)

Anti-Mouse IgG (whole molecule) - FITC antibody produced in goat (F0257)

Anti-Rabbit IgG (whole molecule) - TRITC (T6778)

Collagenase from *Clostridium histolyticum* (C6885)

D-(+)-Glucose (G6152)

D-Mannitol (63559)

Dextran from from *Leuconostoc mesenteroides* Mr 15000-30000 (D4626)

Dextran from *Leuconostoc* spp. Mr 15000-25000 (31387)

Endothelial Cell Growth Supplement from bovine neural tissue (E2759)

Ethanol (458600)

Fetal Bovine Serum (F7524)

Gelatin (G9391)

Maltodextrin test sticks according to Ph. Eur. (80104-1EA-F)

Medium 199 (M5017)

Monoclonal Anti-β-Catenin antibody produced in mouse, clone 6F9 (C7082)

Paraformaldehyde (P6148)

Normal Human Serum (H4522)

Phosphate Buffered Saline tablets (P4417)

Sodium Bicarbonate (S8875)

Sodium Chloride (S3160/63)

Streptomycin Sulfate salt (S9137)

Tetramethylrhodamine isothiocyanate – Dextran (T1162)

Triton, t-Octylphenoxypolythoxy ethanol X-100 (T8787)

Fisher Scientific

Acetone (A/0560/17)

D-Glucose anhydrous (G/0500/53)

Iso-Pentane (P/1030/17)

Propan-2-ol (P/7490/17)

Toluidine Blue O (BP107-10)

Xylene (W/010/17)

R&D Systems

Monoclonal Mouse Anti-Human CD31/PECAM-1 Antibody (BBA7)

Monoclonal Mouse Anti-Human VEGF Antibody (MAB293)

Monoclonal Mouse Anti-Human $VEGF_{165}b$ Antibody (MAB3045)

Substrate - HRP (DY998)

Substrate Reagent Pack (DY999)

Life Technologies (Gibco and Invitrogen)

Fungizone (15290-018)

Medium 199 X 1 (22340-020)

Occludin, Rabbit Polyclonal Antibody (71-1500)

Trypsin-EDTA, 0.05% (25300)

Raymond A. Lamb

DPX (Lamb/DPX)

Eosin 1% (Lamb/100/D)

Haematoxylin (Harris) (Lamb/230/D)

OCT embedding medium (Lamb/OCT)

Pharmacy stores

Novorapid (100U/ml)

Penicillin 600μg/vial

Vector Laboratories, Inc

ImmEdge Hydrophobic Barrier Pen (H-4000)

Vectashield Mounting Medium for Fluorescence (H-1000)

BD Biosciences

Purified Mouse Anti Human CD144 (555661)

Costar, Corning

Transwell Permeable Supports, 12 mm inserts (3460)

CP Pharmaceuticals

Monoparin 5000 IU in 1ml

KM Skipp

22mm cork disks

NHS Logistics

Sutures Dexon II (FVP189)

Santa Cruz Biotechnology

Rabbit anti-human PECAM-1 (C-20) Antibody: sc-1505R

Scientific Laboratory Supplies

12 well plates (353043)

VYGON

Lipid Resistant 3-way Stopcock (875.00)

Appendix 2
Diabetic donors of tissue

This is the diabetic donor information referred to in section 4.3.2.

Donor	Duration of disease	Age of mother	Treatment	Complications
Patient A	8 years	20 years old	Humolog mix 2x daily	No retinopathy
Patient B	21 years	35 years old	Novorapid 3x daily, detemri	No retinopathy
Patient C	8 years	32 years old	Insulintard x2 daily	No retinopathy
Patient D	10 years	25 years old	Actrapid	No retinopathy

Donor	HbA_{1c} at term	Gender of baby	Weight of baby	Gestation at birth
Patient A	65mmol/mol	Male	3.37kg	38 weeks
Patient B	45mmol/mol	Male	2.89kg	37 weeks
Patient C	68mmol/mol	Male	4.30kg	37 weeks
Patient D	97mmol/mol	Female	4.00kg	38 weeks

Appendix 3
Glucose concentration in perfused media samples from fetal and maternal circuits – HemoCue Glucose 201+ analyser measurements

These are the HemoCue Glucose 201+ analyser results from the sampling of perfusion media, as described in section 5.3.2.

Standards (perfusion media with known (5mM and 15mM) concentrations of glucose):

- 5mM glucose readings: 4.5mM, 7mM, 8.6mM
- 15mM glucose readings: 14.5mM, 20.5mM, 20.6mM

Note the inaccuracy and high variation in the standards.

End of,		Euglycaemia (5mm glucose) Fetal (mM)	Maternal (mM)
1st hour	Placenta 1	8.0	8.8
	Placenta 2	8.7	8.2
	Placenta 3	9.0	8.5
2nd hour	Placenta 1	7.4	7.9
	Placenta 2	6.5	5.9
	Placenta 3	6.5	6.3
3rd hour	Placenta 1	8.0	8.8
	Placenta 2	6.5	5.9
	Placenta 3	6.2	6.5

End of,		Hyperglycaemia (5mM glucose Fetal (mM)	Maternal (mM)
1st hour	Placenta 1	5.5	14.6
	Placenta 2	9.1	9.50
	Placenta 3	7.0	10.5
2nd hour	Placenta 1	5.9	14.3
	Placenta 2	9.6	15.7
	Placenta 3	8.2	13.4
3rd hour	Placenta 1	9.4	18.5
	Placenta 2	6.3	14.0
	Placenta 3	7.2	12.9

Appendix 4
Supplementary methods and results

These are the supplementary methods and results referred to in section 6.2.2.4.

A4.1 Methods

A4.1.1 Insulin 30 minute perfusion

Term placentae (n=6; delivered by Caesarean section) were taken from normal pregnancies undergoing term elective Caesarean sections in Queen's Medical Centre, Nottingham, with informed patient consent and ethical approval. Lobules were chosen and cannulated as described in Chapter 5. Briefly, both maternal and fetal circulations were independently perfused with perfusion media which simulated physiological conditions regarding osmolarity, temperature and vessel pressure, and from delivery of the placenta to complete cannulation no longer than 30 minutes elapsed. After a 20 minute equilibration period with oxygenated perfusate (M199 with added albumin, heparin, dextran (20,000Mr) and sodium bicarbonate, as described in Chapter 5), fetal media was switched to one which contained 25μU/ml insulin and the lobules were perfused for a further 30 minutes in an open (non-recirculated media) circuit. In the last 10 minutes, TRITC-dextran (76Mr; 0.5mg/ml) was added to the fetal circulation. In control perfusions, the perfusion media did not contain added insulin. Placental lobules were fixed with 1% w/v p-formaldehyde (30 minute perfusion) and prepared for immunohistochemistry.

A4.1.2 Immunofluorescence

The immunofluorescence protocol performed was the same as the one described in the previous chapters. Primary antibody against VEGFb (MAB3045, R&D Systems, 5μg/ml) was used in conjunction with an goat anti-mouse human secondary antibody (F-0257, Sigma-Aldrich, 20μg/ml).

A4.1.3 Analysis of data

Analysis of immunopositive profiles was performed initially by counting the number of profiles immunoreactive to VEGFb, but differently from previous chapters, the immunoreactivity to VEGFb of the trophoblast and mesenchyme was also studied. In addition, the vascular profiles counted were divided into stem villi, intermediate villi, and terminal villi, to observe whether there was a different localisation of VEGFb in insulin perfused placenta.

Mann Whitney-U tests were used for statistical analyses.

A4.2 Results

A4.2.1 VEGFb immunolocalisation

After the normal (without added insulin) placental perfusion, VEGFb was localised in 50.2% of vessels, in 51.0% of the trophoblast layer, and in the mesenchyme of 80.8% of stem villi,. After the insulin perfusion, VEGFb was found in 46% of vessels, in 48.9% of the trophoblast layer, and in 81.7% of stem villi mesenchyme, (Figure A4.1). This resulted in no significant differences ($p>0.05$) between the normal and insulin perfusions.

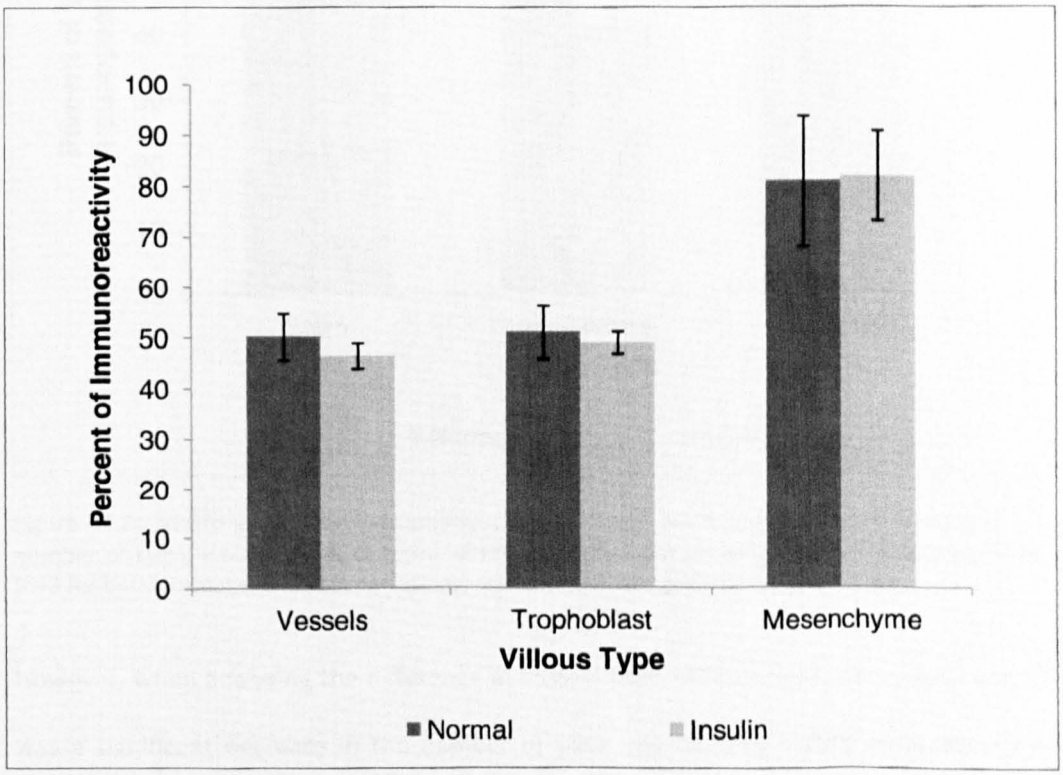


Figure A4.1: Effect of insulin perfusion on total VEGFb immunoreactivity. Insulin perfusion had no effect ($p>0.05$) on the total number of vessels, trophoblast or stem villous stroma tissues showing VEGFb immunoreactivity. Data ($n=3$ for both normal and insulin perfusions) was analysed using Mann-Whitney U test.

Further analysis revealed that VEGFb was present in 54% of trophoblast surrounding stem villi, 46% of trophoblast surrounding intermediate villi, and 54% of trophoblast surrounding terminal villi of normal perfused placentae. In insulin perfused placentae, VEGFb was present in trophoblast in the following percentages: surrounding stem villi 53.6%, surrounding the

intermediate villi 41.5%, surrounding the terminal villi 52.0% (Figure A4.2). Again, there were no significant differences in the trophoblast immunoreactivity between these different groups ($p>0.05$).

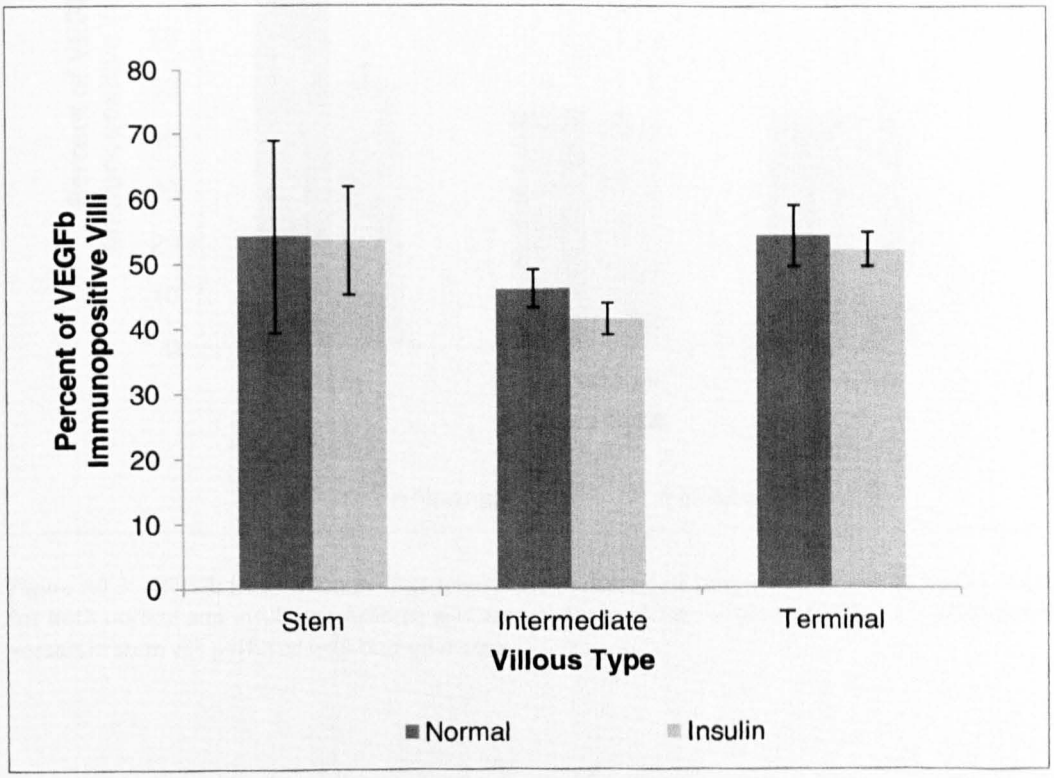


Figure A4.2: VEGFb localisation in trophoblast tissue. There was no significant difference ($p>0.05$) in the number of stem, intermediate, or terminal villous trophoblast showing VEGFb immunoreactivity. Data ($n=3$ for both normal and insulin perfusions) was analysed using Mann-Whitney U test.

However, when analysing the difference in vessels from different villi, we observed that there was a significant decrease in the number of stem villi showing VEGFb immunoreactivity, as normal perfused placentae showed 75.3% of vessels immunopositive for VEGFb, whilst insulin perfused placentae showed only 51.2% of VEGFb immunopositive vessels ($p<0.05$). The percentage of VEGFb immunopositive vessels in both intermediate villi (45.7% in normal placentae, 43.7% in insulin perfused placentae) and terminal villi (45.4% in normal placentae and 46.1% in insulin perfused placentae) was not significantly different between normal and insulin groups ($p>0.05$, Figure A4.3).

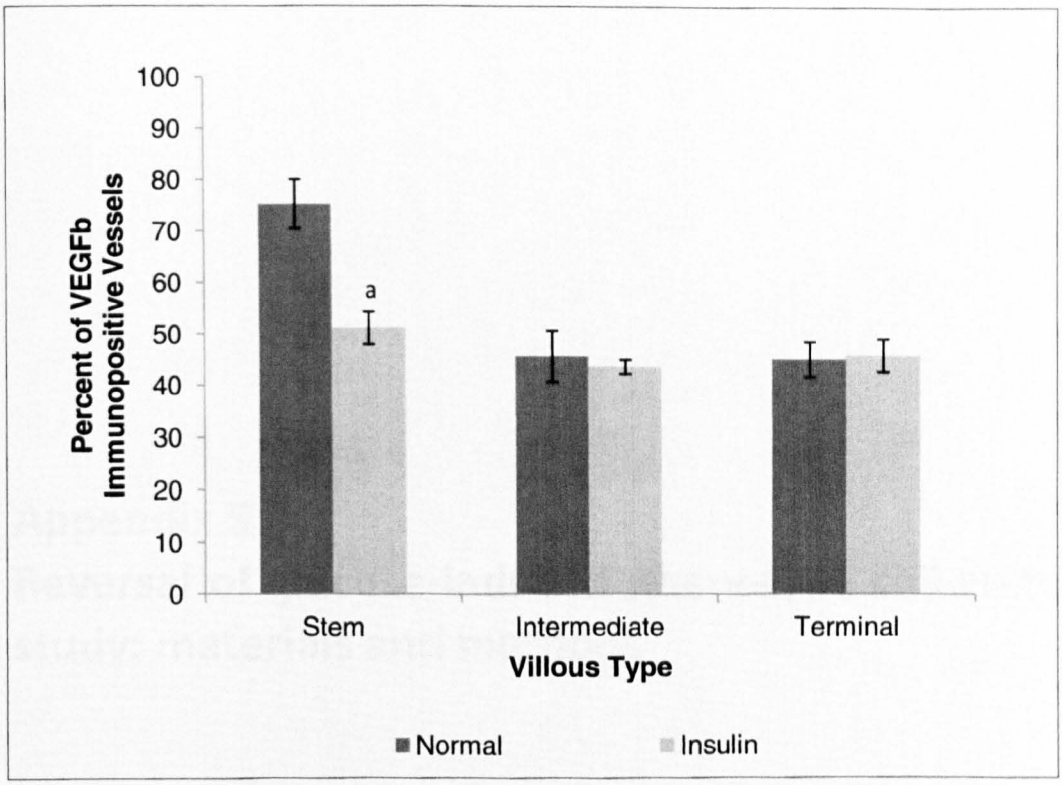


Figure A4.3: VEGFb localisation in large (stem) and microvessels (intermediate and terminal). Data (n=3 for both normal and insulin perfusions) was analysed using Mann-Whitney U test. a: $p<0.05$ relative to vessels in stem villi perfused with normal media.

A4.3 Conclusion

There was a significant decrease in the number of large vessels (found in stem villi) immunopositive for VEGFb in response to insulin perfusion compared to normal perfusion ($p<0.05$, Figure A4.3). Microvessels (intermediate and terminal) did not show any differences in VEGFb expression. The mesenchymal layer surrounding the large vessels of the stem villi and the trophoblast layer did not seem to be affected in terms of VEGFb expression by the hyperinsulinaemia. The trophoblast is the tissue furthest away from the fetal circulation, and thus in contact with the maternal circulation, and therefore any changes affecting the trophoblast may affect the maternal environment.

Appendix 5
Reversal of glucose-induced phenotype cell culture study: materials and methods

These are the materials and methods referred to in section 6.2.5.1.

Human umbilical vein endothelial cells (HUVEC) were isolated from 3 separate placentae, pooled in a single T25 flask and grown in growth medium M5 (Medium 199 with 5% heat inactivated fetal bovine serum (FBS), 100IU/ml penicillin, 100g/ml streptomycin, 2μg/ml fungizone, 150μg/ml endothelial cell growth supplement and 50μg/ml heparin) at 37°C and 5% CO_2 in air.

At 90-100% confluence, HUVECs were detached and seeded onto transwells (12mm, 0.4μm) at a cell density per well of 10^5, with 200μl of M5 in the apical chamber and 700μl of M5 in the basal chamber. When confluence was achieved, the media was changed to M0 containing TRITC-albumen tracer (68kDa, 50mM) containing 15mM glucose for 3h. The media was sampled (triplicate aliquots of 20μl) from the basal chamber every hour, with immediate replacement of the sampled media. The transendothelial electrical resistance (TEER), a measure of endothelial permeability, was measured with an EVOM resistance meter (World Precision Instruments).

After the 3h hyperglycaemic incubation, the media was then immediately replaced with M0 containing TRITC-albumen tracer containing 5mM glucose for a further hour, repeating the TEER measurement and media sampling at the end of this period. The media was then replaced again with M0 containing 15mM glucose and TRITC tracer for a further 3h. Again, the media was sampled (triplicate aliquots of 20μl) from the basal chamber every hour, with immediate replacement of the sampled media.

At the end of this second 3h incubation, the TEER was calculated, and the experiment terminated. 20μl of the media which had been replaced at every step of the experiment (first 3h hyperglycaemic media, euglycaemic media, and second hyperglycaemic media) was then analysed with a DYNEX Microtiter Plate MFX Fluorometer (excitation = 530nm, emission =

590nm), and the concentration of the tracer in the media was then calculated. Leakage on TRITC-albumin was also calculated for transwells without cells as a control.

Appendix 6
Publications

Published review

- L. Leach, A. Taylor and F. Sciota (2009) *Vascular dysfunction in the diabetic placenta: causes and consequences.* *Journal of Anatomy*, Vol. 215, No. 1, pp. 69-76.

Published abstracts

- Flavia Sciota and Lopa Leach. *Effects of glucose on expression of VEGF165a/VEGF165b splice variants in human normal and diabetic placental microvessels.* British Microcirculation Society conference, University of Exeter, April 2010.
- Flavia Sciota and Lopa Leach. *Effects of glucose on the expression of VEGF splice variants in normal and diabetic placenta*, International Federation of Placental Associations (IFPA) Adelaide, Australia conference, October 2009.
- Flavia Sciota and Lopa Leach. *Hyperglycaemia increases Vascular Endothelial Growth Factor (VEGF) expression and vascular leakage in human placental vasculature*, British Microcirculation Society, University of Birmingham conference, April 2009.
- Flavia Sciota and Lopa Leach. *Insulin perfusion decreases expression of the splice variant $VEGF_{165}b$ in large vessels of the human term chorionic stem villi but not in microvessels*, European Microcirculation Society Budapest, Hungary conference, August 2008.
- Flavia Sciota, Dalbir Dhiraj and Lopa Leach. Effects of hyperglycaemia and hypoxia on the vascular endothelium of the placenta, Anatomical Society of Great Britain and Ireland conference, University of Nottingham conference, July 2008.
- Flavia Sciota and Lopa Leach. *Effects of hyperglycaemia on Vascular Endothelial Growth Factor (VEGF) and Vascular Endothelial-cadherin (VE-cadherin) expression in a placental villous explant culture*, British Microcirculation Society, King's College London conference, April 2008.
- Flavia Sciota and Lopa Leach. *Effect of hyperglycaemia on vascular endothelial growth factor (VEGF) and vascular-endothelial-cadherin (VE-cadherin) expression in a placental villous explant culture*, Anatomical Society of Great Britain and Ireland, University of Oxford conference, January 2008.
- Flavia Sciota and Lopa Leach. *Role of VEGF in Diabetic Angiogenesis: Evaluation of Human Placental Villous Explant Cultures*, Anatomical Society of Great Britain and Ireland, Durham University conference, July 2007.

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