

The Effects of Undernutrition and Dexamethasone Treatment on Cultured Rat Neonatal Cardiomyocytes

By

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Declaration

I declare that the contents of this thesis are my own work unless otherwise stated, and that this thesis has not been presented to this or any other university in support of any degree other than that for which I am now a candidate.

Signed.....

Date.....

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Abstract

It is well-established that undernutrition during pregnancy increases offspring's risk of disease in later life. Studies have found that maternal protein restriction during rodent gestation programmes premature hypertrophic growth and decreased mitotic index of cardiomyocytes, as well as impaired contractile ability of the heart. All of these are predisposing factors to cardiovascular disease. To date, few studies have focused on the underlying mechanisms that lead to such alterations in the structure and function of the cardiovascular system. This study aimed to investigate if protein deficiency during gestation impacts upon cardiomyocyte proliferation, and glucose uptake and transport *in vitro*, and if programming effects can be demonstrated in culture.

Methods. Trial 1: Ten pregnant wistar rats were fed either a control or low protein (MLP) diet throughout gestation. Pups were culled on the day of birth and hearts were taken to isolate cardiomyocytes. After 10 days in culture (baseline) some cardiomyocytes were treated with 100nM and 10µM of dexamethasone (Dex) for 48 hours (Day 2). Both untreated and Dex treated cultures were analysed for proliferation and differentiation rates, and glucose uptake. Trial 2: Gestational diet and culturing of cardiomyocyte cells was the same as in trial one. Cultures were treated with 10nM and 100nM Dex and analysed at the same time points for proliferation and differentiation rates, cell apoptosis and mRNA expression of glucose transporters. **Results.** In trial one, MLP diet increased day 2 binucleation. High concentrations of Dex increased insulin stimulated glucose uptake in MLP neonatal cardiomyocytes indicating they had an increased sensitivity to glucocorticoids. In trial two, Dex treatment decreased expression of GLUT 4 and increased expression of glucocorticoid receptor (GR) mRNA.

Conclusion. Results indicate possible remodelling of the heart in rat offspring exposed to MLP diet *in utero*, but it remains unclear as to what mechanism underpins MLP cultures increased sensitivity to glucocorticoids. There were some similarities to the findings of *in vivo* studies, but responses varied between trials. This indicates that

cell culture is unlikely to become a viable alternative model, but it is a useful tool for exploring focused mechanisms of fetal programming.

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List of Abbreviations

β -ME	β -mercaptoethanol
μ g	Microgram
μ l	Microlitre
11 β -HSD-2	11 β -hydroxysteroid dehydrogenase type 2
2-dG	2-deoxyglucose
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
APO*E	Apolipoprotein E
AT ₁	Angiotensin II receptor type 1
AT ₂	Angiotensin II receptor type 2
ATP	Adenosine Triphosphate
Bax	Bcl2-associated X protein
BHF	British Heart Foundation
B1c-2	β -cell lymphoma 2
BMI	Body Mass Index
BRdu	Bromodeoxyuridine
BSA	Bovine Serum Albumin
Ca ²⁺	Calcium
CBX	Carbenoxolene
CHD	Coronary Heart Disease
CM-HBBS	Calcium and magnesium free Hanks Balanced Salt Solution
CVD	Cardiovascular Disease
Dex	Dexamethasone
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
GC	Glucocorticoid
GLUT 1	Glucose transporter 1
GLUT 4	Glucose transporter 4
GPDH	Glycerol-3-Phosphate Dehydrogenase
GR	Glucocorticoid Receptor
H3-2-DG	2-deoxy-D-[2,6- ³ H]glucose
HPA	Hypothalamic-Pituitary-Axis

IR	Insulin Receptor
IRS-1	Insulin receptor substrate-1
L-15 Medium	Leibovitz-L15 Media
LDL	Low-density Lipoprotein
LV	Left Ventricle
mg	Milligram
min	Minutes
ml	Millilitre
MLP	Maternal Low Protein
MMLV	Moloney Murine Leukemia Virus
MR	Mineralocorticoid Receptor
mRNA	Messenger Ribonucleic Acid
Na ²⁺	Sodium
NR3C1	Nuclear receptor subfamily 3, group C, member 1
NTC	Non-template Control
P/S	Penicillin-streptomycin
PBS	Phosphate Buffered Saline
PCKζ	Protein-kinase C-zeta
PCR	Polymerase Chain Reaction
PI3-kinase	Phosphatidylinositol 3-kinase
PMSF	Phenylmethane Sulfonyl Fluoride
PPAR	Peroxisomal Proliferators-Activated Receptor
PVD	Peripheral Vascular Disease
PVN	Paraventricular Hypothalamic Nucleus
PXR	Pregnane X Receptor
QC	Quality Control
rpm	Revolutions per minute
RT-PCR	Real time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SERCA 2a	Sarco(endo)plasmic reticulum ATPase 2a
SLC2A1	Solute carrier family 2, member 1
SLC2A4	Solute carrier family 2, member 4
TAT	Tyrosine Aminotransferase
Tris HCl	Tris Hydrochloric Acid
VNM	Ventromedial Hypothalamic Nucleus
WWII	World War Two

1 Chapter 1

Introduction

1.1 Cardiovascular Disease (CVD)

Cardiovascular diseases (CVD) are diseases of the heart and circulatory system. They are the UK's biggest cause of death, responsible for 30% of premature deaths in men, and 22% in women in 2006. This is estimated to cost the UK's economy around £30.7 billion a year, putting a huge strain on the nation's health system (British Heart Foundation (BHF) Coronary Heart Disease Statistics, 2008). One of the main forms of CVD is coronary heart disease (CHD), which accounts for approximately 19% of deaths in men and 14% in women, and is on the rise (BHF, Coronary Heart Disease Statistics, 2008). Stroke is another outcome of CVD accounting for a quarter of CVD related deaths.

CHD occurs when there is occlusion of the coronary artery by an atherosclerotic plaque consisting of LDL cholesterol (British Nutrition Foundation, 1993, Moss *et al.*, 1999). This obstructs the blood supply to the heart, leading to a deficiency of nutrients and oxygen to tissue, and impairing contractile ability (British Nutrition Foundation, 1993).

Stroke is a cerebrovascular disease, and occurs when there is a disturbance of the blood supply to the brain (Frayn & Stanner, 2005). There are two types, the first is ischemic stroke, where a blood clot forms in the heart, becomes dislodged and travels to the brain where it blocks an artery. The reduced blood supply may lead to permanent damage to the brain. The second is known as haemorrhagic stroke, where a blood vessel ruptures in the brain, releasing blood. Increased blood pressure is a common risk factor for developing this type of stroke (Frayn & Stanner, 2005).

1.1.1 Onset of CHD

There are three main events that lead to occlusion of arteries, injury to the coronary artery wall, atherosclerosis, and thrombosis. (British Nutrition Foundation, 1993, Rifkind, 1987). Normally, the lumen of the artery can respond to minor injuries/lesions caused from blood flow, but repeated wear from damaged lipid particles and free radicals can lead to more severe injuries. To repair the artery wall, platelets adhere to the damaged area, which causes a set of reactions to increase cell multiplication. This leads to a thickening of the inner wall due to collagen production and deposition of dead cell material. Thickening of the wall is increased further by scavenging macrophages removing oxidised lipid (LDL) particles (British Nutrition Foundation, 1993, Frayn & Stanner, 2005, Brown & Goldstein, 1996). This is not a regulated process so macrophages can take up large amounts of lipid, and then become overloaded (British Nutrition Foundation, 1993, Frayn & Stanner, 2005). These overloaded macrophages form foam cells, which accumulate in the artery forming a yellow raised spot, which later develops into fatty streaks (Frayn & Stanner, 2005). A blood clotting mechanism is now triggered and white blood cells, such as T-lymphocytes and blood monocytes attach to the atherosclerotic region (British Nutrition Foundation, 1993, Frayn & Stanner, 2005). Monocytes differentiate into macrophages and increase the uptake of oxidised LDL. Platelets then attach to the lesion and growth factors encourage smooth muscle proliferation on the artery walls forming a fibrous cap. This can then become hard and protrude into the artery, blocking blood flow (British Nutrition Foundation, 1993, Frayn & Stanner, 2005). Some plaques become weak and unstable, and can rupture releasing the contents of the lesion. The coagulation cascade leads to a thrombus being formed, which leads to the already narrowed artery being further blocked. Some plaques can completely block the artery leading to myocardial infarction (British Nutrition Foundation, 1993, Frayn & Stanner, 2005).

There are many factors attributing to increased risk of developing CVD. Some are uncontrollable by an individual such as genetic inheritance, as well as race, gender and age. Other risk factors are due to an individual's life style choice, such as, poor diet, lack of exercise, excessive alcohol consumption and smoking (BHF Coronary Heart Disease Statistics, 2008, Brown & Goldstein, 1996).

1.1.2 Uncontrollable/Background Risk Factors

CVD are diseases associated with old age, but are the most common causes of premature deaths (death before 75 years) in Britain (BHF, Coronary Heart Disease Statistics, 2008). In the UK, the Scottish population and Northern England have higher mortality due to CHD in comparison to Southern areas (BHF, Coronary Heart Disease Statistics, 2008). The North-South divide in health was also noted in a study completed by Barker in the early 1900's, where death rates due to low birth weight (a precursor of CVD) were higher in Northern industrial towns (Barker, 1997). This is partly due to socio-economic differences across the UK. In the late 1990's, the BHF estimated that due to social class inequalities, 5000 working class lives were lost each year amongst men aged 20-64 years.

This is not just a problem affecting the UK, but also various ethnic groups living in the UK, as well as populations internationally (BHF Coronary Heart Disease Statistics, 2008). Countries in Eastern and Central Europe generally have higher death rates from CHD in comparison to the UK. The Ukraine, for example, has experienced an increase in mortality from CHD between the years 1990 and 2000 (BHF Coronary Heart Disease Statistics, 2008). Ethnic groups, such as South Asians living in the UK, are more predisposed to develop heart problems (BHF Coronary Heart Disease Statistics, 2008), and have a higher premature death rates. This is similar amongst

Pakistani women who have 146% higher death rate, and Bangladeshi men, who have 112% higher death rate compared to the average for people living in the England and Wales (BHF Coronary Heart Disease Statistics, 2008).

1.1.3 Controllable/Lifestyle Risk Factors

Lifestyle choices, such as poor diet and lack of exercise increase the risk of developing coronary vascular diseases, as well as other life threatening conditions including diabetes and obesity. According to the BHF only 13% of British men, and 15% of women eat their daily recommended five portions of fruit and vegetables, and most of the population are consuming more than the recommended levels of saturated fat (BHF, Coronary Heart Disease Statistics, 2008). In 2002, the World Health Organisation suggested that due to low fruit and vegetable consumption (<600g/day) approximately 30% of the Western population suffered with CHD, and 20% with strokes. In 2006, 24% of the UK's population was classified as obese, with 32% of women and 43% of men being overweight (BHF, Coronary Heart Disease Statistics, 2008). Obesity is defined using the body mass index (BMI) measure. To be classified as obese an individual has to have a BMI of over 30. This is associated with high blood pressure, increased plasma insulin and cholesterol levels, all of which increase the possibility of developing CHD (British Nutrition Foundation, 1993, Brown & Goldstein, 1996).

In 2006, a UK health survey showed that only 40% of men and 28% of woman achieved the recommended amount of exercise of five 30 minute sessions of aerobic activity a week (BHF Coronary Heart Disease Statistics, 2008). It is also becoming a common problem in the under 15's where approximately only 65% are fulfilling the recommended quota. Figure 1.1 illustrates the effects that low levels of exercise can

have on an individual's risk of developing CVD. Smokers increase their risk of developing CHD by 60% in comparison to non-smokers. It is estimated that smoking caused approximately 25,000 deaths from coronary heart disease alone in 2000 (BHF Coronary Heart Disease Statistics, 2008). Smoking leads to peripheral vascular disease (PVD), which is narrowing of peripheral arteries in areas other than the main blood supply, such as the leg. Smoking leads to various physiological responses detailed in Figure 1.2, which are similar to those seen in CHD. In severe cases this can lead to loss of limbs (Frayn & Stanner, 2005).

Figure 1.1 – Consequences of poor or lack of exercise that can lead to cardiac failure

(Modified from British Nutrition Foundation, 1993)

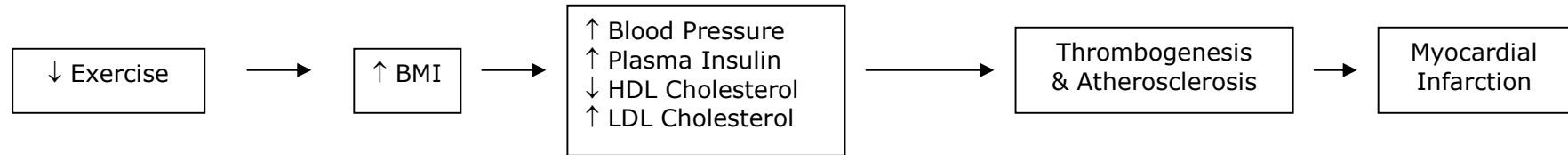
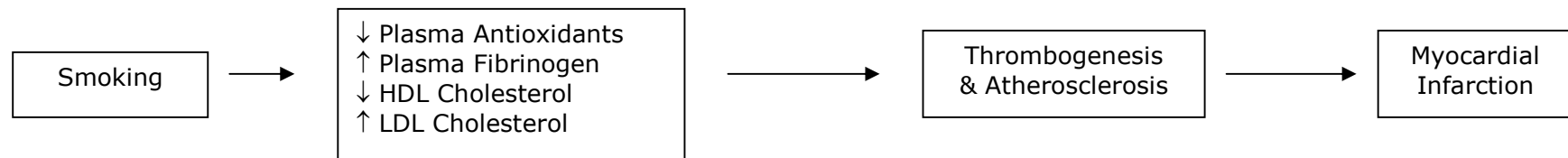


Figure 1.2 - Consequences of smoking that can lead to cardiac failure

(Modified from British Nutrition Foundation, 1993)



1.2 Hypertension

High blood pressure is a symptom-less illness affecting 31% of men and 28% of women in England in 2006 (BHF Coronary Heart Statistics, 2008, Levick, 2000). In 40-69 year old adults, each 10mmHg in diastolic blood pressure and 20mmHg increase in systolic blood pressure doubles the risk of death from CHD (BHF Coronary Heart Statistics, 2008). The World Health Report in 2002 stated that 50% of CHD cases and 22% of heart attacks are caused by hypertension due to an increase in systolic blood pressure (Interheart, BHF Coronary Heart Statistics, 2008).

Blood pressure is dependent on the equilibrium between cardiac output and peripheral resistance (Levick, 2000). Hypertension is defined as "chronic, usually progressive, raised arterial pressure", and is normally diagnosed as a resting pressure in patients under 50 years of 140/90mmHg. In patients over 50 it is diagnosed when pressure rises above 160/95mmHg (Levick, 2000, BHF Coronary Heart Statistics, 2008). There are two types of hypertension, primary and secondary. Primary, also known as essential hypertension, accounts for approximately 90% of cases that have no definable cause. Development of primary hypertension can be slow or rapid, and if left untreated can lead to various diseases including cardiac failure, cerebral damage and renal damage. Secondary (non-essential) hypertension is quite uncommon and includes Conn's syndrome and renal artery stenosis (Levick, 2000).

An individual with high blood pressure experiences moderately normal cardiac output with raised peripheral resistance, but in the early stages of hypertension development, cardiac output is raised and peripheral resistance is normal (Levick, 2000, Langley-Evans *et al.*, 1994). If the problem remains untreated it can then affect other organs including the kidneys. In the heart this can lead to hypertrophic growth of the smooth muscle, raising blood pressure as the inner walls of the arteries begin to narrow.

Eventually it will lead to compensatory hypertrophy of the left ventricle wall due the heart having to work harder (Levick, 2000).

1.3 Fetal Programming

It is understood that pregnant women should eat a balanced diet including all the essential food groups, minerals and vitamins (macro- and micro-nutrients). It is not so well known by the general public though, that small alterations in dietary intake can have a lasting effect on offspring in later life.

Fetal programming, also known as the Fetal Origins of Health and Disease, refers to the theory that the *in utero* environment influences the growth and development of the fetus. Evidence suggests that maternal influences such as poor nutrition and stress during fetal development, lead to irreversible adaptations in the structure and function of organs, which predispose the offspring to the onset of various diseases in later life (Godfrey & Barker, 2000). This hypothesis was first defined by Barker in 1980's and has since been researched extensively using both epidemiological and experimental approaches. For this study, nutritional insults to the fetus during development, and the effects these have on risk of coronary heart disease are the main areas of interest.

The hypothesis evolved from evidence that low birth weight, high placental weight and infant head circumference at birth are related to offspring developing cardiovascular problems in later life (Campbell *et al.*, 1996, Godfrey *et al.*, 1996, Godfrey & Barker, 2000). Low birth weight is also an indicator to the onset of other disorders including hypertension, glucose intolerance and diabetes (Nyirenda *et al.*, 1998).

Barker's 1986 Hertfordshire study found that small birth size was associated with increased incidence of CVD related deaths, as well as type II diabetes and insulin resistance. The study investigated interactions between intrauterine environment, adult diet and lifestyle, and disease onset. As well as low birth weight associated with increased risk of disease, it was also noted that birth weight and weight at one year was positively associated with BMI and fat mass.

Low birth weight also increases an individual's chance of developing hypertension (Campbell *et al.*, 1996), and is thought that adult blood pressure is "set" *in utero*. The Aberdeen pregnancy study (Campbell *et al.*, 1996) monitored women at seven months of gestation, and recorded food consumption during a one week period. For the first time, a link between maternal consumption of animal proteins and carbohydrates, and offspring low birth weight was found. Higher maternal protein intake led to low birth weight. Low protein and high carbohydrate intake was associated with increased blood pressure and low placental weight. High protein and low carbohydrate intake was also predictive of hypertension in the offspring. Godfrey *et al.* (1996) found in a study of Southampton women that the same nutrients were associated with markers of fetal growth. An increased carbohydrate intake in early pregnancy was predictive of the offspring having low placental and birth weight, and the same was seen in dairy and meat protein restricted females in late gestation.

Another study that found a link between nutritional intake during pregnancy and offspring's later risk of various diseases, and is one of the main studies that contributed to the development of the fetal origins hypothesis, was the WWII Dutch famine study completed by Roseboom *et al.*, 2001. Previous to the wartime famine the population were well nourished, with an average energy intake of approximately 2000 calories a day. Due to a reduction of war time rations, calorie intake dropped to between 400-800 calories a day for a period of six months. Remarkably, women still managed to conceive and give birth during this time. During the famine, data was

collected on pregnant women whose babies were exposed during early, mid and late gestation. Offspring were then followed up later in life for analysis of their health. It was found that offspring exposed to famine specifically during the first trimester of pregnancy appeared to have a higher risk of CHD in later life (Roseboom *et al.*, 2001) despite being of normal to high birth weight.

As well as these studies corroborating the proposal that maternal undernutrition leads to low birth weight and later onset of cardiovascular problems, they also highlighted that time of insult determines the effects it will have on the offspring. If the fetus is exposed to nutritional deficiencies during a 'critical window' of development then different organs will be affected depending on which organ is developing at that time (Langley-Evans, 2004, Godfrey & Barker 2000, Nyirenda *et al.*, 1998).

Low birth weight is thought to be due to growth retardation due to maternal undernutrition, which in turn leads to fetal undernutrition (Langley-Evans *et al.*, 1996a). During the fetal development of protein restricted pregnant rats, it has been found that brain growth is spared and truncal growth is restricted in the final weeks of gestation leading to a shorter body, and reduced body weight in the offspring at birth (Langley-Evans *et al.*, 1996a). The fetus adapts to the undernutrition to allow short-term survival even though this can lead to irreversible adaptations that can be detrimental in adult life (Godfrey & Barker, 2000). These adaptations could then permanently alter the way in which organs develop, therefore programming the physiology and metabolism of the offspring (Barker & Osmond, 1986).

1.4 Criticisms of the Fetal Programming Hypothesis

Despite the evidence that supports the fetal programming hypothesis, there have been robust criticisms of the epidemiological evidence that shaped the concept. In studies such as the Dutch famine, there is a heavy reliance upon historic records that may be inaccurate, and may not have recorded the necessary information. This could be because tracking down individuals for investigation into the onset of adult disease is difficult, and the study size is not seen as sufficient to gain a thorough conclusion. Roseboom *et al.* (2001) stated that birth weight may be a poor indicator for evaluating nutritional events during pregnancy. Not all offspring have an altered birth weight due to maternal nutrient restriction, but such restrictions could still have health implications on the offspring.

One recent study by Langley-Evans & Langley-Evans (2003) in Northampton also found evidence that conflicted with Barkers low birth weight link to onset of disease theory. It was suggested that low birth weight, increased placental weight, thinness at birth and large head circumference of the offspring, predict diseases such as diabetes and CVD. In this study, maternal nutrient intake in pregnant women during the first and third trimester was measured to investigate its effects on offspring's birth and placental weight as well as infant proportions. There was found to be no relationship between maternal diet in the first and third trimester of gestation and offspring birth or placental weight. Only thinness at birth was associated with maternal carbohydrate intake. Mathews *et al.* (1999) also found no significant relationship between maternal diet and offspring birth or placental weights.

A study completed by Huxley *et al.* (2004a) found evidence to suggest that there is no relationship between birth weight and an offspring's later risk of CVD. It also conflicted with evidence that individuals born into deprived backgrounds developed hypertension as adults (Barker & Osmond, 1986). Investigations by Huxley *et al.* (2004a) were

completed on the offspring from women who were pregnant during WWII, similar to that in the Dutch famine study. The pregnant women underwent biochemical tests completed to analyse markers of nutritional intake and an interview to establish socio-economic standing. The offspring went through a similar process when located a number of years later looking for precursors of coronary heart disease. It was found that there was no link between the offspring's birth weight and the development of hypertension in later life, and that individuals from higher social backgrounds had higher blood pressure. This demonstrates the inconsistency in findings across studies, and that birth weight cannot necessarily be used as a marker of fetal undernutrition.

The programming concept has also been criticised on the basis of inconsistency in findings and a lack of control for confounding factors in epidemiological studies (Huxley *et al.*, 2004a). Early studies of associations between birth weight and blood pressure often overlooked confounding factors, which have the potential to have an effect on disease onset. The impact of factors such as parental social status and blood pressure, and offspring's social-economic status, lifestyle in later life were not fully assessed by the majority of retrospective cohort studies (Huxley, 2004b).

Meta-analyses are being more commonly used in epidemiological investigations, reviewing data from several studies and providing a more robust and accurate outcome. Studies by Huxley (2004b) have found that there is variation between studies of differing subject size. Large studies (≥ 3000 subjects) have less significant data indicating weaker associations between birth weight and blood pressure. Smaller studies (≤ 1000 subjects) on the other hand, appear to have more significant data. Favour is often given to publication of these smaller studies as they have high impact findings, so are being more frequently reported. Meta-analysis is a useful tool in the elimination of this publication bias.

Huxley (2004b) suggests that human studies finding casual links between birth weight and disease onset are good for forming hypotheses, but need further investigation using animal models of programming to support them. Animal studies provide a means to eliminate discrepancies between studies and investigate the mechanisms of programming.

1.5 Animal Models of programming

Even though human studies provide the best insight into fetal programming and how nutritional intake can affect the offspring's risk to diseases, they alone cannot fully support the hypothesis. It is clear that criticisms of the epidemiological approaches used have raised significant questions about the validity of assertions made by Barker and others. Development of animal models of programming has been important in demonstrating plausibility of fetal programming by maternal nutrition. Animal models have been developed to explore the theory and back up current findings from human studies. Such models allow investigation of different dietary intakes in pregnancy looking, for example, at specific nutrient restriction, which is not feasible in human studies. These experiments can also begin to explore the underpinning mechanisms and pathways involved in programming.

Various models of dietary alteration during pregnancy have been established in animals including rat, pig and sheep with alterations to a 'normal' well balanced diet. Most experimental diets attempt to demonstrate the biological principles of programming (Langley-Evans, 2004). Experimental models of diet in pregnancy fall into a few categories such as variance in total food intake including dietary excess and restriction, and altering macro- and micro- nutrients such as calcium.

1.5.1 Restricted Food Intake Models

Global undernutrition is a commonly used approach to investigate the impact of maternal undernutrition on later CVD risk. This approach is favoured as it produces fetal growth retardation (Langley-Evans, 2004). Some of the first studies of total dietary restriction using animal models were completed by Persson & Jansson (1992), and Woodall (1996). Persson & Jansson developed a guinea pig model to investigate food restriction *in utero*. Blood flow was restricted to one horn of the guinea pig's uterus depriving pups of energy and nutrients, and the other horn had no restriction. It was found that pups subject to restriction were growth-restricted compared to the unrestricted pups, and these pups developed higher blood pressure. This demonstrated that there was a significant association between low birth weight and elevated blood pressure in later life.

Woodall *et al.* (1996) investigated restricted food intake in pregnant rats to establish whether this diet affected blood pressure in offspring later in life. Maternal diet was restricted by 70% compared to control animals, and it was found that these animals also had a lower birth weight and developed hypertension. Both of these studies strongly supported the hypothesis that maternal undernutrition leads to growth retardation, and predispose offspring to develop cardiovascular abnormalities. In contrast to other studies, six month old offspring of pregnant sheep fed 50% of the recommended metabolisable energy intake during days 28-80 of gestation, were found to develop lower resting blood pressure in comparison to controls, and a low nephron number (Gopalakrishnan *et al.*, 2005).

1.5.2 Dietary Excess Models

Increased food consumption above the recommended calorie intake is a driver of human obesity, and is an ever increasing problem in Europe and the US. In 2006, approximately 25% of the UK's population were classed as obese (BHF Coronary Heart Statistics, 2008). Obesity can lead to various life threatening illnesses including type II diabetes and CHD (Khan *et al.*, 2004). Obesity during pregnancy is also becoming more common in the UK and US (Akyol *et al.*, 2009), and is associated with complications such as pre-eclampsia and thrombosis. There is also an increased risk of delivery complications leading to maternal and infant death (Akyol *et al.*, 2009).

Samuelsson *et al.* (2008) explored the hypothesis that obese mice during pregnancy would be more likely to have offspring that developed cardiovascular and metabolic problems in later life. By feeding mice either a control or highly palatable, high fat diet, made up of 16% saturated fat and 30% sugar before, during and after pregnancy, they showed that maternal obesity led to offspring developing hyperphagia, greater adiposity and hypertension.

The cafeteria diet model is becoming commonly used in the investigation of the impact of junk food diet on pregnant rats. Akyol *et al.* (2009) investigated the effects of feeding twelve high fat food items to rats to induce obesity prior to pregnancy. Pre-gestational obesity was achieved with this increased calorie diet leading to increased maternal total body fat. Interestingly, this did not affect reproductive success of the females, but continuation of feeding throughout pregnancy did lead to reduced weight in the offspring at gestational age day 20.

Bayol *et al.* (2008) also investigated the effects of high fat, sugar and salt diet in pregnant rats. The diet was fed throughout gestation and lactation, and then offspring were weaned onto standard chow. Adolescent offspring developed increased fat pad

mass and adipocyte hypertrophy. They also showed raised insulin-like growth factor-1, insulin receptor substrate-1, PPAR γ , leptin and glucose transporters 1 and 3 mRNA expression. They are all involved in the regulation of lipid synthesis, adipocyte differentiation and proliferation, glucose transport and appetite. These increases led to early onset of hyperglycemia, hyperinsulinaemia and hyperlipidemia.

1.5.3 Nutrient Restriction Models

More commonly used models for the study of fetal programming are ones which impose a specific nutrient restriction. Not many human populations undergo famine during pregnancy, rather they lack in specific vitamins, minerals and nutrients. Several models of nutrient restriction, which assess the impact of such deficits, have been developed in different animals including rat, mouse, and guinea pig (Langley-Evans, 2004), which enable studies to examine the effects of relatively small dietary manipulations.

Iron deficiency is a common problem amongst pregnant women, with up to 30% of the UK population affected by it. Global prevalence of iron deficiency is estimated at 2-3 billion, and in developing countries it is seen in 50% of pregnant women (Langley-Evans, 2009). A model has been developed to investigate how maternal anaemia throughout gestation affects the health of the offspring. Gambling *et al.* (2003) completed a study using dietary iron restriction before and during pregnancy. It was found that iron intake at 7.5mg/kg diet, compared to control levels at 50mg/kg diet, significantly increased the offspring's blood pressure, and that hypertension remained into adult life. These rats were also born with anaemia, even though the diet was classed as a mild form of iron deficiency.

Bursey *et al.*, 1983 investigated pregnant rats treated with decreasing concentrations of dietary sodium throughout gestation, ranging from 0.022% to 0.173%, as a model of nutrient restriction. It was found that low levels of sodium in the rats' diet led to dramatic effects on the offspring three weeks postpartum. Females fed the lowest two sodium concentration diets experienced less weight gain during pregnancy, decreased food consumption, and had smaller litters. Offspring had low birth weight, and impaired brain development, resulting in smaller brains in comparison to higher sodium groups. Offspring exposed to the lowest level of sodium did not survive lactation.

1.5.4 Maternal Low Protein (MLP) Model

Langley & Jackson (1994) developed the low protein rat model that has now become well established in the investigation of the effects of undernutrition during gestation upon cardiovascular function. The diet consists of a total protein content of 9% casein compared to a control diet of 18% casein. This is regarded as a mild form of protein restriction, and is the concentration required for a non-pregnant rat (Langley Evans, 2004), pregnant rats require 12% protein (Langley-Evans *et al.*, 1994). To match the control diet total composition, the MLP diet has an increased carbohydrate content consisting of starch and sucrose (Langley Evans, 2004) and has similar energy content.

To date, research on MLP fed rats and mice as a model of maternal under nutrition has provided evidence that this diet leads to offspring developing hypertension (Langley-Evans *et al.*, 1994, Langley-Evans *et al.*, 1996c, Langley-Evans *et al.*, 1999, Harrison & Langley-Evans, 2008), abnormal cardiac output (Cheema *et al.*, 2005), atherosclerosis (Yates *et al.*, 2009) and reduced nephron number (Langley-Evans *et*

al., 1999). Maternal protein restriction therefore predisposes the offspring to develop diseases, including CHD and risk factors for CHD.

MLP diet fed during gestation induces hypertension (blood pressure elevation of 20-30mmHg) as early as 4 weeks old in offspring, independent of maternal blood pressure. Hypertension continues throughout life (Langley-Evans *et al.*, 1994). This increase in offspring blood pressure has recently been identified to pass between generations (Harrison & Langley-Evans, 2008). Raised blood pressure and reduced nephron number were identified in the F1 generation, and this was then seen in the subsequent F2 generation without further dietary manipulation. Decreased nephron number was passed down both maternal and paternal lines. By the F3 generation these alterations in blood pressure and renal function were no longer present (Harrison & Langley-Evans, 2008). Lower nephron number and increased blood pressure have been suggested to be linked. It has been proposed by Mackenzie *et al.* (1995), discussed in Harrison & Langley-Evans (2008), that decreased nephron number increases blood pressure as a compensatory mechanism, maintaining glomerular perfusion. The increase in blood pressure then leads to further losses of nephrons and a vicious cycle begins resulting in hypertension. These transgenerational effects may be explained by female offspring's cardiovascular being altered by maternal diet during their pregnancy. Decreases in vasodilator acetylcholine and beta-adrenoceptor agonist, isoprenaline, were seen in MLP female offspring towards the end of pregnancy (Torrens *et al.*, 2003). These are known to contribute to the onset of hypertension, potentially leading to problems during pregnancy.

As well as the MLP diet causing cardiovascular problems, it also disturbs metabolic and behaviour of rodent offspring (Bellinger *et al.*, 2005). Environmental factors and undernutrition *in utero* can have significant impact on metabolic abilities. Offspring exposed to MLP *in utero*, at 18 months old, developed hypertriglyceridemia and insulin resistance, and a 2-3 fold increase in hepatic triglycerides compared to controls

(Erhuma *et al.*, 2007). These findings along with a depressed expression of PPAR α , which is involved in lipogenesis and lipid storage, are indicative of metabolic syndrome.

Bellinger *et al.* (2005) observed that female offspring fed a MLP diet for one week of gestation (early, mid and late), consumed less fat when put on a self-selection diet of high-fat, high-carbohydrate and high-protein in comparison to controls. Conversely, previous studies have shown that a MLP fed throughout gestation leads to increased consumption of fat. These changes may be mediated by gonadal steroids as changes were only seen in female offspring. It was also interesting to note that the MLP diet had effects before the placenta had been fully established.

Lifespan of MLP offspring, exhibiting low birth weight and increased blood pressure from an early stage, has been suggested to be reduced by 11% compared to control animals (Sayer *et al.*, 2001). The rats in this study were left to die of natural causes assessing only the total lifespan, whereas other studies have investigated the potential of premature aging (Langley-Evans & Sculley, 2005). At birth MLP offspring displayed increased oxidation of hepatic proteins, and in later life, at several time points, male offspring accumulated increased protein carbonyl concentrations in liver, and increased glutathione peroxidase activity. Increased oxidation promotes apoptosis and loss of tissue function, so these findings are indicative of premature aging (Langley-Evans & Sculley, 2005). MLP leads to premature oxidative damage leading to premature aging and a decreased lifespan.

Protein restriction has also been found to have effects in Apo*E Leiden mice that carry a mutation making them prone to developing atherosclerotic lesions when fed high cholesterol diets. MLP diet during gestation combined with atherogenic diet at weaning, led to the offspring developing a significant increase in the formation of an

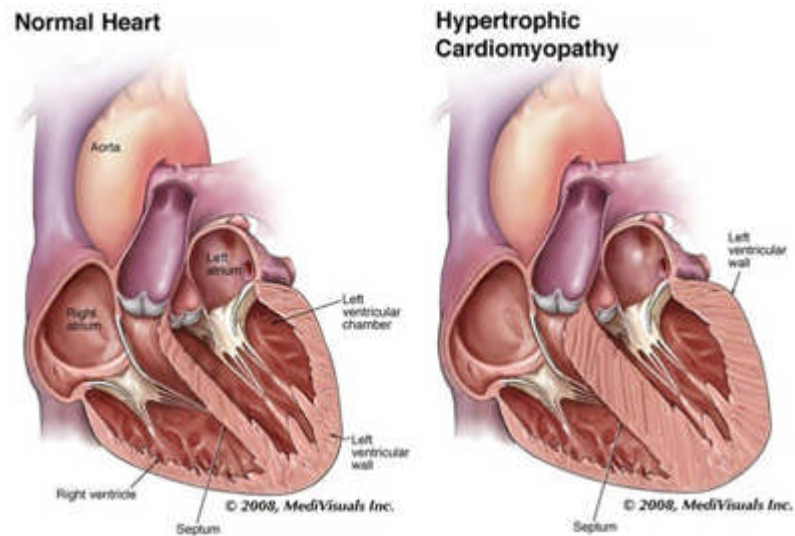
atherosclerotic lesion within the aortic arch, and increased total plasma cholesterol and dyslipidaemia (Yates *et al.*, 2009).

1.6 The Heart

Within this thesis, study will be focused upon programming effects of MLP diet upon the heart. It is therefore useful to consider the anatomy, development and function of this organ.

The heart is a unique organ that every cell in the body is reliant upon. It pumps approximately 8000 litres of blood a day throughout a persons life span. It pumps blood carrying oxygen, nutrients, white blood cell and hormones to their intended destination (Martini *et al.*, 2001b). The mammalian heart is split into four chambers; two atria and two ventricles. The two ventricles pump blood around the body. The right pumps to the pulmonary system, oxygenating the blood, and the left pumps 6-7 times more blood to the rest of the systemic circuit. For this reason the left ventricle is a larger chamber compared to the right, and has a much thicker wall (Martini *et al.*, 2001b).

Figure 1.3 – Normal and Hypertrophic Heart (Washington University, School of Medicine web page)



The human heart develops into its complete structure 21 days after conception and begins to beat (Martini, 2001a), and in the rat it is fully established at 3 weeks postpartum (Rudolph *et al.*, 1974). During development the rat heart uses different energy substrates. At birth there is an increased expression of fatty acid transporters, and glucose transport and oxidation genes are down-regulated (Lavrentyev *et al.*, 2004). This persists until approximately two weeks postpartum when fatty acids are no longer the main sources of energy. At three weeks after birth glucose transport and oxidation genes begin to be up-regulated, and fatty acid uptake is decreased (Lavrentyev *et al.*, 2004). From this point on, glucose transporters (GLUT) 1 and 4 become the main glucose transporters for the heart.

1.6.1 Cardiomyocytes

The muscle cells of the heart are known as cardiomyocytes, and are responsible for producing electrical impulses that control the heart. When cells join together they beat

in unison as excitation spreads from one cell to another (Berne & Levy, 1997). Gap junctions between cells are connected by either connexins (Berne & Levy, 1997) or desmosomes (Levick, 2000), which attach the cell cytosol together. Without this connection ionic currents would not be transmitted from one cell to the next, and so it ensures the cells of the myocardium work together. If cell linkage is broken, in cases of tissue damage, oxygen is not effectively delivered to the cells, resulting in impaired electrical conduction (Levick, 2000).

Cardiac muscle cells fall into two categories, myocytes and the cardiac electrical system (Levick, 2000). Cardiomyocytes form the majority of the cells of the heart and carry out the mechanical work (contracting), but can only contract when electrically stimulated. The electrical supply is provided by a small number of cells that form the cardiac electrical system, which comprises of sinoatrial node, atrio-ventricular node, Purkinje fibres, and conduction bundles. These nerve cells manage the heart beat by initiating and conducting the electrical impulses that stimulate the myocytes (Levick, 2000).

In the rat at birth, cardiomyocytes are approximately 5-6 microns in size, and grow to 15 microns in the adult heart (Rudolph *et al.*, 1974). Human myocytes are approximately 10-20 μ M in diameter, and 50-100 μ M long (Levick, 2000). Cardiomyocyte growth occurs in three phases; proliferation, binucleation and hypertrophy (Ahuja *et al.*, 2007). The two main growth phases in both rats and humans are termed hyperplastic and hypertrophic. Hyperplasia is where the cells undergo mitosis and division, increasing the number of cells in the heart tissue (De Vries *et al.*, 2002). These immature cardiomyocytes are mononucleated, and still have the ability to divide. Once they are fully differentiated the cells are binucleated, and are no longer able to divide. They can however undergo hypertrophic growth, where the cells increase in size and the heart increases in weight (De Vries *et al.*, 2002). During both human and rodent heart maturation, cardiomyocytes undergo both types

of growth (Cheema *et al.*, 2005). Hyperplastic growth occurs in the early stages of development, and hypertrophic occurs in the perinatal stage resulting in a 30-40 fold increase in individual cell size (Sugden *et al.*, 1999, Oparil, 1984). In the rat, postnatally cardiomyocytes are predominantly mononucleated as hyperplasia still occurs in the first few weeks after birth (Cortius *et al.*, 2005), and shortly after, the cells cease to divide (Oparil, 1984).

1.6.2 Hypertrophy

Whilst hypertrophy is a normal element of the growth of the heart, allowing an increase in size, adult hypertrophy is also associated with disease. Hypertrophic growth in adult life is a compensatory mechanism following insult, or when the heart has to work harder than normal to pump blood, such as during hypertension or myocardial infarction (Figure 1.3). This growth allows the heart to continue to work at its normal rate, giving the same cardiac output as before the incident, adapting to its increased demands (Santalucia *et al.*, 2005, Liao *et al.*, 2002, Sugden *et al.*, 1999). However, if this type of growth continues, whether it is throughout the heart, or just ventricular, for a prolonged period, it can have detrimental effects (Santalucia *et al.*, 2005, Sugden *et al.*, 1999). Hypertrophy can lead to remodelling of the heart, permanently affecting its ability to dilate and contract (Katz, 2000). There are also metabolic effects. In the damaged adult heart when hypertrophy has occurred, glucose oxidation is increased even though heart failure has been linked to a reduction in glucose transporters (Santalucia *et al.*, 2005). The expression of ventricular GLUT 1, basal glucose uptake is increased, and GLUT 4, insulin regulated uptake is decreased (Liao *et al.*, 2002, Santalucia *et al.*, 2005).

The mechanisms and pathways that underlie cardiac hypertrophy are currently poorly understood. A study completed by Montessuit & Thornburn (1999) investigated the effects hypertrophic agonists had on rat neonatal ventricular cardiac myocyte cells. It was found that agonists induced expression of GLUT 1, demonstrating that the heart requires a high basal energy intake to compensate for work overload. It has been suggested that cardiomyocyte mitosis (hyperplastic growth) does occur in adult life, which could provide a means for the recovery of the heart following heart damage (Anversa *et al.*, 1998).

1.7 Tissue Remodelling as a Mechanism for Fetal Programming

The mechanisms which underpin programming of disease risk are largely unidentified, but it is likely that changes to physiological function are due to tissue remodelling (Langley-Evans, 2009). Organ development is complete at birth, with a surge of cortisol in human tissues that ensure organs are matured in preparation for life. In the rat, some organ development continues shortly postpartum, for example, the heart continues to undergo hypertrophic growth for a short period in neonatal life. At this stage any deficits are irreversible, improper growth of an organ for instance, cannot be reversed or corrected.

Tissue remodelling is the process where by organs and tissues are permanently altered in their structure, and hence function. A reduction in cell number or type could have detrimental effects on an organ, altering the number of functional units leading to an increased risk of disease (Langley-Evans, 2004). Such alterations have been frequently found to occur in offspring that have experienced some type of insult *in utero*, such as dietary restriction or stress. Feeding rats a low protein diet, for example, during gestation, impacts on kidney function of the fetus. Mild protein

restriction mid-gestation increases blood pressure and reduces the number of nephrons in the kidney (Langley-Evans *et al.*, 1999). It is argued that having fewer nephrons in the kidney increases local blood pressure in order to maintain renal perfusions over time, and with further nephron loss, this drives up systemic blood pressure. Other rodent studies have found that protein restriction during pregnancy decreases the number of pancreatic β -cells, which permanently changes glucose homeostasis. This was seen more severely, along with growth defects, in offspring that were exposed to protein deficiency during both gestation and lactation (Heywood *et al.*, 2004).

Remodelling has also been identified in the brain tissue in the offspring of rats fed MLP during pregnancy and lactation (Plagemann *et al.*, 2000). The hypothalamus is sensitive to hormonal, metabolic and nutritional alterations during development. Protein deficiency altered the organisation of hypothalamic regulators of body weight and metabolism leading to low body weight, hypoglycaemia and hypoinsulinemia in offspring at weaning age. Ventromedial Hypothalamic Nucleus (VNM) and Paraventricular Hypothalamic Nucleus (PVN), the two main nuclei involved in the regulation of food intake and weight gain, developed a higher overall mass of neurons, and the VNM increased in volume. These alterations may be permanent, therefore offspring of this maternal diet would have permanent disorganisation and dysfunction of the feeding centres of the hypothalamus (Plagemann *et al.*, 2000). This is of interest in the context of the altered appetite and feeding behaviours of such animals (Bellinger *et al.*, 2005)

To date, few studies have investigated remodelling specifically of the heart and whether permanent alterations to structure predispose offspring of undernourished mothers to develop cardiovascular abnormalities in adult life. It is well established that maternal low protein diet during pregnancy induces hypertension in the offspring in adult life (Langley-Evans, 2004). Cheema *et al.* (2005) used this rat model to observe

if this diet caused structural and functional changes to the heart of the developing fetus. It was found that the cardiomyocytes underwent hypertrophy, a compensatory mechanism leading to hypertension, causing left ventricular wall thickening, a known predisposing factor of heart disease. The reason for the cells undergoing this type of growth was due to a threefold increase in cell apoptosis, producing a decrease in cell number, which needed to be replaced by increasing cell size. This in turn also reduced the ejection fraction (left ventricular end-diastolic volume), which strongly indicates left ventricular remodelling. Cheema *et al.* (2005) stated that as aging is a known driver of such for changes to the heart, that hearts exposed to MLP diet *in utero* could be undergoing premature ageing (Cheema *et al.*, 2005).

A maternal low protein diet is also known to cause growth retardation of the developing fetus, which is thought to be a crude marker for the onset of CVD in later life (Langley-Evans & Nwagwu, 1998). The reason for this link could be due to a reduced supply of nutrients affecting cell mitosis in organs and tissues. This growth retardation affects all organs excluding the brain, and in particular, it is thought that growth restriction during gestation could lead to a loss of cardiomyocytes of the heart (Cortius *et al.*, 2005). Cardiomyocytes of low protein offspring underwent normal hyperplasia during fetal growth, but hypertrophic growth occurred at a much earlier stage compared to control animals (Cortius *et al.*, 2005). Hearts were found to have a reduced number of cell nuclei and an overall decreased number of cardiomyocytes, as well as a significantly reduced cardiac tissue volume and weight. As hyperplasia does not occur for very long postpartum, these lower numbers of heart cells are likely to remain throughout adult life (Cortius *et al.*, 2005). As the heart has not developed as a 'normal' heart would, a period of catch up growth takes place, which is in the form of hypertrophic growth (Cortius *et al.*, 2005). A premature halt to hyperplastic growth means that the heart will not have enough functional units in comparison to a control heart, potentially compromising later function. This is analogous to what is observed with nephron number in the kidney (Langley-Evans *et al.*, 1999).

Aroutiounova *et al.* (2009) supported this finding when investigating the ability of cardiomyocytes in neonatal rats to replicate. Prior to mating and during pregnancy, rats were fed either a 9% or 18% casein diet, and following birth they were put on standard chow throughout the suckling period. The maternal low protein diet decreased the rate of mitosis in neonates, suggesting that the diet may limit cell division. This would explain the low number of cells found at birth by Cortius *et al.* (2005), as well as the increased apoptosis reported by Cheema *et al.* (2005). One to two weeks postpartum, mitosis began to increase in MLP animals, and by one month there was no difference between controls and low protein animals. This strongly suggests that chow diet enables the cells to recover and restart replication (Aroutiounova *et al.*, 2009). This study did not however examine whether the earlier period of abnormal growth impacted upon function.

Such abnormalities in the heart may predispose offspring of protein restricted mothers to develop hypertension (Langley-Evans *et al.*, 1995, Langley-Evans *et al.*, 1996a, Langley-Evans *et al.*, 1999,) and associated cardiovascular disease. These disorders also cause the heart to respond with another bout of hypertrophic growth (Oparil, 1984). Due to the fact that the heart has already gone through several episodes of hypertrophic growth in early life in hypertensive animals, the capacity for this type of growth is much reduced in adult life. This decrease in the hearts capability to increase tissue mass and poor contractile ability could lead to heart failure. This supports Cheema *et al.*'s (2005) suggestion that the exposure to MLP in fetal life, leads to the heart undergoing premature aging. Consistent with this, Elmes *et al.* (2007) have found, using the Langendorff perfusion technique, that rats exposed to MLP diet during development have an impaired recovery of left ventricle pressure following ischemic reperfusion. This effect was more severe in males than in females. These offspring also developed hypertension. In addition, the low protein diet altered the hearts response to β -agonist stimulation in males (Elmes *et al.*, 2009). Stimulation of the

cardiac β -adrenergic system by β -agonists may contribute to development of heart failure, as they were found to delay the recovery to basal cardiac function.

Following on from Cheema *et al.*'s (2005) work, where decreased contractile function was caused by exposure to the MLP diet, the mechanisms of energy utilisation of the neonatal heart were investigated as a possible reason for apparent remodelling. Using the same model of maternal protein restriction, the expression of genes regulating phospholipid metabolism was assessed by Tappia and colleagues (2005). Expression of MEF-2C (controls glucose supply and utilisation genes) was reduced, whilst PPAR- α (involved in the regulation of fatty acid oxidation) was increased. There was also evidence of increased apoptosis in this study. However, these changes were not to a level sufficient to alter cardiomyocyte function. This indicates that cell apoptosis is a more likely cause for a reduced contractile ability (Tappia *et al.*, 2005).

Tappia *et al.* (2009) investigated if calcium channels were affected by maternal protein deficiency, as this could play a role in the programming of poor contractile function. MLP diet was found to affect various components of the regulation of cardiomyocyte calcium homeostasis. This included reducing mRNA expression of the L-type calcium channel and increasing Na^{2+} - Ca^{2+} exchanger expression, as well as depressing ryanodine receptor protein content and SERCA 2a expression that may be involved in cardiac contractile mechanisms. However, it was not found that this had a direct impact on function.

It is becoming clear that, as with other organs, the rat heart undergoes remodelling of structure in fetuses exposed to maternal protein restriction. It is possible to speculate on how this may change later function and hence risk of CVD. It is of considerable interest however, to determine the events that link maternal undernutrition to permanent changes in organ structure. Although this may simply reflect nutrient deprivation during phases of growth and maturation, a number of studies have

suggested that more complex scenarios operate. These include disturbances of the normal process that regulate the balance of endocrine signals between mother and fetus.

1.8 Glucocorticoids

Glucocorticoids (GC) are steroids primarily involved in mediating stress-responses, but are also involved in regulating metabolic and cardiovascular functions. In humans, cortisol is the main glucocorticoid (Whitworth *et al.*, 2005) and is essential for maintenance of blood pressure. Natural occurring excess of glucocorticoids results in Cushing's syndrome, when cortisol is over-secreted causing hypertension (Whitworth *et al.*, 2005). Both systolic and diastolic pressures are elevated and can lead to death from CVD, and even in treated patients mortality rates are high (Whitworth *et al.*, 2005). In the rat, corticosterone is the main glucocorticoid. They regulate gene transcription via the nuclear hormone receptor (glucocorticoid receptor), and disturbance of the GC axis in the developing fetus may lead to later abnormalities in glucose metabolism (Nyirenda *et al.*, 1998).

In the case of the developing fetus, GC's are involved in the maturation of organs and tissues, especially in preparing the lungs for postnatal life (Slotkin *et al.*, 1991, Drake & Seckl, 2004). Prior to a normal human birth there is a surge of cortisol to the fetus (Liggins, 1994) to promote the maturation of vital organs such as heart, gut and kidneys. For this reason, synthetic GC's, such as dexamethasone, are frequently used in women who are likely to have a preterm delivery. Due to GC's affects on organ development, they have become extensively used during child birth and in newborns (Drake & Seckl, 2004). Excess of glucocorticoids during development can however, have detrimental effects if the fetus is exposed too early. GC's are known to restrict

growth leading to lower birth weight and alterations in cellular development and function (Slotkin *et al.*, 1991, Langley-Evans *et al.*, 1997b).

1.8.1 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD-2)

Glucocorticoids during pregnancy can freely cross the placenta, so the fetus needs to be protected from high concentrations of maternal GC's, so that the fetal hypothalamic-pituitary-adrenal axis can develop independently (De Vries *et al.*, 2002). Fetal concentrations of GC are approximately 5-10 times lower in comparison to maternal levels in humans (Nyirenda *et al.*, 1998) and can be as much as 100-1000 fold in the rat (Drake & Seckl, 2004). The enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD-2) acts as the barrier between the mother and fetus's steroids, and metabolises GC's as they pass across the placenta. In humans, cortisol is converted to inert cortisone, and in rats corticosterone is converted to 11-dehydrocorticosterone (Benediktsson *et al.*, 1993). However, this barrier is not always effective and slight changes in enzyme activity can have significant impacts on the fetus due to the difference in concentrations of GC. It has been found that if high levels of maternal steroids reach the fetus, the overexposure can lead to growth retardation of the offspring (Nyirenda *et al.*, 1998, Langley-Evans, 1997b). This increase can also lead to premature maturation of organs and tissues (effectively tissue remodelling). Such alterations can lead to disease onset in later life, thus suggesting that GC overexposure can have permanent programming effects.

11 β -HSD-2 is known to be down-regulated at rat gestational age 20 by MLP diet (Langley-Evans & Nwagwu, 1998). Langley-Evans *et al.*, (1996c) found that expression of 11 β -HSD-2 was reduced in placentas of pregnant rats fed a low protein diet, which also led to the offspring developing hypertension in adult life. It was also

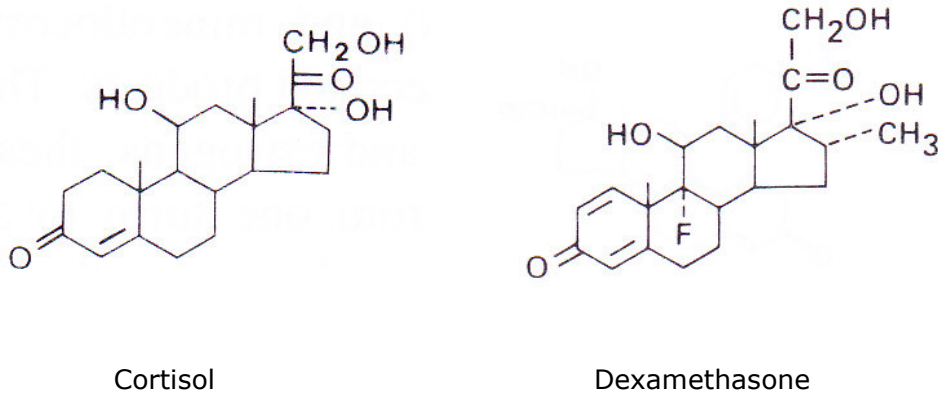
found that if mothers fed this diet were treated with the inhibitor of GC synthesis, metyrapone, hypertension was prevented from occurring in the offspring (Langley-Evans *et al.*, 1996, McMullen & Langley-Evans, 2005).

Timing of GC exposure seems to have different impacts on the fetus, for example, rats treated with dexamethasone specifically in the last week of pregnancy had reduced birth weight and developed hypertension (Levitt *et al.*, 1996). Inhibition of 11 β -HSD-2 using carbenoxolone (CBX), raised blood pressure and decreased birth weight in rat offspring regardless of timing of treatment. However, greatest increase in blood pressure was noted when mothers received CBX in the last week of pregnancy (Langley-Evans, 1997b). It is thought that normal silencing of 11 β -HSD-2 occurs at different times in different organs depending on when maturation is to occur. In mice, there is a silencing of 11 β -HSD-2 around mid-gestation, which could be to allow GC to have effects in time for birth (Brown *et al.*, 1996).

1.8.2 Dexamethasone

Dexamethasone (Dex) is a synthetic glucocorticoid used as an alternative to the natural occurring steroid cortisol. It is used in the treatment of preterm births in humans (Drake & Seckl, 2004), and is commonly used in rodent models of disease. Dex is a more potent glucocorticoid in comparison to human cortisol and has different chemical structure (Kamphuis *et al.*, 2007) as illustrated in Figure 1.4 (Cook & Beanstall, 1987, Wakeling, 1987). It is poorly metabolised by 11 β -HSD-2, so easily crosses the placenta (De Blasio *et al.*, 2007, Dodric *et al.*, 1999, Benediktsson *et al.*, 1993). This leads to greater active glucocorticoid exposure to the fetus in comparison to cortisol, of which only approximately 10-20% in humans passes to the fetus (Blasio *et al.*, 2007).

Figure 1.4 – Chemical structure of cortisol and dexamethasone (Cook & Beanstall, 1987, Wakeling, 1987)



In comparison to natural occurring glucocorticoids in the rat, Dex has a high binding affinity to glucocorticoid receptor (GR), but not the mineralocorticoid receptor (MR) (De Blasio *et al.*, 2007). It has also been noted that it does not completely bind to these receptors in bovine tissue (Dodric *et al.*, 1999). Studies discussed in Dodric *et al.* (1999) have suggested that Dex has a high affinity for the pregnane X receptor (PXR), which does not bind to natural occurring GC's.

Dex is known to promote differentiation of cells, inhibiting cell mitosis in early development, as well as inducing growth retardation, alteration of the HPA axis and increased blood pressure (Dodric *et al.*, 1999). This can have detrimental effects on the fetal heart as it prevents further growth, resulting in fewer and potentially smaller cells (De Vries *et al.*, 2002, Slotkin *et al.*, 1991). In rodent models of programming, Dex has been found to have effects towards the end of gestation, leading to growth retardation linked to high blood pressure of the offspring in later life (Levitt *et al.*, 1996). In other studies Dex administered at gestational days 17, 18 and 19 in pregnant rats led to a reduction in the number of cells in both the heart and kidney (Slotkin *et al.*, 1991), but it did not affect the overall size of these organs. Low doses

of Dex (0.2mg/kg) increased the relative cell size and packing density in the kidney suggesting an increase in hypertrophic growth.

Dodric *et al.* (1999) also found that treatment of rats in the last third of pregnancy with dexamethasone led to increased blood pressure 16 weeks after birth. There appear to be windows of sensitivity to Dex treatment, which result in different long-term problems (Dodric *et al.*, 1999). In some cases Dex has been identified to reduce lifespan (Kamphuis *et al.*, 2007).

Dexamethasone has also been found to alter the expression of the glucose transporters GLUT 1 and 4, in treated rats. Dex administered mid-gestation induced growth retardation and hypertension in offspring, which in turn caused up-regulated GLUT 1 in males (Langdown *et al.*, 2001). It may however not be Dex directly causing an increase in the glucose transporter, but a response to hypertension.

1.9 Glucocorticoid hypothesis as a Mechanism for Fetal Programming

During development, growth of the fetus is predominantly determined by genetics, but it is thought that the *in utero* environment may also have an effect (Langley-Evans & Nwagwu, 1998). The link between low birth weight and hypertension has been suggested to be due to fetal overexposure to maternal glucocorticoids, as they are known to retard fetal growth (Benediktsson *et al.*, 1993, Edwards *et al.*, 2001, Langley-Evans *et al.*, 1997b, Langley-Evans & Nwagwu, 1998). The hypothesis proposed suggests that glucocorticoids passing from the maternal system across the placenta expose the fetus to abnormally high concentrations. This will then lead to growth restriction and thus alteration in tissue structure and function (Godfrey & Barker, 2000, Edwards *et al.*, 2001). These permanent changes then predispose the

offspring to develop cardiovascular, metabolic and behavioural abnormalities (Drake *et al.*, 2004). The reason for increased GC's passing across the placenta is through alteration of placental function, (Edwards *et al.*, 2001) caused by a reduction in the expression and activity of 11 β -HSD-2 (Langley-Evans, 1996c, Langley-Evans & Nwagwu, 1998). The MLP diet has been suggested to cause a decrease in 11 β -HSD-2 in the placenta (Langley-Evans, 1996c); the mechanism of this, however, is unknown.

Many animal studies have been completed to investigate this theory, and the most frequently reported finding from administering GC's during pregnancy is a reduction in birth weight, suggesting growth retardation of the fetus, and hypertension. Benediktsson *et al.* (1993), Langley-Evans (1997a), Langley-Evans & Nwagwu (1998) all reported that pregnant rats administered glucocorticoids, such as dexamethasone, led to low birth weight and hypertension in the offspring. Benediktsson *et al.* (1993) also reported that rat placental 11 β -HSD-2 was positively correlated with offspring birth weight and negatively with placental birth weight.

Langley-Evans & Nwagwu (1998) investigated the effects of MLP diet fed at specific time points throughout gestation on placental and fetal growth. The study also examined markers of glucocorticoid sensitivity including activity of the GC-inducible enzymes TAT and GPDH. Offspring were born with a low birth weight, and at 4 weeks postpartum they had developed hypertension. Gestational age 15-22 of MLP animals appeared to be the most sensitive time of GC exposure, leading to increased brain GPDH at birth and elevated hepatic TAT in neonates. It is thought that MLP diet led to prolonged exposure to GC's, which may lead to permanent down-regulation effects of genes.

Intergenerational effects of glucocorticoid exposure are beginning to be investigated (Drake *et al.*, 2005). Dex administered during the third week of rat gestation leads to low birth weight, hypertension and glucose intolerance in the F1 generation. Low birth

weight was then passed onto F2 males, but this effect was not seen in F3 generations, similar to that seen in the Harrison & Langley-Evans (2008) study of effects of MLP.

Fetal exposure to GC's early on in development are also thought to affect the function of the hypothalamic-pituitary-axis (HPA), and this can have profound effects on the central nervous system and other tissues including the cardiovascular (Langley-Evans, 1997b). Langley-Evans *et al.* (1996b) hypothesised that MLP diet fed to pregnant rats prior to and during gestation would result in permanent alterations in the HPA axis. The diet was shown to cause hypertension in the offspring as well as alterations in HPA-related gene expression. There was an increased activity of corticosterone-sensitive enzymes related to low hormone concentrations, and increased GR binding capacity and receptor numbers in the hippocampus of MLP male offspring. MLP diet decreases 11 β -HSD-2, which led to increased sensitivity of the offspring to circulating glucocorticoids. This was likely to be a life-long effect.

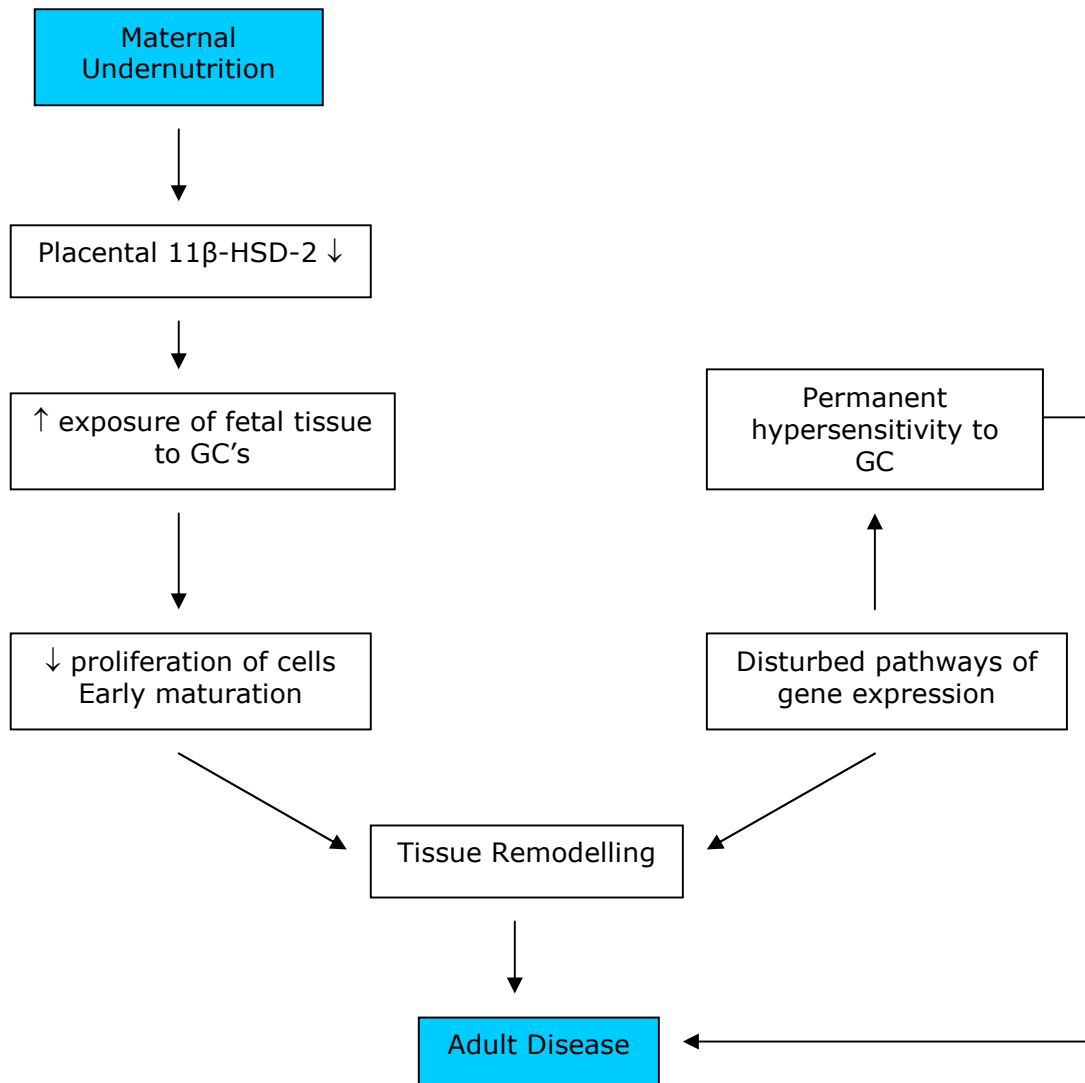
Nyirenda *et al.* (1998) found giving dexamethasone to pregnant rats in the last week of pregnancy led to a 10% reduction in birth weight, hyper-insulinaemia and hyperglycaemia in different forms, as well as an increased expression of hepatic glucocorticoid receptor. Dex administered at any other time point in pregnancy had no effect, which demonstrates that GC's have specific effects at different time points of development.

De Vries *et al.* (2002) completed a study on newborn rats to investigate how Dex treatment on days 1, 2 and 3 postnatally, would affect pups one, eight, and forty-five weeks later. It was found that Dex caused permanent structural differences in the heart. In treated animals, heart weight was significantly lower, and the protein:DNA content of the cells was higher in comparison to controls, indicating hypertrophy had occurred. Increased collagen content of the cardiomyocytes was also found, which suggested premature degradation. Low DNA content indicated that fewer myocyte

divisions occurred. From this study it was suggested that hypertrophy occurs in order for the heart to maintain function, as there is no alternative for the growth of the heart following GC insult. Glucocorticoids could inhibit mitosis of the cells, preventing growth of the developing heart, leading to a reduced number of myocytes.

Glucocorticoids effects *in utero* have been seen in other species as well as that rat. In the ovine fetal kidney, dexamethasone has been found to programme the renin-angiotensin system (Moritz *et al.*, 2002). Dex increased AT₁ and AT₂ receptors in fetal kidneys, and in this group angiotensin II decreased glomerular filtration rate. Sloboda *et al.* (2002) reported that administering betamethasone to pregnant ewes at days 104, 111 and 118 of gestation affected glucose homeostasis of the adult offspring. At 125 days of gestation fetuses exposed to this treatment had increased expression of hepatic 11 β -HSD-1, which is involved in regulation of local tissue levels of GC's. They also exhibited decreased expression of corticosteroid-binding protein, which regulated free bioactive GC's, but GR expression was unaffected.

Figure 1.5 - Flow diagram showing the glucocorticoid hypothesis



1.10 Glucose Transporter Genes

Glucose is the body's primary energy source and is an essential substrate for the metabolism of almost all cells. It is transported down a concentration gradient when energy is needed (Watson *et al.*, 2004). Glucose transporters are the proteins involved in the uptake of glucose into the cell, enabling cells to function. The two main class I glucose transporters responsible for the hearts uptake of energy are glucose

transporter 1 and 4 (GLUT 1 and 4) (Lavrentyev *et al.*, 2004). These are part of a large family of 13 genes all involved in glucose uptake in different tissue types (Huang & Czech, 2007). These transporters are specific transport proteins which facilitate normal levels of glucose in the body, at around 5-6mM. They control this even when high levels, or lack of sugar have been consumed, preventing hyper- or hypoglycaemia from occurring (Huang & Czech, 2007).

1.10.1 Glucose Transporter 1 (GLUT 1)

GLUT 1 is also known as SLC2A1 (solute carrier family 2, member 1). Its main role in the heart is to allow cardiac basal transport of glucose across the plasma membrane of cells. It is predominantly distributed in fetal tissue, and starts to be down-regulated in early postnatal life (Studelska *et al.*, 1992). In the adult heart however, it still accounts for approximately 30% of the cardiac uptake of glucose (Laybutt *et al.*, 1997). During adult life it is highly expressed in the endothelial cells of barrier tissues such as the blood-brain barrier (Dobrogowska & Vorbrodt, 1999, Maher *et al.*, 1994). Expression increases when glucose levels are low in the body therefore allowing more glucose into the cells, and vice versa, always maintaining a constant level of uptake (Gould & Holman, 1993).

GLUT 1 is thought to have “protective” properties in the heart, as demonstrated in a study completed by Liao *et al.* (2002). Hypertrophy caused by heart failure was induced in transgenic mice with over-expression of cardiac GLUT 1. It was found that these mice had increased survival rates compared to wild type mice, due to the increase in basal glucose uptake. This suggests that a rise in the expression of GLUT 1 increases the glucose uptake of the cells, enabling the heart to cope with increased haemodynamic demands.

1.10.2 Glucose Transporter 4 (GLUT 4)

GLUT 4, also known as SLC2A4 (solute carrier family 2, member 4), is found in various tissues in the body, including adipose tissue. It can mainly be found in striated muscle such as the heart. It is thought that it is stored in specialised insulin responsive intracellular vesicles (Watson *et al.*, 2004), and is redistributed to the plasma membrane in response to insulin (Huang & Czech, 2007). Insulin stimulation leads to a 20-fold increase in GLUT 4 in the plasma membrane (Watson *et al.*, 2004). Glucose transport into the cell depends mainly on the number of GLUT 4 receptors on the membrane. Therefore, if there is a surge in glucose concentrations, insulin will be activated, which in turn activates GLUT 4 expression on the plasma membrane, increasing glucose uptake in the cell. GLUT 4 works by trafficking glucose into the muscle where it is stored as glycogen and then metabolised. This mechanism of glucose removal controls the levels of glucose in the cells allowing normal function, maintaining homeostasis (Huang & Czech, 2007). This transporter has low expression during fetal development, and begins to be up-regulated in early postnatal life to become the main glucose transporter in the heart (Studelska *et al.*, 1992), maintaining glucose balance in cells. Due to this, glucose uptake becomes more susceptible to changes in response to insulin concentrations.

Various studies have been completed to assess the outcomes of altered GLUT 4 regulation. Commonly, studies use a model of GLUT 4 over-expression, or knockout mice for the investigation of GLUT 4's effects on glucose metabolism in skeletal muscle, obesity, and type II diabetes (Tsao *et al.*, 2001, Zisman *et al.*, 2000). GLUT 4 deficiencies can lead to growth retardation, decreased sensitivity to insulin stimulation, and cardiac hypertrophy in mice (Katz *et al.*, 1995). GLUT 4 knockout mice also exhibited an increased in glucose transporter 1, thought to be a possible compensatory mechanism to maintain a normal glucose levels. They developed hyperinsulinaemia following food intake. Cardiac hypertrophy was noted in early life

and led to increased mortality (Katz *et al.*, 1995) demonstrating that GLUT 4 is vital for normal physiological homeostasis.

1.10.3 Glucocorticoid Receptor (GR)

The Glucocorticoid Receptor is a member of the nuclear receptor subfamily 3, group C, member 1 (NR3C1) of ligand-activated transcription factors (Seckl *et al.*, 2004). It is expressed in most fetal tissues from an early stage and is highly expressed in the placenta (Seckl *et al.*, 2004), as well as in every cell in the adult body. Its main role is acting as the receptor that glucocorticoids, including Dex and cortisol, bind to. It is thought that these receptors are involved in the majority of glucocorticoid effects on the body (Cole *et al.*, 1995), although GC can also bind to the mineralocorticoid receptor (MR), particularly in late gestational stages in the rat (Seckl *et al.*, 2004). The level of expression is different in every tissue, and in some cases expression varies regionally within specific tissues (Herman *et al.*, 1989).

Since glucocorticoids have a very important role in the body, especially during fetal development, alterations in GR expression can have a significant impact on development, growth and metabolism. GR's are expressed in most fetal tissue (Drake & Seckl, 2004), and in mice it has been found to expressed from approximately day 9 of gestation (Cole *et al.*, 1995). Reduction in GR can lead to abnormalities in adult blood pressure (Lillycrop *et al.*, 2005), and there is evidence to suggest that events during fetal growth can programme GR gene transcription in certain tissues or organs (McCormick *et al.*, 2000). Lillycrop *et al.* (2005) found that MLP diet fed during gestation has implications for GR expression in the offspring. MLP diet increased GR methylation status in the offspring's liver at weaning. These changes are known to remain into adult life thus affecting blood pressure regulation and risk of developing

CVD (Lillycrop *et al.*, 2005). Expression of GR during fetal development is however, still poorly understood.

1.11 Cell culture vs animal studies

Animal models as described in this thesis have been crucial in the advancement of understanding of fetal programming, and have been the basis of a vast array of research. Such advancements in scientific understanding could not have taken place without these animal models. There is, however a need to reduce animal work as a main experimental model. Ethically the use of animals in science is questionable and current emphasis is on replacement, refinement and reduction.

There are various advantages and disadvantages in using cell culture as an alternative experimental model. Using primary culture and cell lines has the potential to reduce the number of animals currently used in scientific trials, as well as reducing cost. Scientists are always exploring new methods to move away from animal work, or at least ways to reduce the numbers used and reduce animal suffering. Cell culture would eliminate housekeeping and husbandry costs associated with animal care, and possibly result in quicker trials.

Using culture could also increase the number of endpoint measurements. For example, in the study described in this thesis, one litter of neonatal rat hearts produced millions of cells enabling numerous experiments and measurements to be completed, as well as many replicates. Cells can be manipulated in ways that animals cannot due to ethical restrictions. For example, cells cultured in plates can be treated with toxic levels of drugs, or hormones to monitor responses, or even add several treatments

simultaneously to one culture. It is desirable to develop culture models to become as standard as animal models, such as the MLP rat model of programmed CVD.

However, there are restrictions associated with using culture instead of animals. The main problem is that culture does not allow experiments to investigate how body systems and organs interact, or how each system impacts on the other. Also, some animal models currently used would take a considerable amount of time to establish in culture. This is an area that still requires work to develop cell culture into a feasible alternative to animal trials. For the time being cell culture will be used alongside animal trials with the aim to reduce the numbers used in the future.

1.12 Aims

This study has been designed to investigate the impact that fetal exposure to a maternal low protein diet has on primary cultures of neonatal cardiomyocytes. Animal trials and primary cell culture work will be completed to explore glucose uptake of cardiomyocytes, and expression of genes involved in glucose metabolism, exploring the contribution of glucocorticoids.

The primary aim of this project is to show if programming can be demonstrated in cell culture. Do neonatal cardiomyocytes insulted with low protein and control diet during pregnancy hold a memory of maternal diet? The project will investigate if effects of maternal diet on the heart, such as a reduced nuclear number get passed on through cell division onto daughter cells. Secondly, the project is designed to investigate whether synthetic glucocorticoids affect the responses of the cultures. Are proliferation rates, glucose uptake, and gene expression affected by addition of dexamethasone into culture? Addressing these questions may begin to explain the mechanisms that are involved in fetal programming leading to cardiovascular disease.

At this present time there is major interest in the potential contribution of epigenetic markers to developmental programming. Undernutrition appears to reset such markers, producing permanent changes in gene expression (Lillycrop *et al.*, 2005). GC exposure also appears to have the potential to act in this manner. The transgenerational effects of MLP and Dex reported previously (Drake & Seckl, 2004, Harrison & Langley-Evans, 2008) seem consistent with an epigenetic mechanism. The finding of programmed effects upon cultured cells would add further support to this concept.

1.13 Hypotheses

The studies described in this thesis have been performed to test the following hypotheses.

1. Primary cultures of neonatal cardiomyocytes will retain a cellular memory of maternal diet, and manifest differences in metabolic function, rates of proliferation and apoptosis that are associated with maternal protein restriction.
2. Maternal undernutrition will programme expression of genes involved in glucose uptake.
3. Maternal protein restriction will increase sensitivity to synthetic glucocorticoids in cultured cardiomyocytes.
4. Expression of glucocorticoid receptor in cultured cells will be programmed by maternal protein restriction.

These studies will enable development of new approaches to the study of fetal programming, and add to understanding of how the diet contributes to tissue remodelling and sensitivity to glucocorticoid signal in early life. Various lines of investigation will be undertaken to test these hypotheses. Proliferation, differentiation and cell apoptosis of the cardiomyocytes will be measured by determination of protein and DNA content and binucleation in cultures. Functional responses of the cardiomyocytes to synthetic glucocorticoids will be measured by assessing glucose uptake. Gene expression will be assessed by measuring glucose transporter 1 and 4 and glucocorticoid receptor mRNA expression in cultures.

2 Chapter 2

Materials and Methods

All experimental procedures described were conducted under license and in accordance with the Home Office Animals (Scientific Procedures) Act 1986.

2.1 Animals

Experimental animals were Wistar strain rats supplied by Harlan Laboratories (Bicester, Oxon, UK). All animals were housed in wire mesh and Perspex cages, in groups of two per cage, in a controlled environment (21°C, 55% humidity, 12:12 hour light: dark cycle) until the start of trial. Prior to mating stud males and females were fed *ad libitum* standard, non-purified, laboratory chow diet (Harlan, UK) and had free access to water.

2.1.1 Maternal Protocol

Individual virgin females, weighing between 210-260g were caged in wire bottomed mating cages with a stock stud male. Mating was confirmed by appearance of a semen plug on the cage floor and this day was denoted Day 0 of pregnancy. Pregnant females were then individually housed throughout gestation and fed an experimental diet of either control (18% casein) or low protein (9% casein), and water *ad libitum*. See Table 2.1 for the composition of diets (Langley-Evans *et al.*, 1994).

To prepare diet, dry ingredients were weighed out and mixed thoroughly before adding the corn oil. Once this was mixed in, water was added until the mixture

became dough-like. Balls of diet were baked in an oven at 60°C for 48 hours, and then frozen at -20°C prior to use.

Table 2.1 - Composition of Diets

Ingredient	Control Diet - 18% Casein (grams/kg)	Low Protein Diet - 9% Casein (grams/kg)
Casein	180	90
Cornstarch	425	485
SolkaFlok (Cellulose)	50	50
Sucrose	213	243
Choline Chloride	2	2
DL-Methionine	5	5
AIN-Minerals	20	20
AIN-Vitamins	5	5
Corn Oil	100	100

2.1.2 Neonatal Protocol

After parturition, the females were culled using a Schedule 1 method (CO₂ asphyxia and cervical dislocation). Eight neonates from each litter (where possible 4 female and 4 male) were also culled using Schedule 1 techniques (cervical dislocation). Each whole pup was placed in a Falcon tube containing 30ml of ice cold calcium and magnesium free Hanks Balanced Salt Solution (CM-HBSS) (Worthington Neonatal Cardiomyocyte Isolation System kit, Lorne Laboratories, UK). Neonatal hearts were dissected out under sterile conditions to reduce the risk of infection of subsequent cell cultures.

2.1.3 Cell Culture Protocol

Cardiomyocytes were seeded onto 24 well plates and grown in a DMEM solution (See Appendix) for ten days. On Day 11 (baseline time point) one set of cells were lysed from the plates and stored at -80°C until analysis. The remaining cells underwent dexamethasone (Sigma, UK) treatment and were incubated until Day 13 of culture (48 hours time point/Day 2) before analysis.

For trial one, cells were treated with 0, 100nM or 10µM dexamethasone. 100nM was within the physiological glucocorticoid range (Flagel *et al.*, 2002), and 10µM was added to consider the maximal cardiomyocyte response. For trial two, cells were treated with 0, 10nM and 100nM, which were entirely within the physiological range.

2.2 Cell Culture

2.2.1 Isolation of Neonatal Cardiomyocytes

This method was adapted from the commercially available Worthington Neonatal Cardiomyocyte Isolation System kit (Lorne Laboratories, UK). Each kit can culture 5-15 hearts per preparation using hearts taken from 1-7 day old rat pups, requiring two days to prepare under sterile conditions, in a cell culture laminar flow hood. All reagents were provided in the kit and were prepared before starting the protocol, see Appendix.

Neonatal pups were culled and dissected as described in Section 2.1.2. The heart of each pup was placed in a sterile Falcon tube containing 20-30ml of CM-HBSS. Once all hearts were dissected, the tube was swirled to clean the hearts and CM-HBSS was removed and discarded. A further 10ml wash of CM-HBSS was added and then removed. The hearts were then placed in a petri dish and using a sterile disposable

scalpel knife cut into approximately 1mm³ pieces. 9ml of CM-HBSS was added to the petri dish, followed by 1ml of trypsin reagent, which was mixed by swirling the dish (final trypsin concentration was 50µg/ml). The petri dish was taped closed and placed in a fridge (2-8°C) overnight for 16-20 hours.

After this time the petri dish was removed from the fridge and kept on ice. The contents were transferred into a sterile 50ml Falcon tube and 1ml of Trypsin Inhibitor reagent was added and mixed. This was incubated in a water bath at approximately 30-37°C for 40 minutes. Following this, 5ml of collagenase reagent was added and incubated at 30-37°C, in a water bath set to shake at 2-4rpm, for a further 40 minutes. Trypsin and collagenase are enzymes that breakdown the extra-cellular matrix of heart tissue.

The solution was then triturated gently 10 times using a serological pipette and cells were left to settle. A fresh 50ml Falcon tube was set up with a cell strainer, and 1ml of filtered L-15 medium was passed through followed by the cell supernatant, leaving a clump of tissue at the base of the first Falcon tube. A further 5ml of L-15 medium was added to this, and again triturated gently 10 times, allowing the cells to settle after. The supernatant was again transferred through the cell strainer and a further 2ml of L15 medium was passed through. The cells were left to stand for 20-60 minutes at room temperature.

After this time they were centrifuged at 94g, for 5 minutes, at 20°C. The supernatant was then removed leaving the pellet at the base of the tube. 5ml of Advanced Dulbecco's modified Eagle medium/F12 solution (DMEM) (Invitrogen/Fisher Scientific, UK), fetal bovine serum (FBS) (Heat inactivated Sigma, UK), penicillin-streptomycin (P/S) (Sigma, UK) and Fungi-zone (GIBCO/Fisher Scientific, UK) solution was added and thoroughly mixed (See Appendix for details of DMEM solution). 100µl of the cell

suspension was taken to complete a cell count and the cells were diluted accordingly with DMEM solution to seed cells at approximately 100,000 cells/ml/well.

24-well plates (Falcon, UK, Polystyrene, non-pyrogenic, TC treated by vacuum gas plasma) were seeded at 100,000 cells/ml/well and left at 37°C (5% CO²) for 24 hours before changing the medium. DMEM solution was then changed every 48 hours after this for 9 days.

2.2.2 Detaching cells from plates to perform a cell count

This method was taken from the Sigma cell culture manual 2008-2009, 3rd Edition. It was partly completed under sterile conditions, see Appendix for reagents.

DMEM solution was removed from each well and washed using sterile PBS. 0.5ml of trypsin (Fisher Scientific, UK) was added, and wells were incubated for 3-4 minutes at 37°C. The plate was tapped on the side and viewed under the microscope to check that the cells had detached. Cells were re-suspended in 1ml/well of DMEM, transferred to an eppendorf tube, and centrifuged at 10,000rpm for 5 minutes at 18°C. The supernatant was removed and replaced with 1ml of DMEM and mixed thoroughly. Approximately 20-40µl was taken to complete the cell count detailed in Section 2.2.3. Counts were completed before the cardiomyocytes were plated, and after 10 and 12 days in culture (baseline and 48 hour time points).

2.2.3 Cell Count/Haemocytometer Protocol

Some of the cell suspension (20 μ l) was taken and gently pipetted into the groove between the haemocytometer and the cover slip. The cell suspension was drawn under the cover slip by capillary action. The haemocytometer was secured on the microscope and the 40x objective used. Cells were counted in either ten of the 1/16mm² squares in the top and bottom grids, or eight 1mm squares on two grids as shown in Figure 2.1, depending on the concentration of the cell suspension. To calculate the total number of cells, the following calculations were completed:

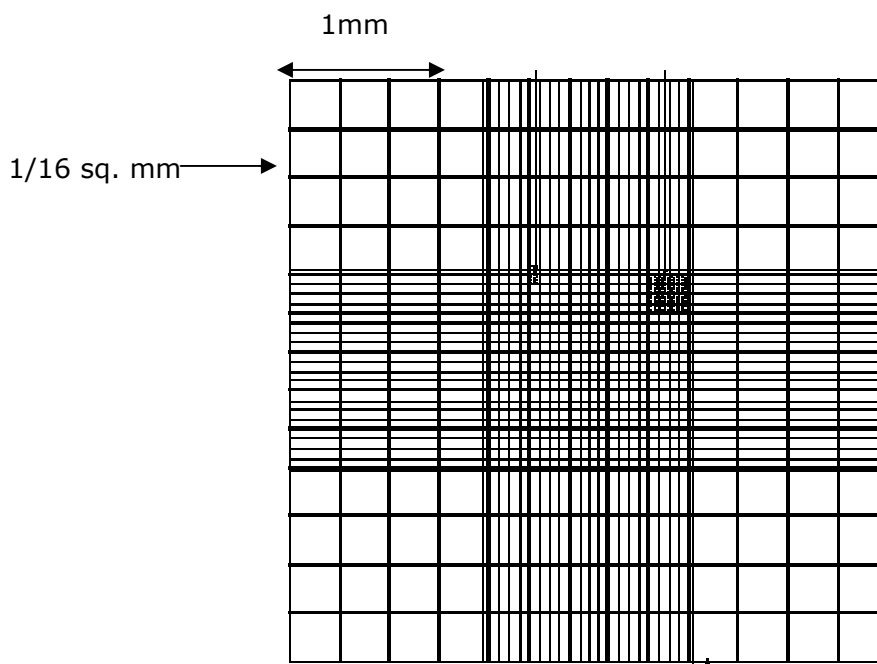
Ten of the 1/16mm² squares

Total number of cells per ml = Average number of cells in two grids x (1.6x10⁵)

Eight 1mm squares

Total number of cells per ml = Average number of cells in two grids x (1x10⁴)

Figure 2.1 – Haemocytometer Grid



2.2.4 Trypan Blue Cell Counting

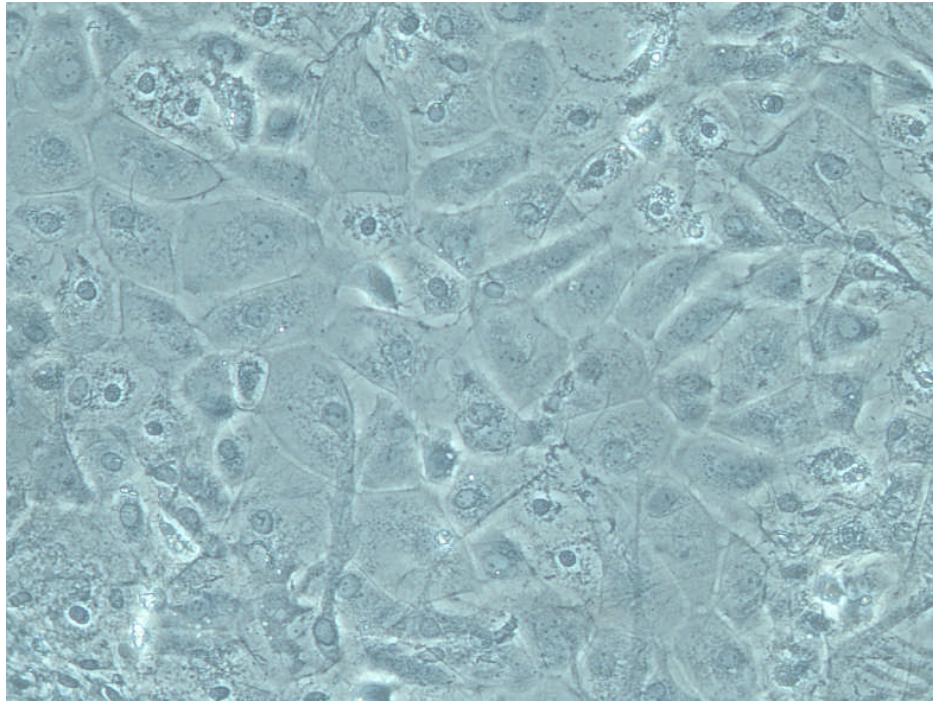
This method was adapted from Sigma cell culture manual 2008-2009, 3rd Edition, and was used to identify dead and live cells. Trypan blue is taken up by the dead cells so they appear blue, and live cells are clear. Before completing the cell counting as detailed above, 20µl of cell suspension was taken and mixed in a separate eppendorf tube with 20µl of trypan blue (Sigma, UK). 20µl of this mixture was taken and counted using the haemocytometer method. Counts were multiplied by 2 to account for the dilution factor.

2.2.5 Cardiomyocyte Staining

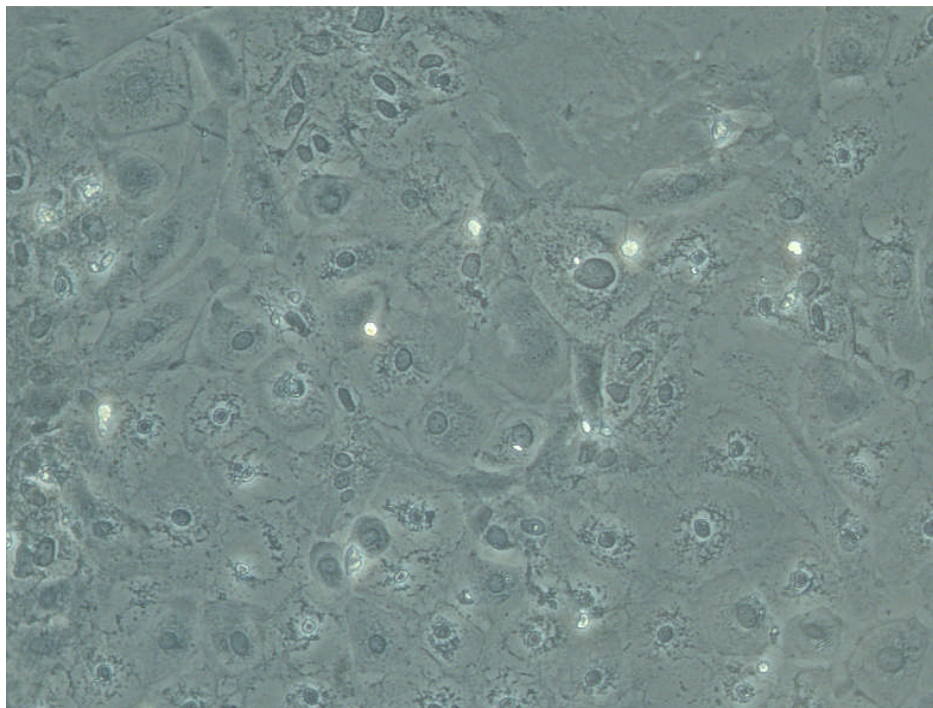
In order to assess the stage of cell differentiation, cultures were stained with a nuclear stain. DMEM solution was carefully removed from each well and 300µl of 1mg/ml bisbenzamide (Sigma, UK) solution was pipetted down the side of the well. This was removed after 4 minutes. Cells were washed with 1ml of 1x PBS solution and imaged using Leica Application Suite (Leica Microsystems Ltd). Binucleated, mononucleated and the total number of cardiomyocytes were counted in five microscope fields per well. All staining was completed in duplicate. In order to determine how many images were needed for accurate counts, 10 images per well were counted in comparison to five, which generated the same answer. It was therefore determined that counting five images per well was representative of the entire well.

Figure 2.2 - Bisbenzamide (1mg/ml) stained cardiomyocytes at a 20x0.3 magnification

Control Culture - 0 Dex



MLP Culture - 100nM Dex



2.3 Chemical Analysis

2.3.1 Cell Lysis for DNA and Bradford Assay

The cell lysis method was taken from Molecular Cloning, A Laboratory Manual, 2nd edition (1989), and the lysis buffer was taken from Cell Biology Catalogue, Millipore, 2007.

DMEM solution was removed from each well and 300µl cell lysis buffer (see Appendix for details) was added and incubated on ice for approximately 20 minutes. Cells were detached from the wells using a cell scraper, and then harvested into a chilled microcentrifuge tube and centrifuged for 2 minutes, at 12,000rpm, 4°C. The supernatant was transferred to a fresh microcentrifuge tube, and frozen at -80°C until analysed.

2.3.2 Determination of Protein - Bradford Assay

Protein was assayed using the Bradford 1976 method. Reagents are described in Appendix. Prior to analysis cell lysates were thoroughly defrosted. 10µl of each sample, standard, blank and QC was added in duplicate to a 96-well, flat bottomed microplate (Sarstedt) and mixed with 200µl of the Bradford reagent. Protein concentration was determined from a standard curve prepared in duplicate from dilutions of 1mg/ml BSA (Sigma, UK) dissolved in water at 0-8µg/ml. The plate was left for 10 minutes and the absorbance was measured at 560nm using a microplate reader (Tecan Sunrise, UK) interfaced to the Magellan Version 4.0 computer package. Trial one inter-assay variation was 2.59%, and intra-assay variation was 0.63%. Trial two inter-assay variation was 1.01%, and intra-assay variation was 0.72%.

2.3.3 Determination of DNA Content – Hoechst Fluorometric Method

This method was adapted from Daxhelet *et al.* (1989). Reagents were prepared as detailed in Appendix. Cell lysates were thoroughly thawed before analysis. 100µl of each sample, standard, blank and QC was added in duplicate to a black 96-well microplate (Costar, UK) along with standards made from a stock calf thymus DNA solution (Sigma, UK) of 1mg/ml. A top standard of 40µg/ml was prepared and from this, serial dilutions were prepared ranging from 0.312 to 20µg/ml. Bisbenzamide (Sigma, UK) dye solution was added to each well at 100µl, and then scanned immediately on the fluorescent plate reader (FLUROstar Optima, BMG Labtech, UK), with an excitation wavelength of 355nm and emission wavelength of 544nm. Trial one inter-assay variation was 7.19%, and intra-assay variation was 3.79%. Trial two inter-assay variation was 4.95%, and intra-assay variation was 2.56%.

2.3.4 Determination of Cell Death

This method was adapted from the commercial Roche Cell Death ELISA^{PLUS} kit. All solutions were provided in the kit and prepared according to the manufacturer's instruction (see Appendix). Cell culture plates were spun at 2000rpm for 10 minutes at room temperature before carefully removing the medium and discarding. Plates were then immediately frozen at -80°C until ready for analysis.

Plates were then thawed for 30 minutes before adding 200µl of lysis buffer, which was then incubated for a further 30 minutes at 15°C to 25°C. The whole plate was centrifuged at approximately 2000rpm for 10 minutes at room temperature, and the supernatant removed. To a 96-well Streptavidin coated plate, 20µl of each sample was carefully added in duplicate, along with a positive control, background control and QC.

80µl of the immunoreagent was added to each well. The plate was covered and incubated on a plate shaker at 300rpm, for 2 hours, at a temperature of 15°C to 25°C. The solution was then thoroughly removed and each well was rinsed 3 times with 250-300µl of incubation buffer. 100µl ABTS solution was added to each well and incubated on a plate shaker at 250rpm for 10 minutes. This was adequate time for the colour to develop sufficiently for photometric analysis. 100µl of ABTS Stop Solution was added, and at this stage 100µl of ABTS Stop Solution was added to 2 separate wells to use as a blank. The plate was read immediately on a fluorescent plate reader (Tecan Sunrise, UK), using a wavelength of 405nm with a reference wavelength of approximately 490nm. Sample data was calculated as a ratio against the quality control. The inter-assay variation for this assay was 3%, and the intra-assay variation was 2%.

2.3.5 Glucose Uptake Assay

Basal and insulin-stimulated glucose uptake was determined using a radioisotope-based method adapted from Kozma *et al.* (1993). Basal cultures were not treated before glucose uptake was measured, and insulin-stimulated cells were incubated in insulin for 30 minutes before glucose uptake was measured. Reagents were prepared as detailed in Appendix. Cells were removed from the incubator and carefully washed twice in 1ml of warm (37°C) PBS. For baseline plates, 1ml of PBS containing 200nM of insulin (from bovine pancreas, Sigma, UK) was added to two of the wells, and to another two wells 1ml 1x PBS was added. For 48 hour plates, 1ml of PBS per well containing 200nM of insulin was added to six of the wells, and to another six wells 1ml 1x PBS was added as shown in Figure 2.3. To two separate wells containing no cells, 1x PBS was added. These wells were used as blanks. The cells were then incubated for 30 minutes at 37°C and again washed twice with 1ml of warm PBS.

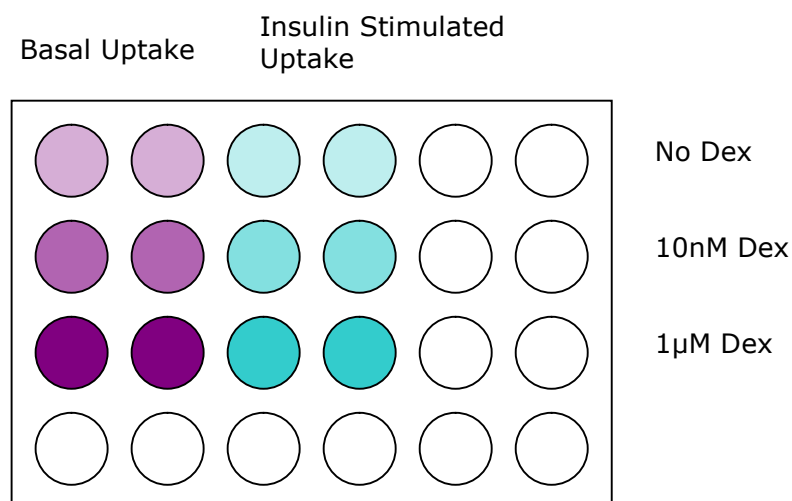
After washing, 1ml of warm PBS containing 0.1mM 2-deoxyglucose (2-dG) (Sigma, UK) and 1 μ Ci 2-deoxy-D-[2,6-³H]glucose (³H-2-DG) (GE Healthcare Ltd, UK) was added to all wells for 5 minutes at room temperature. The assay was terminated rapidly by washing each well 3 times with 1ml of ice cold PBS. Cells were left to lyse for 1 hour, at room temperature, in 0.4ml of 1% SDS. Each well was then scraped with the pipette tip and the contents were transferred into a scintillation vial insert, and 4ml of Emulsifier Scintillator Plus (Perkin and Elmer, UK) was added and mixed thoroughly. Inserts were placed in a vial and counted immediately on a liquid scintillation analyser (TRI-CARB 2100 TR).

Calculation of Uptake

$$\frac{\text{dpm} \times 12}{10.12 \times 10^3} = \text{pmoles/hour}$$

$$\frac{\text{pmoles/hour} \times 10^6}{\text{Cell Count}} = \text{pmoles/hour/million cells}$$

Figure 2.3 – 24 well, Day 2 cell culture plate setup



2.4 Real-time PCR

2.4.1 Cell Preparation for RNA extraction

For this section of the trial, cardiomyocytes were seeded at 200,000 cells/well in 12 well plates to ensure there were enough cells to achieve adequate amounts of RNA, for cDNA synthesis. Cultures were grown exactly the same as 24 well plates and treated with 2ml of dexamethasone in DMEM to cover the larger well size. DMEM was removed, and each cell culture well was carefully washed with 1ml of sterile PBS (Sigma, UK). This was removed and 200µl of PBS was then added. The well was scraped using a cell scraper, and then with a P1000 pipette tip before cells were placed in a sterile eppendorf tube and snap frozen in liquid nitrogen and stored at -80°C. For each day and treatment group, three wells of cells were prepared.

2.4.2 RNA Extraction and DNase Treatment

This method was modified from the commercial Qiagen RNeasy Mini kit. All solutions were provided in the kit and prepared according to the manufacturer's instruction, see Appendix.

The cells were defrosted and all three wells were mixed to make one cell suspension for each time point and treatment group. To each sample, 800µl of RLT buffer plus β -mercaptoethanol (β -ME) solution was added and mixed well by pipetting several times. Then, 1400µl of 70% ethanol was added and, again, mixed thoroughly by pipetting. 700µl of this solution was passed through an RNeasy spin column placed in a 2ml

collection tube, and spun for 15 seconds at 10,000rpm. This was repeated until all of the cell suspension solution was used, discarding the flow through after each spin.

At this stage the samples were DNase treated to remove any DNA contamination. To each sample, 350µl of Buffer RW1 was added and centrifuged for 15 seconds at 10,000rpm to wash the spin column membrane, discarding the flow through. 80µl of the incubation mix was then added, ensuring it was added directly to the membrane. This was left for 15 minutes at room temperature. Following this, another 350µl of Buffer RW1 was added to the spin column, and centrifuged for 15 seconds at 10,000rpm, again, discarding flow through.

Another wash step was completed using 500µl buffer RPE and centrifuging for 15 seconds at 10,000rpm, the flow through was discarded. A second aliquot of 500µl buffer RPE was washed through by centrifuging for 2 minutes at 10,000rpm. This step ensured that no ethanol was carried over during RNA elution, as residual ethanol may interfere with downstream reactions.

Using a fresh collection tube, the column was spun for another 1 minute at 10,000rpm to eliminate any possible carryover of Buffer RPE, and prevent any residual flow-through remains on the outside of the spin column. The column was then transferred to a sterile eppendorf tube, and 30µl of RNase free water was added directly to the membrane, and centrifuged for 1 minute at 10,000rpm to elute the RNA. This was then frozen at -80°C until ready for cDNA synthesis.

2.4.3 RNA Quantity and Quality

RNA quantity was measured using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, UK), which measures the sample by creating a column of liquid and passing light through it, producing a concentration expressed as ng/ μ l.

Samples stored at -80°C were placed immediately on ice to defrost. They were mixed thoroughly using a pipette, and 1.5 μ l of each sample was loaded onto the nanodrop for analysis, in duplicate. The RNA quality was determined from the 260nm:280nm ratio, which should fall between 1.8 to 2.0.

Before synthesising cDNA from the RNA, samples were standardised to 20ng/ μ l by diluting with RNase free water. Samples that were below 20ng/ μ l were added in a higher volume to make up to this concentration. Once all were standardised, samples were examined to ensure they were not contaminated and only produced one product. As the RNA concentration was too low to run on an Agarose gel, RNA samples were run on a Roche 480 lightcycler with the β -actin housekeeper gene, and each of the samples cDNA to check this. RNA samples should appear negative, meaning they are not contaminated with DNA, and cDNA should have one product. See section 2.4.6 for the RT-PCR step. Samples were then stored at -80°C until required for analysis.

2.4.4 cDNA synthesis/Reverse Transcriptase

RNA is converted to cDNA by reverse transcription. Complementary base pairing of the RNA strand forms the double stranded cDNA molecule. Due to some RNA samples having a higher concentration than 20ng/ μ l and some lower, different volumes of RNA and primers were added at this stage. For samples with initial RNA concentration of

$\geq 20\text{ng}/\mu\text{l}$, $10\mu\text{l}$ of diluted RNA was added along with $1\mu\text{l}$ of random primers, and $4\mu\text{l}$ of water, to each reaction tube. For samples with initial RNA concentration of $<20\text{ng}/\mu\text{l}$, $1\mu\text{l}$ random primers was added along with a specific volume of neat RNA to make up to the required concentration of $20\text{ng}/\mu\text{l}$. For samples to be used in the standard curve pool and non-template control (NTC), $5\mu\text{l}$ of neat RNA or water was added, with $1\mu\text{l}$ of random primers and $9\mu\text{l}$ of water.

Samples were loaded into a 96 tube plate along with primers and water, as detailed above, and then loaded onto the PCR machine (Gene Amp PCR System 9700, Applied Biosystems) to incubate for 5 minutes at 70°C to denature the structure, and then immediately put on ice. To each tube the following was then added: $5\mu\text{l}$ MMLV reverse transcriptase buffer x5 (Promega, UK), $1.25\mu\text{l}$ of nucleotides (10mM each) (Promega, UK), $0.5\mu\text{l}$ of RNase inhibitor (Promega, UK), $1\mu\text{l}$ of MMLV reverse transcriptase (Promega, UK), $2.25\mu\text{l}$ of RNase free water. A master mix of these reagents was made up prior to use and $10\mu\text{l}$ of this was added to each sample.

The samples were then incubated at room temperature for 10 minutes, centrifuged for approximately 5 minutes at 1000rpm . They were then returned to the PCR machine to incubate at 42°C for 60mins to transcribe the complementary strand to the RNA, and then held at 4°C . All samples and the NTC had $75\mu\text{l}$ of RNase free water added, except for the standards. These were then frozen at -20°C until ready for further analysis.

2.4.5 Standards

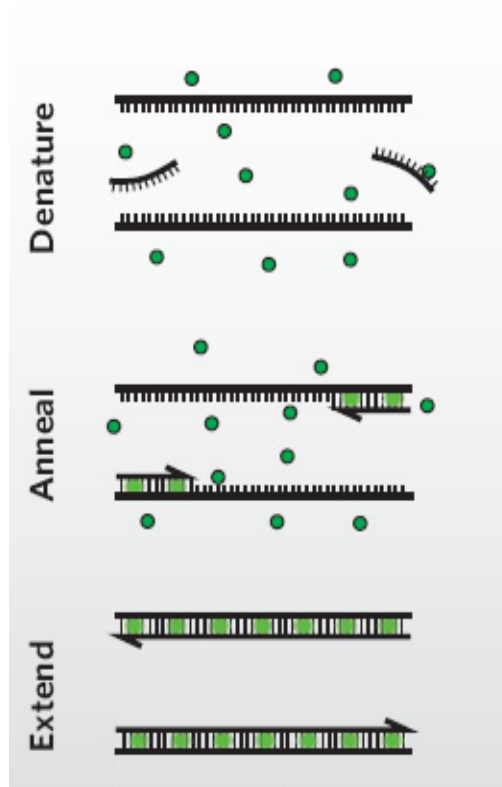
Five of the highest concentrated RNA samples had cDNA synthesised separately using neat RNA to achieve a concentrated cDNA pool. These five samples were pooled and added to $25\mu\text{l}$ of water. This pool was then serially diluted using RNase free water

(Sigma, UK) ranging between 0.016-0.5 to produce a standard curve. This was run on every plate with each gene of interest.

2.4.6 Real time PCR

Real time PCR is the process where cDNA is exponentially amplified using primers, which are complementary to a defined sequence on the DNA strands. In this thesis, SYBR green was used as the dye that binds to the double stranded product and fluoresces. As illustrated in the diagram below (Figure 2.4), cDNA is heated to high temperatures of 95°C to denature the DNA separating the two strands. It is then cooled to 60°C allowing the primers to anneal to the complementary site on the strands. By increasing the temperature to 72°C enables the enzymes to extend the DNA strand from the primer region. SYBR green binds to the amplified DNA and the fluorescence increases as the DNA amplification is increased, and this degree of amplification is quantified.

Figure 2.4 – Three main steps of polymerase chain reaction (PCR)



Double stranded DNA is denatured, primers then anneal to complementary site on the DNA strands and they are then extended using enzymes. SYBR green (shown by the green dots) binds to double stranded DNA and fluoresces.

<http://images.the-scientist.com/supplementary/html/36978/sybr.jpg>

Primers were diluted with RNase free water (Sigma, UK) according to the manufacturer's instructions. From this a 1:10 dilution was made of each primer (forward and reverse) in a sterile eppendorf, which were used to make the master mix for each gene.

The master mix was prepared on ice before loading the 384 well plate, using SYBR green (Roche, UK) as the dye, which binds to any double stranded product. Each master mix contained: 7.5 μ l SYBR master mix, 0.45 μ l forward primer (10 μ M), 0.45 μ l reverse primer (10 μ M) and 1.6 μ l molecular grade water, which was made up for all reactions. cDNA was fully defrosted and 5 μ l of each sample, standard, and blank (no template control) was added along with 10 μ l of master mix. Each reaction was completed in triplicate, and this was repeated for all genes.

Once the plate was set up, it was sealed with an adhesive cover, mixed and centrifuged for approximately 3 minutes at 1000rpm. The plate was then run on the Roche Light Cycler-480 machine for 45 amplification cycles. Pre-incubation at 95°C for 5 minutes is completed to activate enzymes in the master mix, and then 45 cycles consisting of 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 15 seconds. Crossing points were calculated using the Lightcycler 480 software, version 1.5. Samples were measured off the standard curve and gene expression normalised to Cyclophilin A, which was used as the housekeeper gene.

2.4.7 Design of primers for Rat Genes

Primer sets were required for rat β -Actin, Cyclophilin A, Glucose transporter 1 (GLUT 1), Glucose transporter 4 (GLUT 4), and Glucocorticoid Receptor (GR). Primers were designed using Primer 3 (Biology Workbench), as well as Primer Express (Table 2.2). Ensembl was used to find the genomic sequences and was also used to blast the designed primers. The sequences were found under the following accession numbers; β -Actin, NM_031144, Cyclophilin A, NM_017101, GLUT 1, NM_138827.1, GLUT 4, NM_012751.1 and GR, NM_012576.2.

Table 2.2 – Primer sets used for measurements of gene expression

Gene	Forward Primer	Reverse Primer
β -Actin	TCAGGTCATCACTATCGGCA Tm=57.3°C GC Content=50% Length=20bp	CGGATGTCAACGTCACACTT Tm=57.3°C GC Content=50% Length=20bp
Cyclophilin A	TGATGGCGAGCCCTTGG Tm=57.6°C GC Content=64.7% Length=17bp	TCTGCTGTCTTTGGAACCTTGTC Tm=58.9°C GC Content=43.5% Length=23bp
GLUT 1 aka SLC2a1	AGGTGTCACCCACAGCTCTT Tm=59.4°C GC Content=55% Length=20bp	AGAGGCCACAAGTCTGCATT Tm=57.3°C GC Content=50% Length=20bp
GLUT 4 aka Slc2a4	TCCCTTCAGTTTGGCTATAACATTG Tm=59.7°C GC Content=40% Length=25bp	CTACCCAGCCAAGTTGCATTG Tm=59.8°C GC Content=52.4% Length=21bp
GR aka Nr3c1	GTCAGAACTGGCAACGGTTT Tm=57.3°C GC Content=50% Length=20bp	TCCAAAAATGTCTGGAAGCA Tm=53.2°C GC Content=40% Length=20bp

2.5 Statistical Methods

Data is shown as the mean \pm SEM throughout the thesis. SPSS (version 15.0/16.0) was used to complete all analyses. Results were considered statistically significant when $P < 0.05$.

Coefficient of Variation

Intra-assay coefficient of variation was calculated to compare data from duplicate samples on the same plate.

Inter-assay coefficient was calculated to compare data from the same sample on different plates.

$$CV (\%) = \frac{\text{Standard Error} \times 100}{\text{Mean}}$$

Baseline/Day 0 (10 days in culture) data for both trial one and two was analysed using an independent t-test looking at the difference between the two diet groups. Each endpoint measurement was analysed separately against diet. Day 2 (12 days in culture) data for both trial one and two was analysed using a two way ANOVA looking at significance of diet and dexamethasone on cultures, using an LSD test for post hoc analysis. Baseline and Day 2 data were analysed separately and not statistically compared.

3 Chapter 3

Results

3.1 Trial One – Impact of maternal protein restriction on neonatal cardiomyocyte glucose uptake.

The aim of this trial was to identify responses of neonatal cardiomyocytes to increasing concentrations of dexamethasone in culture. Ten pregnant females were fed either a control (n=5) or low protein, MLP, (n=5) diet during gestation and neonatal hearts were used to culture cardiomyocytes. These cultures were grown for ten days, at which point some were taken to complete baseline analysis, and others were treated with different concentrations of dexamethasone (0, 100nM and 10µM), and left for a further 48 hours (day 2 cells) before analyses.

Maternal weight gain and food intake were measured throughout gestation. Neonatal cardiomyocytes were cultured and used for the analysis of protein concentration, DNA concentration, glucose uptake and cell number, including percentage of mononucleated and binucleated cells, for all time points. Cells were challenged with dexamethasone to examine responsiveness to glucocorticoids.

3.1.1 Maternal weight gain and litter sizes

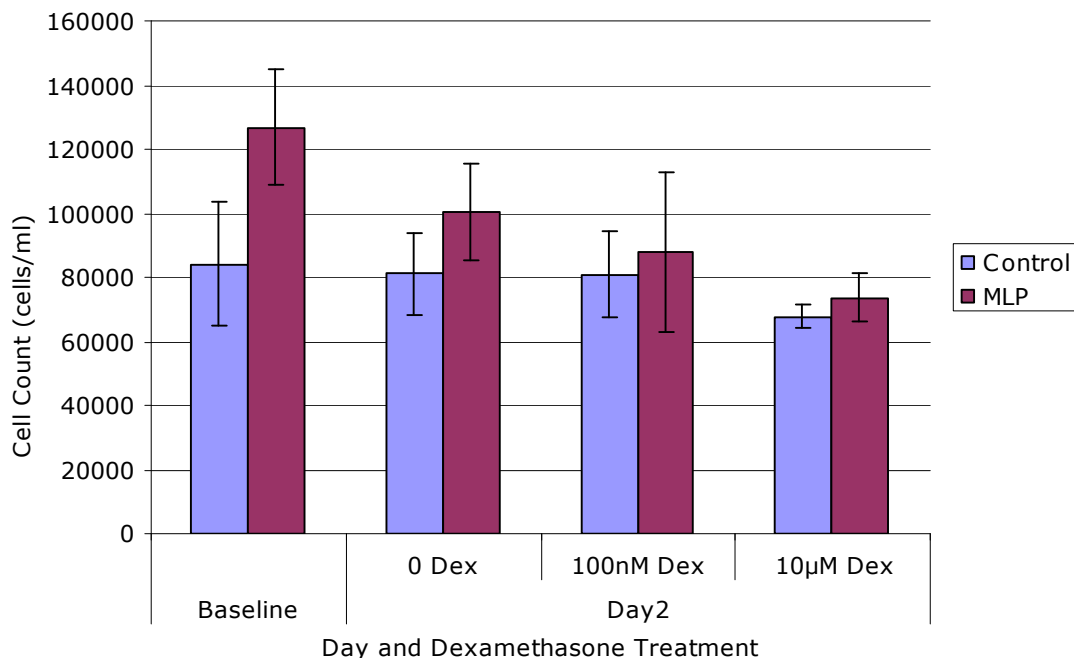
Low protein females appeared to consume more during pregnancy compared to controls, but this was found to not be significantly different. Maternal weight gain and litter size were also found to not be affected by diet during pregnancy (Table 3.1).

3.1.2 Cell Counts

Cell count was used in this study as a measure of cell proliferation. Cell counts were completed on duplicate wells from each litter at both time points, and for all three treatment stages.

All cultures were seeded at a density of 100,000 cells/ml/well in 24-well plates. As shown in Figure 3.1, baseline counts (i.e. day 11) tended to be higher in MLP cultures in comparison to controls. However, an independent t-test found no significant difference between the dietary groups. Cell number decreased in MLP cultures after 2 days of Dex treatment, but in controls remained unaffected. There appeared to be a slight effect of the increasing Dex concentration in both groups, but a two-way ANOVA showed no significant effects.

Figure 3.1 - Cell count of control and MLP cultured cardiomyocytes



See Table 3.1 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

Table 3.1 - Maternal weight gain, food intake and litter sizes

Maternal Diet	Control	Low Protein	P value
N	5	5	
Initial weight (g)	232.1 ± 8.9	235.9 ± 8.4	NS
Final Weight (g)	327.7 ± 18.6	334.3 ± 10.5	NS
Weight Gain GA 1-7 (g)	28.9 ± 1.8	33.1 ± 2.9	NS
Weight Gain GA 8-14 (g)	27.6 ± 3.9	35.0 ± 5.2	NS
Weight Gain GA 15-21 (g)	41.7 ± 5.0	43.2 ± 3.2	NS
Food Intake (g)	410.2 ± 34.2	489.3 ± 13.1	NS
Food Intake GA 1-7 (g)	167.6 ± 8.8	195.7 ± 8.0	NS
Food Intake GA 8-14 (g)	135.6 ± 20.8	166.9 ± 5.5	NS
Food Intake GA 15-21 (g)	107.0 ± 13.2	127.6 ± 6.2	NS
Litter Size	11.2 ± 1.0	9.8 ± 0.9	NS

Pregnant dams were fed either a control (18% casein) or low protein (MLP, 9% casein) diet throughout gestation. Eight neonates per litter were culled on day of parturition, when their hearts were dissected out. Cardiomyocytes were cultured for 10 days. At this point some were treated for 48 hours with increasing concentrations of dexamethasone. Data is shown as a mean \pm SEM for n litters. An independent t-test showed that there was no significant effect of diet on maternal food intake, weight gain or litter size. NS, not significant.

3.1.3 Binucleated and Mononucleated cell number

Cell staining to identify the number of mononucleated and binucleated cells was completed to assess the stage of cell differentiation. Immature cardiomyocytes will

tend to be mononucleated, but in the mature heart we would expect to see a greater proportion of binucleated cells (Corstius *et al.*, 2005). Table 3.2 shows that overall there was a greater number of mononucleated cells in comparison to binucleated cells across all time points and treatment groups. This indicates that the cardiomyocytes were still dividing and proliferating in the cultures. It was noted that there was no effect of diet on the proportions of mononucleated and binucleated cells at baseline. This is consistent with the findings of Corstius *et al.* (2005). However, maternal diet did have a statistically significant effect on the number of mononucleated and binucleated cells in dexamethasone treated cultures. Treatment of the cells with dexamethasone per se, did not significantly impact upon the proportion of mono- and binucleated cells. However, ANOVA indicated a significant effect of maternal diet ($P < 0.05$) in the Dex treated cells. Cultures from MLP litters had a significantly greater proportion of binucleated cells, particularly in cultures treated with 100nM Dex.

Table 3.2 – Effect of diet and Dex on proportions of binucleated and mononucleated cells

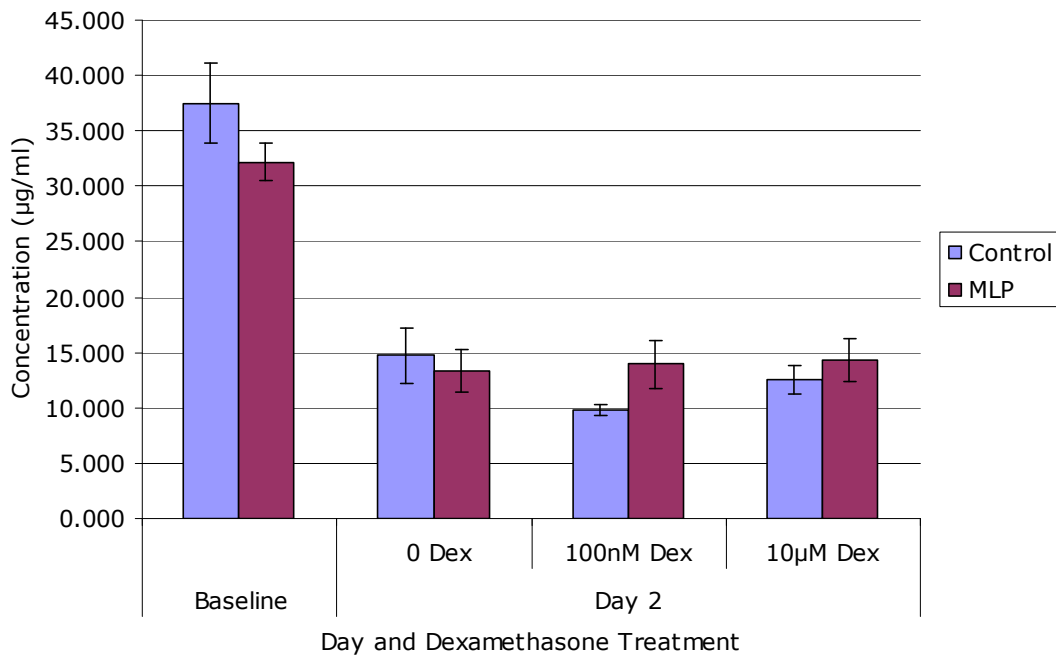
Day	Diet	Dex treatment	% Mononucleated Cells	SE	% Binucleated Cells	SE
Baseline	Control	0	90	1.8	9	1.5
	MLP	0	93	1.7	7	1.7
Day 2	Control	0	94	1.1	6	1.1
		100nM	91	2.6	9	2.7
		10 μ M	92	1.6	8	1.6
Day 2	MLP	0	87*	3.7	13*	3.7
		100nM	83*	4.9	17*	4.9
		10 μ M	87*	3.6	13*	3.6

See Table 3.1 for full legend. Data is shown as a percentage mean \pm SEM for cells taken at Day 0 and 2 of culture growth and treatment. There was no significant effect of diet on baseline cultures. A two-way ANOVA showed that there was a significant effect of diet on the percentage of mononucleated and binucleated cells at day 2 ($P=0.042$), but no effect of dexamethasone. * indicates the significant difference between control and MLP.

3.1.4 Protein Concentration

Protein concentration in the cardiomyocyte cultures was used as a proxy for cell size. As shown in Figure 3.2, diet did not affect the protein content of the cells after ten days in culture (baseline). Baseline protein content however was approximately double the concentration observed in all dexamethasone treated cells. A two-way ANOVA of the protein content of cells after two further days in culture showed no statistical effect of diet or Dex treatment.

Figure 3.2 - Protein content in cell cultures from control or MLP treated rats.

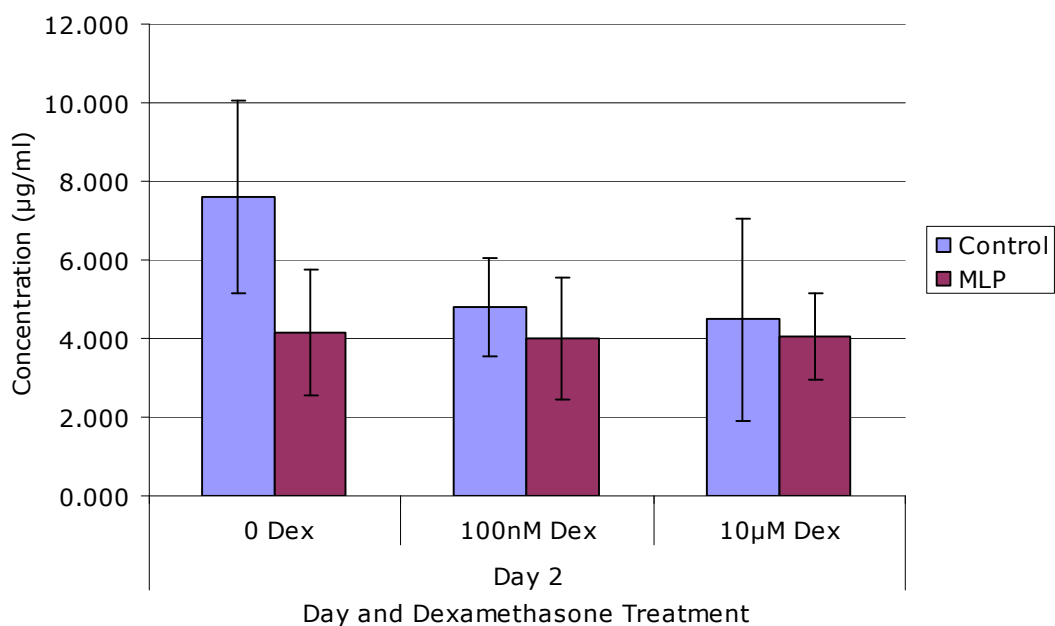


See Table 3.1 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.1.5 DNA Concentration

DNA concentration was measured, alongside the cell counts, to assess cell proliferation in the cardiomyocyte cultures when exposed to different Dex treatments. As shown in Figure 3.3, there was no baseline data available. This was because the DNA concentrations of these cultures were below the detection limits of the assay. Untreated day 2 cultures in the control diet group appeared to have an increased DNA content in comparison to 100nM and 10 μ M Dex cultures. DNA content in the MLP group remained consistent, and it was found that overall the DNA concentration was not affected by maternal diet or Dex treatment.

Figure 3.3 - DNA concentration of day 2, treated neonatal cardiomyocyte cultures

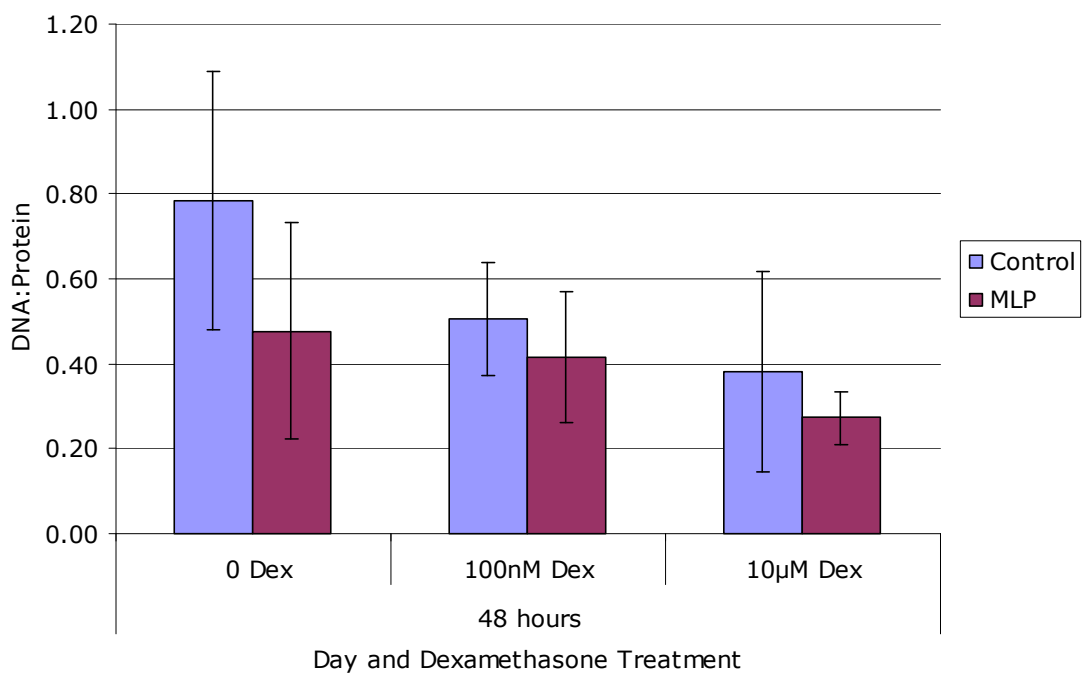


See Table 3.1 for full legend. Data is shown as a mean \pm SEM. A two-way ANOVA indicated that there was no significant effect of diet or dexamethasone treatment on the cells.

3.1.6 DNA:Protein Ratio

The DNA:protein ratio (Figure 3.4) was calculated by dividing DNA content by protein, and this was calculated to give a further indication of cell size. The higher the ratio, the larger the cells and vice versa. Due to the DNA baseline data being below the fluorescence detection limit, a ratio for baseline cells could not be calculated. A two-way ANOVA showed no statistical effect of either diet or Dex treatment on the DNA:protein ratio in the cardiomyocyte cultures. However, there did appear to be a slight trend, as the Dex concentration increased, the DNA:protein ratio decreased (NS).

Figure 3.4 - DNA:Protein Ratio of day 2, treated neonatal cardiomyocyte cultures

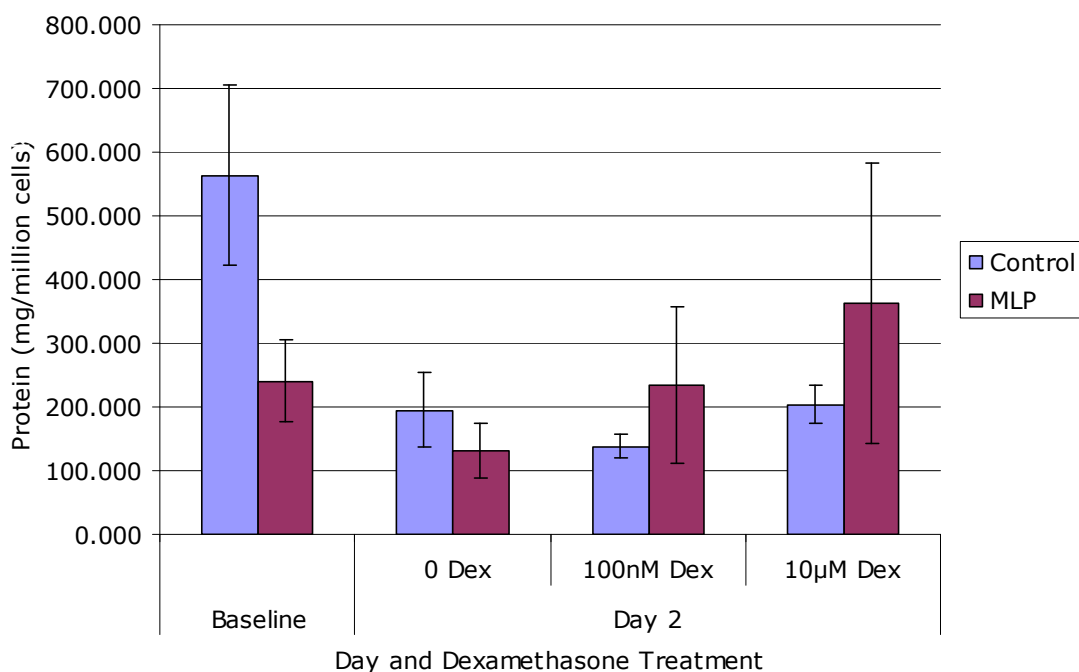


See table 3.1 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells.

3.1.7 Protein:cell count Ratio

Protein:cell count ratio (Figure 3.5) was calculated as an alternative indication of cell size. A two-way ANOVA showed no statistical effect of either diet or Dex treatment on the protein:cell count ratio in the cardiomyocyte cultures. However, for MLP cells in the day 2 cultures, this measure appeared to increase with Dex treatment, whereas baseline cells tended towards a higher ratio in control compared to MLP (NS).

Figure 3.5 - Protein:cell count ratio in control and MLP cultures



See table 3.1 for full legend. Data is shown as a mean \pm SEM and expressed as protein:cell count ratio per million cells. There was no significant effect of diet or dexamethasone treatment on the cells.

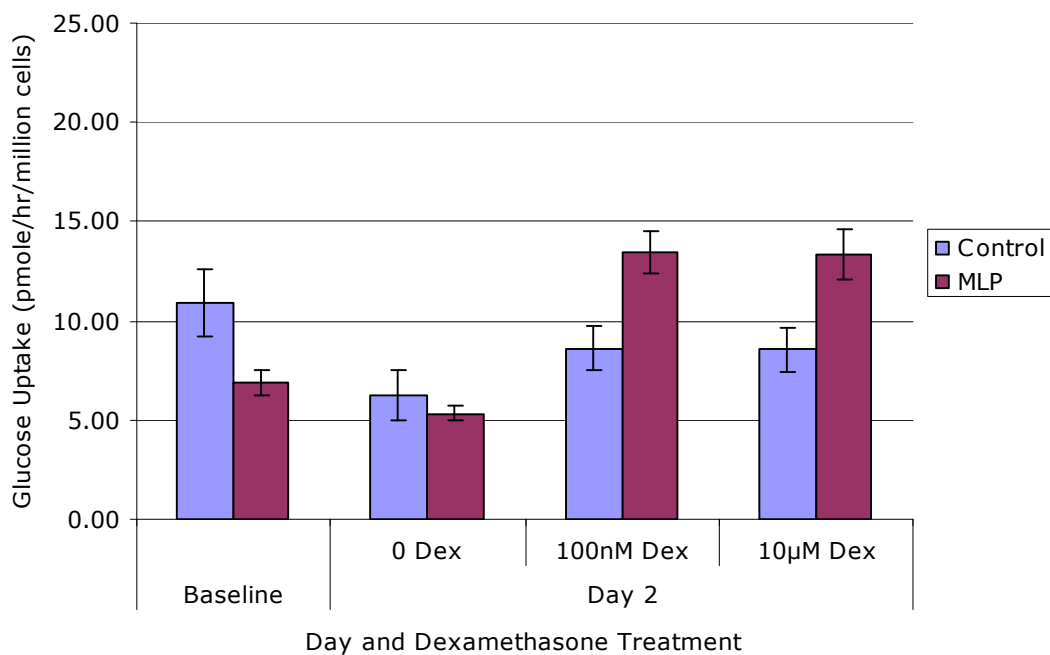
3.1.8 Glucose Uptake Assay

Glucose uptake was determined to gauge the metabolic activity of the cardiomyocyte cells in culture. It demonstrated the metabolic function of the cells, their demand for

and uptake of glucose, which is the main energy source required to power cellular processes. Uptake was expressed as pmole/hour/million cells, and was determined in both the basal and insulin stimulated state.

At baseline, basal glucose uptake appeared to be greater in the control cultures compared to the MLP cultures as shown in Figure 3.6A; however an independent t-test showed no significant effect of diet. Basal glucose uptake at day 2 was also found to be unaffected by diet. Although uptake appeared increased by Dex treatment at both doses, this affect did not achieve statistical significance.

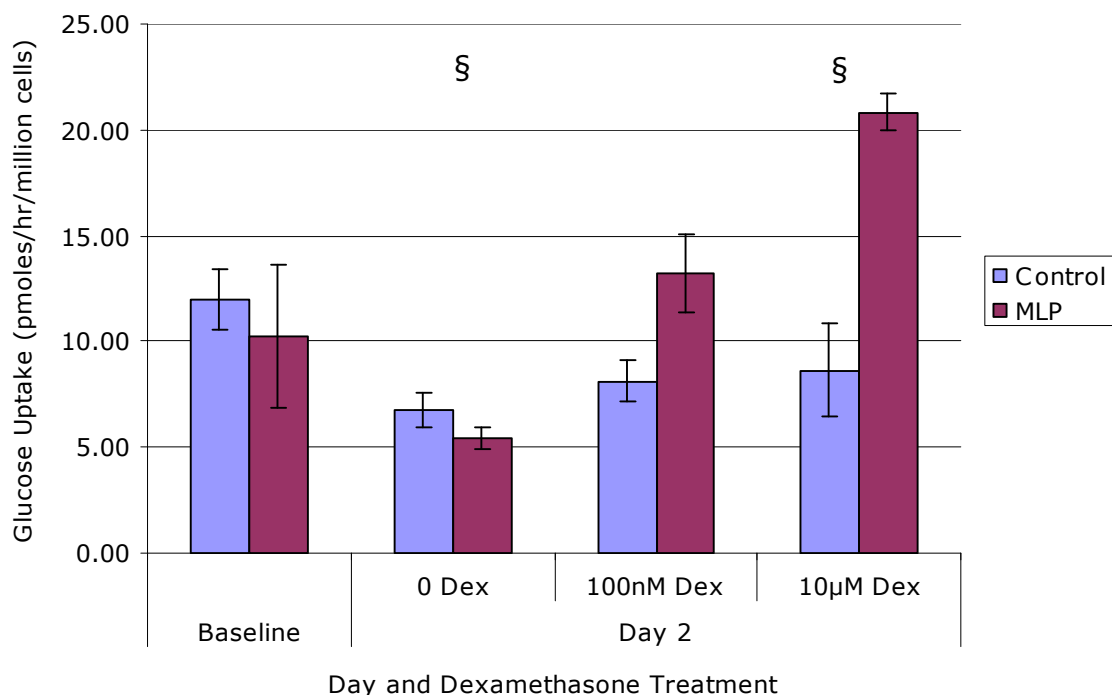
Figure 3.6A - Day 0 and Day 2 basal glucose uptake in control and MLP neonatal cardiomyocyte cultures



See Table 3.1 for full legend. Data is shown as a mean (expressed as pmole/hour/million cells) \pm SEM. An independent t-test and two-way ANOVA showed that there was no significant effect of diet or dexamethasone treatment on the cells.

Insulin stimulated glucose uptake (Figure 3.6B), at the baseline time point was similar in the two diet groups. At day 2 however, insulin stimulated glucose uptake was found to be affected by both diet ($P < 0.05$) and Dex treatments ($P < 0.05$), but there was no interaction between the two. As shown in Figure 3.6B, in the absence of dexamethasone (Day 2, 0 Dex), insulin stimulated glucose uptake was lower in the MLP groups than control (approximately 21% lower, $P < 0.05$). Dexamethasone treatment at either dose increased insulin stimulated glucose uptake. This effect was exaggerated in the MLP cultures with a 4-fold increase noted at 10 μ M Dex, compared to just 27% increase in the control cultures. In Dex treated cultures insulin stimulated glucose uptake was markedly higher in MLP than in the control group.

Figure 3.6B - Day 0 and day 2 insulin-stimulated glucose uptake in control and MLP neonatal cardiomyocyte cultures



See Table 3.1 for full legend. Data is shown as a mean (expressed as pmole/hour/million cells) \pm SEM. A two-way ANOVA showed that there was a significant effect of diet ($P = 0.03$) and Dex ($P = 0.013$) (independently) on the insulin stimulated glucose uptake. \S indicates the significant difference between 0 Dex and 10 μ M treated cells.

3.2 Trial Two – Impact of maternal protein restriction on neonatal cardiomyocyte gene expression.

For the second trial, ten pregnant females were fed either a control (n=5) or low protein (n=5) diet during gestation and neonatal pup hearts obtained to culture cardiomyocytes. These cultures were grown for ten days when some were taken to complete baseline analysis, and other cultures were treated with different concentrations of dexamethasone (0, 10nM and 100nM), and left for a further 48 hours (day 2 cells) before analysis.

Maternal weight and food intake were measured throughout gestation. Neonatal cardiomyocyte cells were cultured and used for the analysis of protein and DNA content, glucose transporter expression, cell death and cell number, including percentage of mononucleated and binucleated cells, for all time points. As with the first trial, glucocorticoid response was assessed by challenging cells with varying concentrations of dexamethasone. In trial one, cells were treated with one concentration that was within physiologically range, 100nM, and the second dose, 10 μ M, was selected with the intention of eliciting maximal responses. In this trial, both concentrations were selected to be within the physiologically relevant range.

The results from trial one showed that there was an effect of diet and Dex on insulin-stimulated glucose uptake. Due to this finding, mRNA expression of GLUT 1, GLUT 4, and glucocorticoid receptor (GR) was investigated to see if changes in gene expression could explain increased glucose uptake when cardiomyocytes are stimulated with insulin. Additionally, cell death in the cardiomyocyte cultures was investigated by running a cell death ELISA, and determining the ratio of dead and live cells.

3.2.1 Maternal weight gain and litter sizes

Maternal weight gain, total food intake and litter size were not affected by the diet fed during pregnancy (Table 3.3). Weekly food intake was also not significantly affected by maternal diet.

3.2.2 Cell Count

In this trial, cell count was completed using the Trypan blue method to identify dead and live cells. Total cell count was a sum of these two measurements per cell culture well. As with the first trial, all cultures were seeded at a density of 100,000 cells/ml/well in 24-well plates, and were measured in duplicate for both time points and all treatment groups.

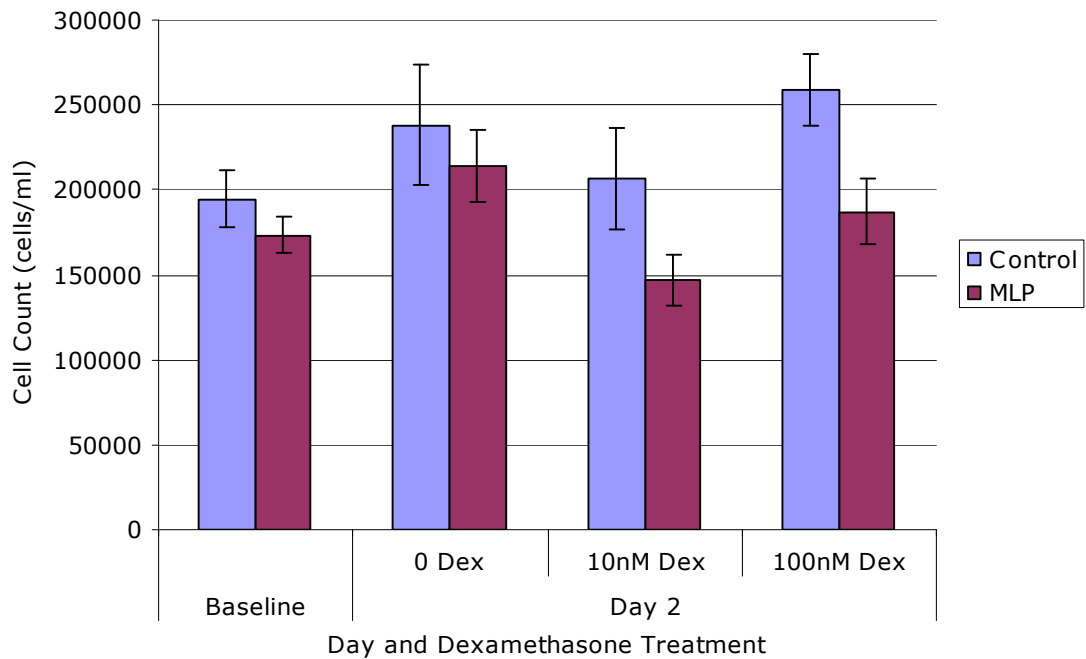
As shown in Figure 3.7 all counts were similar across diet groups and Dex treatments, with MLP counts being slightly lower compared to control. An independent t-test showed no significant effect of diet on the total cell count at the baseline stage (after 10 days in culture). There was no significant effect of diet or Dex, and no interaction between the two, in day 2 cells. However, cell counts in this trial were higher in comparison to the first trial (Figure 3.1).

Table 3.3 – Maternal weight gain, food intake and litter size

Maternal Diet	Control	Low Protein	P value
N	5	5	/
Initial weight (g)	226.8 ± 6.1	211.2 ± 5.2	NS
Final Weight (g)	312.2 ± 7.6	302.2 ± 10.0	NS
Weight Gain GA 1-7 (g)	19.4 ± 3.8	27.5 ± 3.2	NS
Weight Gain GA 8-14 (g)	23.0 ± 2.8	22.4 ± 1.7	NS
Weight Gain GA 15-21 (g)	47.2 ± 3.2	55.1 ± 12.2	NS
Food Intake (g)	431.6 ± 16.5	420.8 ± 10.5	NS
Food Intake GA 1-7 (g)	152.5 ± 3.9	150.5 ± 7.3	NS
Food Intake GA 8-14 (g)	126.5 ± 10.0	144.8 ± 2.6	NS
Food Intake GA 15-21 (g)	152.7 ± 10.3	125.4 ± 7.1	NS
Litter Size	8.0 ± 1.0	10.0 ± 1.0	NS

Pregnant dams were fed either a control (18% casein) or low protein (MLP, 9% casein) diet throughout gestation. Eight neonates per litter were culled on day of parturition, when their hearts were dissected out. Cardiomyocytes were cultured for 10 days. At this point some were treated for 48 hours with increasing concentrations of dexamethasone. Data is shown as a mean ± SEM for n litters. An independent t-test showed that there was no significant effect of diet on maternal food intake, weight gain or litter size. NS, not significant.

Figure 3.7 - Total cell count in cultures of neonatal cardiomyocytes

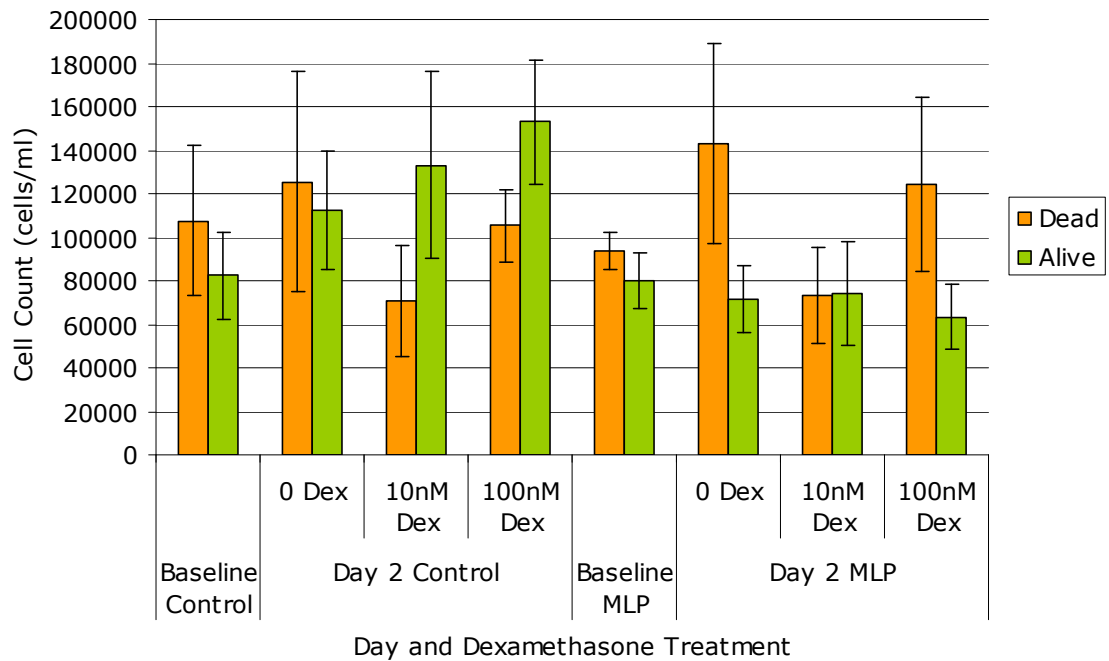


See Table 3.3 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.2.3 Dead and Live cell counts

Using the trypan blue method as detailed in Chapter 2, cells were counted, identifying dead and live cells per cell culture well. There was no significant effect of either diet or dexamethasone on the proportions of live to dead cells (Figure 3.8). Performing the cell counts with trypan blue indicated that between 35% and 65% of cells in culture were dead. There was a tendency for fewer cells to survive in the MLP cultures, but this did not achieve statistical significance.

Figure 3.8 - Dead and live cell counts of control and MLP cultured cardiomyocytes



See Table 3.3 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.2.4 Mononucleated and binucleated cell counts

Table 3.4 shows that most cardiomyocytes in culture were mononucleated, indicating that cells were still dividing and were not fully differentiated. The percentage of binucleated cells across both days and treatment groups were very similar. Maternal diet had no effect on baseline cultures, and an ANOVA of day 2 cultures indicated that differentiation was not affected by diet or Dex treatment.

Table 3.4 – Effect of diet and Dex on proportions of binucleated and mononucleated cells

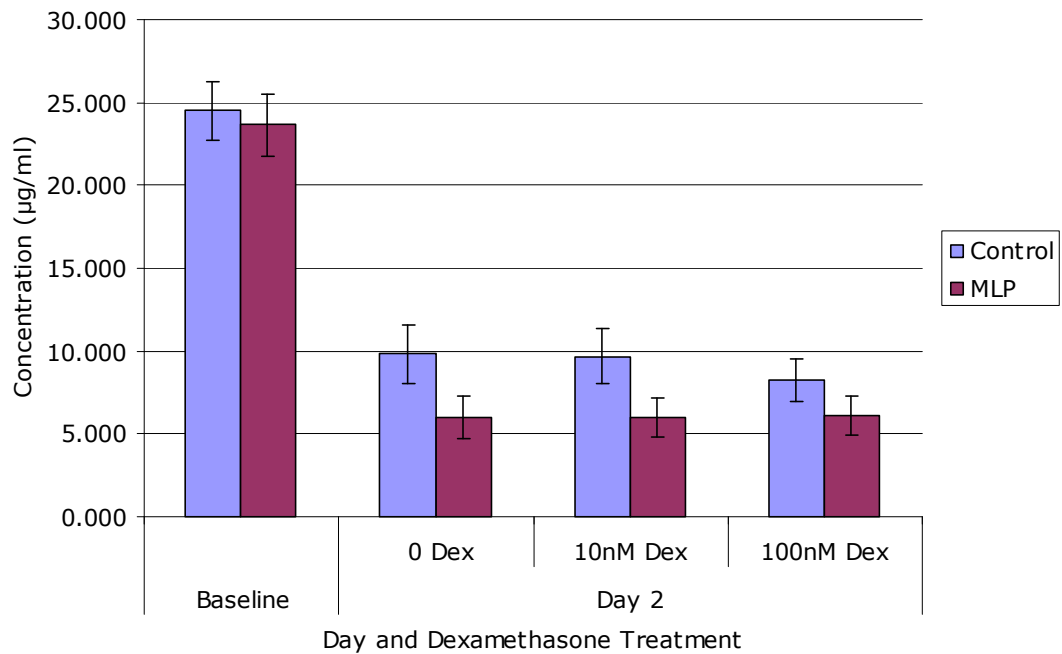
Day	Diet	Dex treatment	% Mononucleated Cells	SE	% Binucleated Cells	SE
Baseline	Control	0	91	1.3	9	1.3
	MLP	0	92	1.1	8	1.1
Day 2	Control	0	92	1.0	8	1.0
		10nM	90	1.4	10	1.4
		100nM	94	0.8	6	0.8
Day 2	MLP	0	92	1.2	8	1.2
		10nM	90	1.5	10	1.5
		100nM	91	1.1	9	1.1

See Table 3.3 for full legend. An independent t-test showed that diet had no significant effect on the percentage of mono and binucleated cells at the baseline level. Dexamethasone treated cells were also not affected by diet or dex, and there was no interaction between the two. NS, not significant.

3.2.5 Protein Concentration

As shown in Figure 3.9, diet did not affect the protein content of the cells after ten days in culture (baseline). Baseline protein concentration was approximately double the concentration observed in all dexamethasone treated cells. A two-way ANOVA of the cells after two further days in culture showed no statistical effect of diet or Dex treatment.

Figure 3.9 – Protein concentration of control and MLP cultured cardiomyocytes

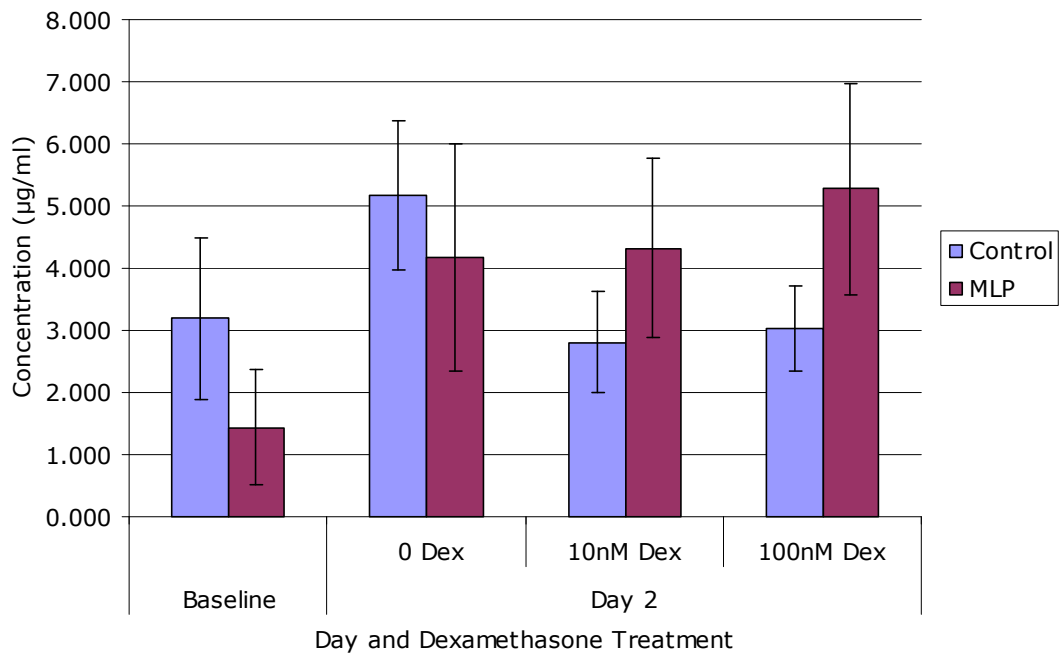


See Table 3.3 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.2.6 DNA Concentration

DNA concentration was measured to assess cell proliferation rates of cells in response to Dex treatment. Overall, the cardiomyocytes DNA concentration was quite low, suggesting that the samples were falling towards the lower end of the detection limit. As shown in Figure 3.10, DNA concentration in the baseline cultures was found to not be affected by diet. Day 2 MLP cultures appeared to show an increase in DNA concentration with increasing Dex treatment but this effect of diet did not achieve statistical significance ($P=0.06$).

Figure 3.10 - DNA concentration of control and MLP cultured cardiomyocytes

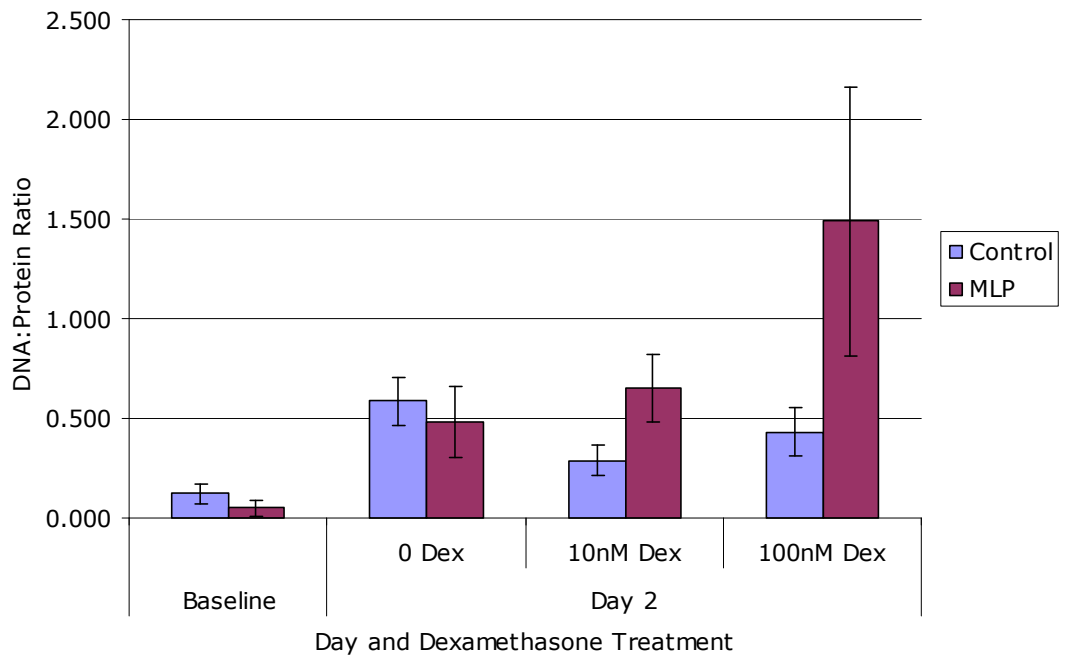


See Table 3.3 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.2.7 DNA:Protein Ratio

DNA:Protein ratio was calculated by dividing DNA content by protein to give an indication of cell size. As seen in Figure 3.11 the baseline ratio was almost half of that seen in day 2 cultures. An independent t-test found that diet had no significant effect on the ratio at any level. However, the P-value for baseline cultures was 0.065 suggesting that there is a trend in the data. In the day 2 control cultures the ratio appeared to be fairly consistent in comparison to MLP litters where the ratio increased with Dex increments. ANOVA showed no significant affect of either diet or Dex, and no interaction between the two.

Figure 3.11 - DNA:protein ratio of cultured neonatal cardiomyocytes



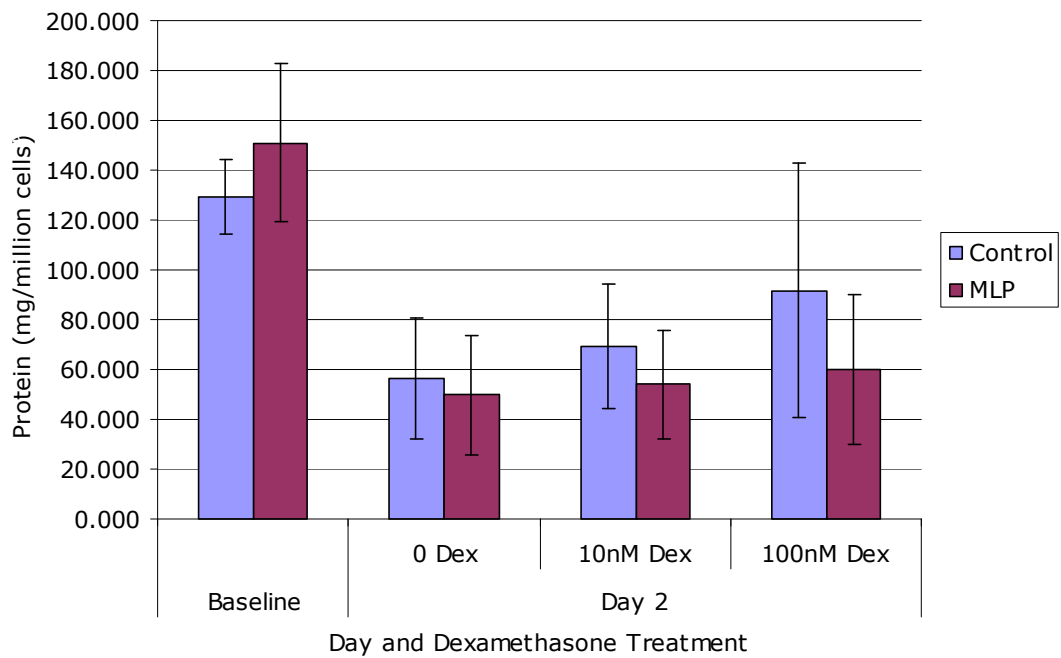
See Table 3.1 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.2.8 Protein:Total Cell Count Ratio

Protein:total cell count ratio was calculated as another indicator of cell size. The ratio was expressed as protein:total cell count per million cells.

Baseline data appeared to be approximately double that of the day 2 treated cells (Figure 3.12). There was no significant effect of diet or dexamethasone treatment upon protein concentration noted at either time point.

Figure 3.12 - Protein:total cell count ratio of control and MLP cultured cardiomyocytes

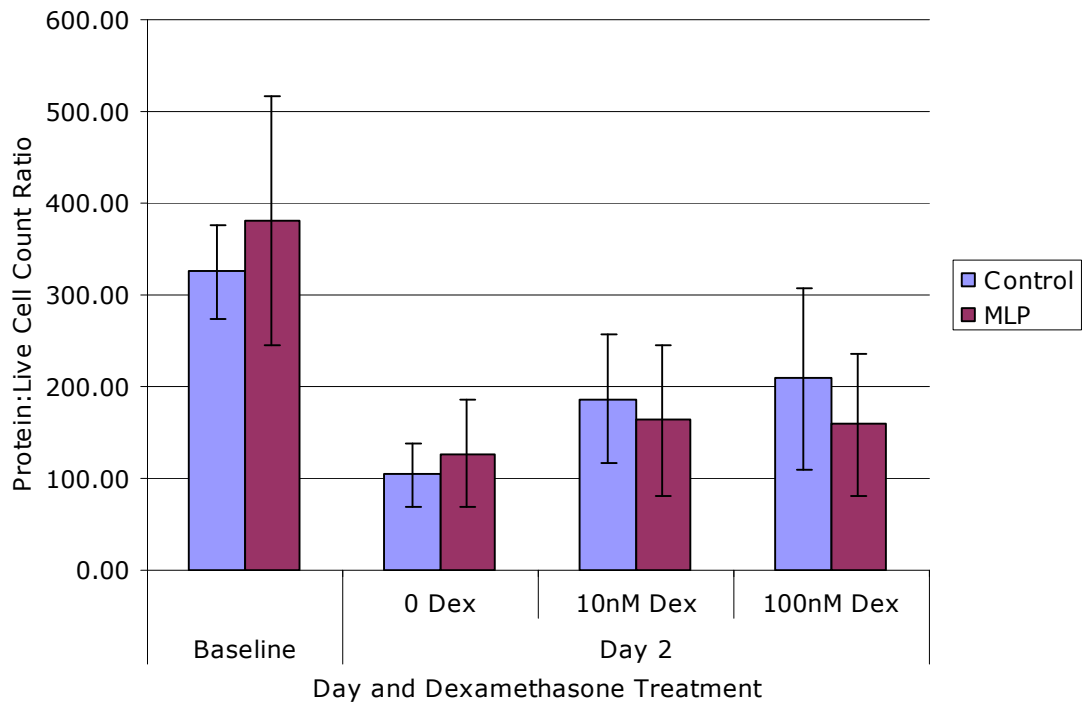


See Table 3.3 for full legend. Data is shown as a mean \pm SEM, and expressed as protein:total cell count ratio per million cells. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.2.9 Protein:Live Cell Count Ratio

Protein:live cell count ratio was another indicator of cell size and was expressed as protein:live cell count per million cells. As with the total cell count ratio, the baseline data was approximately double that of the day 2 treated cells, but was not significantly affected by diet (Figure 3.13). Protein:live cell count ratio showed a similar pattern to that seen for the protein:total cell count ratio in all days and treatments except for 0 Dex. Day 2 cultures were found to be significantly affected by diet ($P=0.041$), with control showing a higher ratio in comparison to MLP for the treated cells. Dexamethasone was found to have no effect upon protein:live cell ratio in these cultures.

Figure 3.13 - Protein:live cell count ratio of control and MLP cultured cardiomyocytes



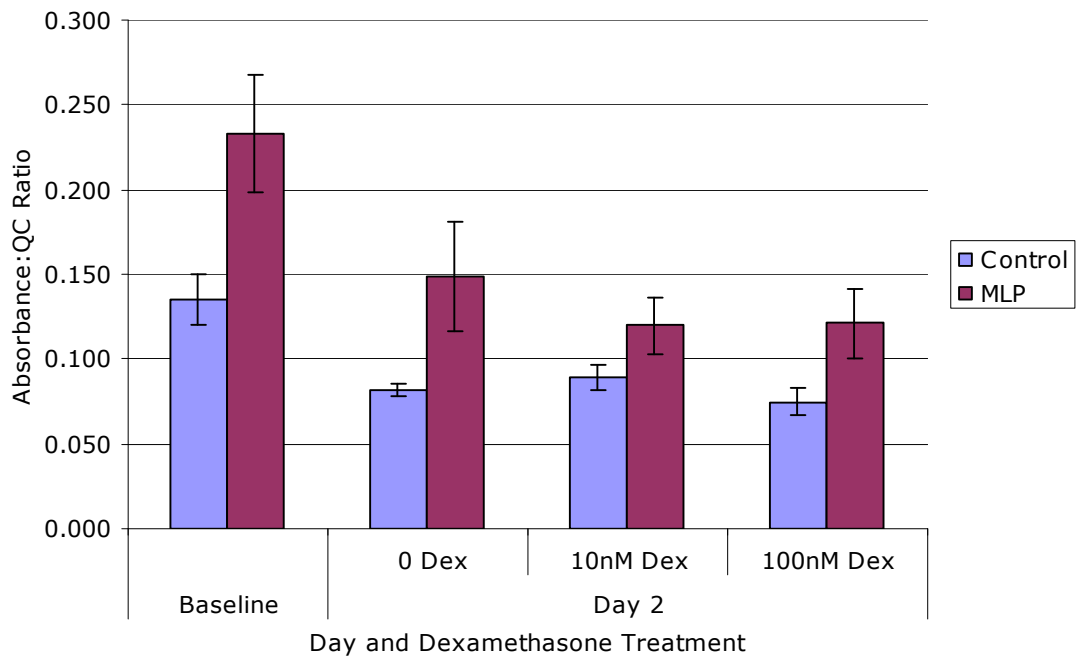
See Table 3.3 for full legend. Data is shown as a mean \pm SEM and expressed as protein:live cell count ratio per million cells. There was no significant effect of diet at the baseline level. Day 2 cells were not affected by dex, but were significantly affected by diet ($P=0.041$).

3.2.10 Cell Death

Cell death is a measure of the number of cells dying in culture, and was used to monitor the response of the cells to the Dex treatments. Cell death was expressed as a ratio against the QC absorbance.

It was found that diet and Dex had no effect on cell death in cultures across any time point or treatment group. It did appear that control cultures have less cell death than MLP (Figure 3.14), but this was not found to be significant.

Figure 3.14 - Cell death ratio of control and MLP neonatal cardiomyocytes



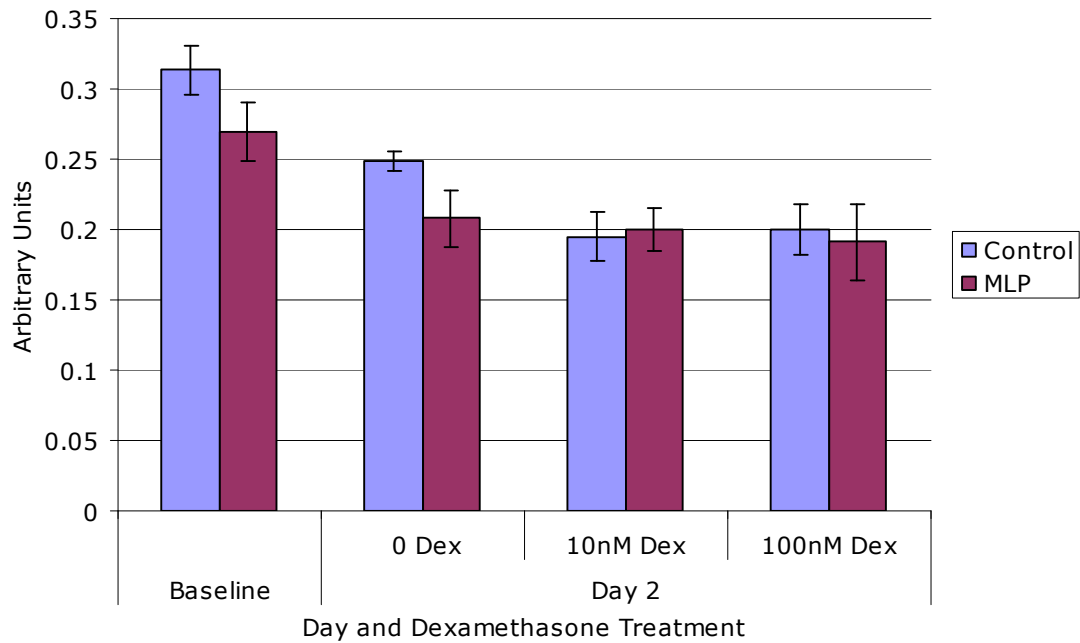
See Table 3.3 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.2.11 Gene expression

Glucose transporter 1 (GLUT 1), glucose transporter 4 (GLUT 4), and glucocorticoid receptor (GR) mRNA expression was determined by RT-PCR to investigate the response of cardiomyocytes to Dex treatment, and assess whether altered expression of these genes was the reason for the increased glucose uptake in insulin-stimulated cardiomyocytes noted in trial one.

Cells were seeded at 200,000 cells per well in 12 well cell culture plates and grown and treated under the same conditions as cells seeded in 24 well plates. Data was normalised to Cyclophilin A, as a housekeeper gene, which was found to have consistent expression across diet groups in baseline cells, and across all Dex concentrations at day 2 (Figure 3.15).

Figure 3.15 - Cyclophilin A mRNA expression of neonatal cardiomyocyte cultures

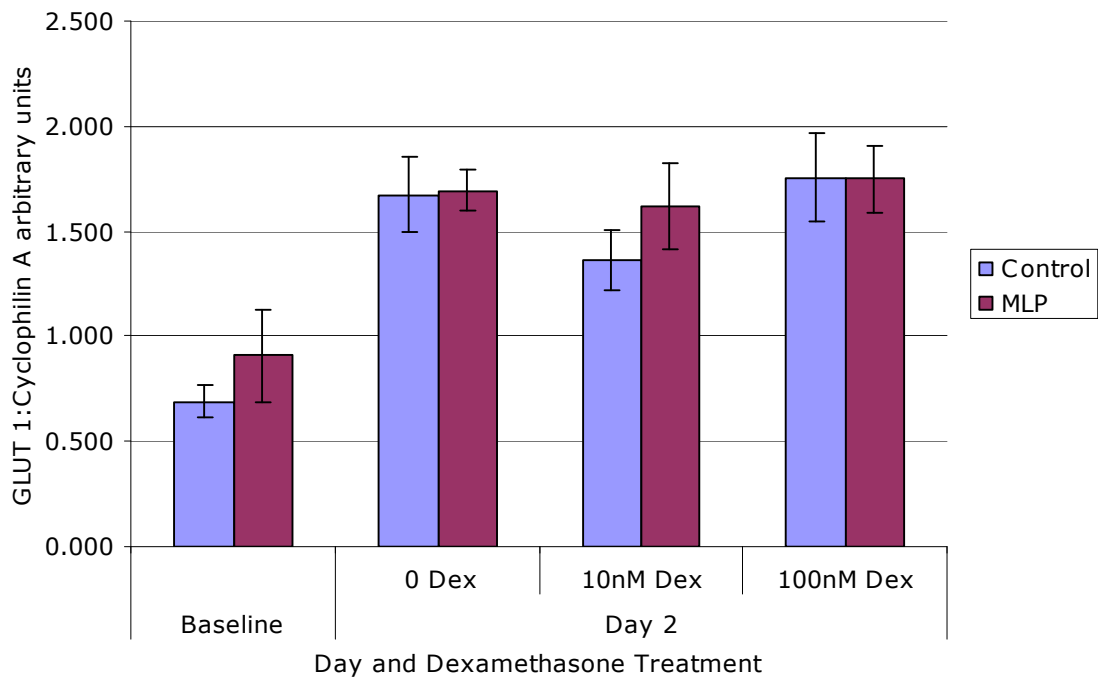


See Table 3.3 for full legend. Data is shown as a mean \pm SEM. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.2.11.1 GLUT 1

As shown in Figure 3.16, mRNA expression of baseline GLUT 1 was not significantly affected by maternal diet. GLUT 1 expression in day 2 cultures was fairly consistent across treatment groups and an ANOVA found no significant affect of either diet or Dex treatment.

Figure 3.16 - GLUT 1 mRNA expression of neonatal cardiomyocyte cultures.

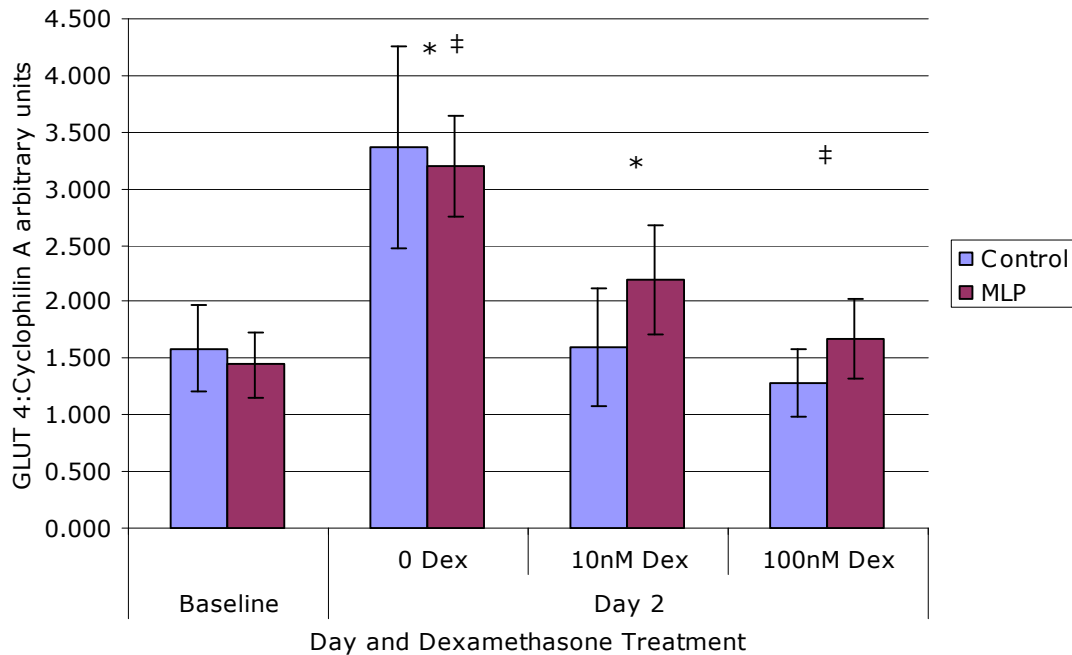


See Table 3.3 for full legend. Data is shown as a mean \pm SEM, and normalised to Cyclophilin A, housekeeper gene. There was no significant effect of diet or dexamethasone treatment on the cells at any time point.

3.2.11.2 GLUT 4

At the baseline stage, an independent t-test found no significant affect of diet on cultures (Figure 3.17). Day 2 cultures also showed no affect of diet, but dexamethasone treatment was found to significantly impact upon GLUT 4 mRNA expression ($P=0.008$). ANOVA showed that 0 Dex cells had a greater expression of GLUT 4 in comparison to 10nM and 100nM treated cultures, with a more marked difference seen at 100nM Dex.

Figure 3.17 - GLUT 4 mRNA expression of neonatal cardiomyocyte cultures



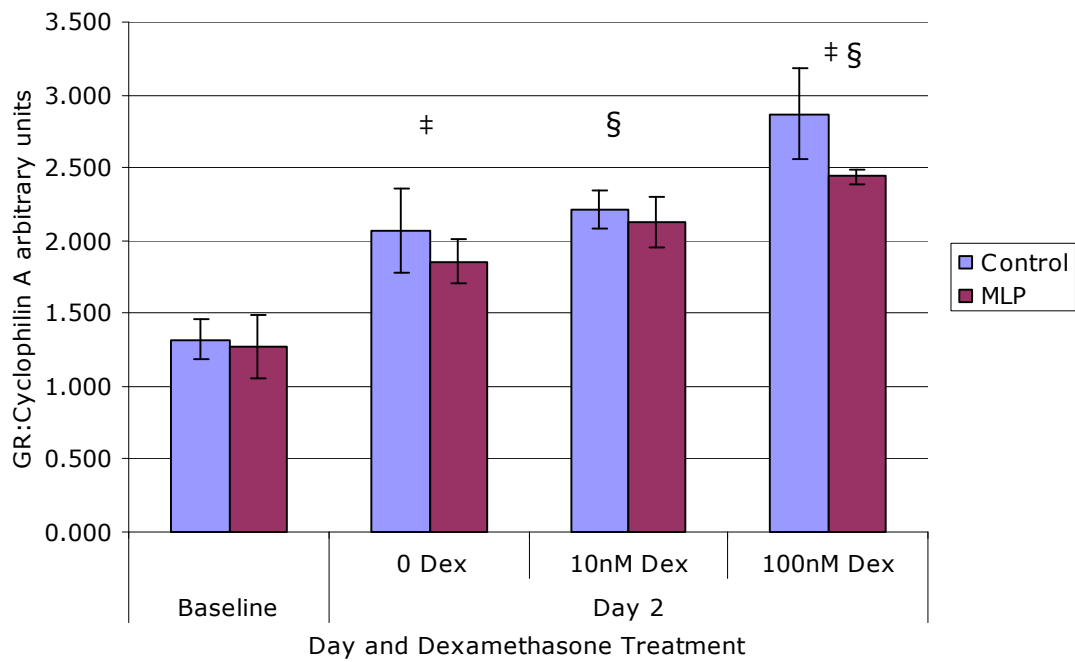
See Table 3.3 for full legend. Data is shown as a mean \pm SEM, and normalised to Cyclophilin A housekeeper gene. There was no significant effect of diet at the baseline level, and no effect of diet on day 2 cells. * indicates significant difference between 0 Dex and 10nM ($P=0.018$), † indicates a significant difference between 0 Dex and 100nM ($P=0.003$), no significant difference between 10nM Dex and 100nM Dex.

3.2.11.3 Glucocorticoid Receptor

From baseline cells through to 100nM Dex treated cells the expression of GR mRNA increased steadily (Figure 3.18).

An independent t-test showed no significant effect of diet on GR expression in the baseline cells. ANOVA indicated no effect of diet and no interaction between diet and Dex on day 2 cells, but Dex had a significant affect on the expression of GR ($P=0.009$). GR expression was significantly higher in the 100nM Dex treated cultures than in 0 or 10nM cultures ($P<0.05$), but the lower dose did not significantly increase expression over the untreated cultures.

Figure 3.18 - Glucocorticoid receptor mRNA expression of neonatal cardiomyocyte cultures



See Table 3.3 for full legend. Data is shown as a mean \pm SEM, and normalised to Cyclophilin A housekeeper gene. There was no significant effect of diet at the baseline level, and no effect of diet on day 2 cells. † indicates significant difference between 0 Dex and 100nM ($P=0.003$), § indicates a significant difference between 10nM Dex and 100nM ($P=0.028$).

4 Chapter 4

Discussion

The fetal programming hypothesis suggests that nutritional deficiencies during fetal development predispose offspring to develop cardiac abnormalities in later life (Campbell *et al.*, 1996, Godfrey *et al.*, 1996, Godfrey & Barker, 2000). Human cohort studies have provided evidence to suggest that undernutrition causes growth retardation (Sydall *et al.*, 2005, Campbell *et al.*, 1996), which is a precursor of cardiovascular disease. However, there are numerous criticisms of the epidemiological evidence, so animal models have been designed to further investigate human findings. They provide the opportunity to explore in more detail the mechanisms that are associated with changes.

The aims of this thesis were to investigate the effects of maternal low protein (MLP) diet on offspring heart structure and function. This model of maternal protein deficiency was developed in the rat by Langley & Jackson (1994), and is a well-established tool for the investigation of nutrient restriction as a contributor to the onset of cardiovascular disease in offspring in adult life (Langley-Evans *et al.*, 1999, Harrison & Langley-Evans, 2008). Hypertension in rodent offspring is one outcome of this diet and has been investigated extensively (Langley & Jackson, 1994, Langley-Evans *et al.*, 1994, Langley-Evans *et al.*, 1996, Langley-Evans *et al.*, 1999). Studies have moved on to investigate specific effects of programming on cardiomyocytes. They have found that a low protein diet affects various aspects of heart structure and function, including a decrease in the contractile ability of the heart (Cheema *et al.*, 2005), evidence of hypertrophy (Cheema *et al.*, 2005, Oparil, 1984), a reduction in cell nuclei number (Corstius *et al.*, 2005), and left ventricle wall thickening (Cheema *et al.*, 2005) as well as impaired recovery of left ventricle pressure following ischemic reperfusion (Elmes *et al.*, 2007). Using primary cultures of neonatal cardiomyocytes

exposed to MLP diet *in utero*, this study investigated if the effects of this diet in cultured cells mimic those seen in animal studies. It also began to explore if synthetic glucocorticoids have programming effects on glucose uptake and the expression of related genes.

4.1 Maternal Effects

In trial one, although MLP animals consumed more during pregnancy it was not significantly different. Similarly in trial two, maternal total food intake during pregnancy was not affected by diet. MLP females may be attempting to increase protein intake by consuming more, but Langley & Jackson (1994), who also noted slight increase in food intake in MLP fed female rats, found that the compensation was not sufficient to offset the protein restriction. In a normal rat pregnancy pre-implantation and implantation occurs in week one (embryonic phase), followed by an organogenesis phase in week two, and finally rapid growth, differentiation and maturation of the fetus in the final week (Bellinger *et al.*, 2005). Due to this, it was thought that the rats would consume more food during the final week of gestation. This current study has similar findings with other studies, which also found that food intake was not affected by the low protein diet (Langley-Evans *et al.*, 1994, Cheema *et al.*, 2005, Tappia *et al.*, 2005).

Maternal weight gain was also not affected by MLP diet in either trial, which is comparable with Langley-Evans *et al.* (1994) who noted no difference in maternal weight gain between the two diet groups. Heywood *et al.* (2004) saw a 14.9% decrease in low protein fed females weight during pregnancy compared to controls. The low protein diet was however a slightly different composition, made up of 6% protein compared to the 9% casein model used in this study. Comparable to other

studies (Langley & Jackson, 1994, Langley-Evans *et al.*, 1994, Corstius *et al.*, 2005) litter size did not differ between the dietary groups in either trial.

4.2 Cardiomyocyte Characteristics

Cell counts were not significantly affected by diet or dexamethasone treatment across any time point in either trial, and remained at a steady count across both day 0 and 2 cultures. There was no decline in counts from day 0 to 2 demonstrating that cultures were not dying. Interestingly, counts were quite varied across the trials, trial two cultures had an overall higher number of cells compared to trial one. Trial two counts were approximately 200,000 cells/ml, and trial one counts were between 8000 and 12,000 cells/ml. The reason for these differences could be due to improvement on technique of isolating and culturing primary cells, or inter-litter variability. Trial two counts did appear to show a possible trend whereby MLP counts were slightly lower to that seen in control. This is similar to the findings in the Corstius *et al.* (2005) study, where offspring exposed to a low protein diet during gestation had a decreased number of cardiomyocytes (1.15×10^7) compared to control animals (1.43×10^7).

Live cell counts and apoptosis were assessed and found to be not significantly different between control and MLP diets. There did appear to be a possible trend, with MLP cultures having a higher apoptosis rate compared to controls, and more dead cells than live. Further investigation of this trend may be worth pursuing as Cheema *et al.* (2005) reported maternal low protein diet increased cardiomyocyte apoptosis by three-fold in five day old neonates. Apoptosis and necrosis are both types of cell death that differ in morphology, mechanism and incidence (Dive *et al.*, 1992). The Roche cell death ELISA^{PLUS} kit used in this thesis measured cytoplasmic histone-associated DNA fragments, which occurs in apoptosis several hours before plasma membrane

breakdown. Apoptosis is natural cell death where cells shrink, and condensation of cytoplasm occurs, activating endogenous endonuclease which cleaves systemic DNA (Dive *et al.*, 1992, Roche, UK). Phagocytes then rapidly engulf these cells preventing inflammatory responses from occurring. For these reasons it is complicated to measure (Dive *et al.*, 1992). Necrosis is the death of living tissue through disease, injury, or disruption of blood supply. The cell membrane is disrupted, which leads to respiratory poisons and hypoxia causing depletion of ATP, loss of metabolic function, and the cell swells and ruptures leading to inflammation. Future studies could measure ATP concentrations in the cells, or markers of inflammation to assess if MLP diet causes tissue damage leading to cell death. The Roche cell death ELISA^{PLUS} can also be used to measure necrosis instead of apoptosis. Dive *et al.* (1992) suggest an alternative method for quantifying apoptosis and necrosis. Multi-parameter flow cytometry measures cell diameter, internal granularity and DNA binding, which has been suggested as a more accurate measure of cell death.

Another alternative measure of cell death is determining apoptosis specific gene expression by western blot or immunohistochemistry (Condorelli *et al.*, 1999). Bcl2-associated X protein (Bax) is a pro-apoptotic gene that has been shown to be up-regulated in ischemic myocardium *in vivo*. β -cell lymphoma 2 (Bcl-2) is an anti-apoptotic gene that under the same circumstances has been shown to be decreased (Condorelli *et al.*, 1999). They both lie upstream in the cell death pathway and play an important role in whether a cell will die (Gross *et al.*, 1999). The ratio of the two genes has been suggested to be an important marker of myocardial cell survival probability (Condorelli *et al.*, 1999). Caspases lie further down-stream to Bax and bcl-2 in the apoptosis pathway, and could also be measured as they play an essential role in cell apoptosis.

Fully differentiated cardiomyocytes are binucleated, and immature cells are mononucleated. In both trials, a greater number of mononucleated cells were seen

compared to binucleated across both dietary groups, indicating that all cultures contained cells which were immature and actively proliferating. In trial one and two, nucleation in baseline cultures was unaffected by diet. In day 2 cultures however, MLP exposure decreased the percentage of mononucleated and increased binucleated cells compared to controls. This suggested that at day 2 MLP diet led to an increased number of fully differentiated cardiomyocytes compared to control cultures. This finding did not appear to be robust however, as in trial two there was no effect of diet on the day 2 cultures. Dexamethasone treatment did not affect the number of nuclei in either trial, demonstrating that, in this study, glucocorticoids had no effects postnatally on cell differentiation in culture. Corstius *et al.*'s (2005) study using the MLP model also found that dexamethasone administered, postnatally, had no effect on nucleation of cardiomyocytes. Consistent with this current study, Corstius *et al.* (2005) found binucleation to be low across both diet groups, but no differences were noted between the control and low protein diets. De Vries *et al.* (2002) reported an increase in myocyte volume, indicative of hypertrophy in rats administered dex in the first three days of neonatal life.

For both trials protein content was not significantly affected by diet or dex at either time point. De Vries *et al.* (2002) investigated protein concentration in left and right ventricles in neonates exposed to standard chow diet during gestation, and administered dexamethasone at days 1, 2 and 3 postpartum. In 7 day old pups, protein tended to be higher in dexamethasone treated animals compared to saline. Interestingly, in this current study, in both trials, baseline cultures had almost double the protein concentration in comparison to the dexamethasone treated cells. This data was not statistically compared, but a possible reason for the noted difference could be due to a lack of fetal bovine serum (FBS) in the growth medium in day 2 cultures. FBS was not added to day 2 culture medium in order to measure the direct effects of dexamethasone without FBS and other medium additives interfering. For future studies, it would be interesting to measure cell proliferation in cultures that have been

grown in medium with and without serum, as it contains growth factors that promote cell proliferation (Sigma, UK).

De Vries *et al.* (2002) also calculated the protein:DNA ratio as an indicator of cell size, and found the ratio to be higher in dexamethasone treated rats compared to saline treated rats at 7 days old. The higher the ratio the larger the cells and vice versa, so the finding suggested that dexamethasone treated animals had larger cells, indicative of hypertrophic growth. Protein:DNA ratio and protein:total cell count ratio were calculated in the current trial, but were not affected by diet or dexamethasone. Protein:live cell count ratio was calculated in trial two and was significantly, but inconsistently affected by MLP diet in day 2 cultures. Even though Dex treatment had no significant effect, untreated day 2 cultures established an increased ratio in the MLP litters in comparison to control, and the opposite was seen in untreated litters. The high ratio seen in untreated MLP cultures indicates larger cells and the low ratio in the MLP treated cultures indicates smaller cells. The reason for this difference is unknown.

Various *in vivo* studies have found MLP diet leads to functional defects in the hearts of offspring in the first few weeks of life. Cheema *et al.* (2005) noted a severe depression in ejection fraction (left ventricular end-diastolic volume) of the heart in the first 2 weeks of neonatal life, with the greatest peak depression seen in 7 day old pups. From 1 to 84 days of age, MLP offspring exhibited increased left ventricular internal diameters during systole and diastole, as well as thinner walls from 3 days to 2 weeks of age, where thickening began and continued into adult life (Cheema *et al.*, 2005). De Vries *et al.* (2002) also noted changes in heart structure and function in 7 day old pups. Heart weight and DNA content was lower compared to controls, with higher protein content and protein:DNA ratio. The mitotic index of cardiomyocytes was low at birth in MLP animals, but then increased one to two weeks postpartum (Aroutiounova *et al.*, 2009). The earliest onset of hypertension however, has been noted in 4 week

old rat pups (Langley-Evans *et al.*, 1994). Functional changes in rat offspring hearts exposed to MLP *in utero*, appear to occur after one week old, and disease state occurs later in life. This suggests that these changes are not a product of a diseased heart, and cultures in this study would not be at a diseased stage.

The measures of cell proliferation assessed in the current study partly address the hypothesis set that MLP diet affects proliferation and differentiation rates. This diet, in some, but not all cases, had an effect on cardiomyocyte cultures demonstrating that it had programming effects on cell growth, proliferation and differentiation, some of which are similar to those seen *in vivo*.

4.3 Glucose uptake and gene expression

Glucose uptake, a measure of cellular function, was not affected by diet or dexamethasone at the basal level, but was affected by dexamethasone treatments when stimulated with insulin. Baseline (Day 0) insulin stimulated glucose uptake was not influenced by maternal diet, but both diet and dexamethasone impacted on day 2 cultures. Following treatment with 10 μ M dexamethasone for 48 hours, the cultures had an increased glucose uptake in response to insulin stimulation. 100nM of dexamethasone on the other hand, had no effect. The effects of insulin stimulation in the 10 μ M dexamethasone treated cells were exaggerated in the MLP cultures, which indicates that these cultures are more sensitive to Dex. This suggests that glucocorticoids elicit enhanced responses in MLP cardiomyocytes compared to control, causing them to take up more glucose when insulin is present. This supports the hypothesis that maternal protein restriction increases sensitivity to synthetic glucocorticoids in cultured cardiomyocytes, but only with concentrations above the physiological range. Maternal low protein cultures treated with 100nM dex did not show an increased sensitivity. This is consistent with *in vivo* findings of maternal

protein deficiency, which *in utero* lead to the offspring developing glucocorticoid sensitivity (Langley-Evans & Nwagwu, 1998, Langley-Evans *et al.*, 1996b).

Overexposure of the fetus to maternal glucocorticoids is thought to be due to decreased expression of placental 11 β -HSD-2, which protects the fetus from maternal GC's (Drake & Seckl, 2004, Benediktsson *et al.*, 1993). MLP diet is known to decrease placental 11 β -HSD-2, leading to increased sensitivity of the offspring to circulating glucocorticoids (Langley-Evans *et al.*, 1996). Langley-Evans *et al.* (1996b) found an increased activity of corticosterone-sensitive enzymes related to low hormone concentrations and increased GR binding capacity and receptor numbers in the hippocampus of MLP male offspring.

Another possible reason for the increase in glucose uptake is that dexamethasone inhibited the final stages of hyperplastic growth, leading to the cardiomyocytes requiring a higher energy intake to undergo hypertrophic growth. Findings from cell proliferation and differentiation however, were not consistent enough to conclude this. The increased glucose uptake appears more likely to be mediated by the altered expression of glucocorticoid receptor leading to GC sensitivity from the low protein diet.

One hypothesis to be addressed by this study was that maternal undernutrition alters the expression of genes involved in regulating glucose uptake. Glucose transporter expression was measured to test this hypothesis, and given the response of insulin-stimulated glucose uptake to dexamethasone it was also of interest to determine whether the glucose transporters were regulated by glucocorticoids. Baseline GLUT 1 expression in MLP and control cultures were not dissimilar. Day 2 treated cultures also showed no significant difference and GLUT 1 expression was constant across all Dex treatments. Interestingly in day 2 cultures, expression of GLUT 1 was approximately 25% higher in both MLP and control groups, in comparison to baseline. Baseline and

day 2 data though, were not statistically compared, but the increase in expression suggests that day 2 cells were undergoing a higher rate of hypertrophic growth. The reason for this however, is unknown. Conversely, Langdown *et al.* (2001), found that dexamethasone administered mid-gestation increased the expression of GLUT 1 in adult offspring. It was thought that this increase was not a direct cause of dexamethasone, but the hypertension that dexamethasone caused, possibly through altered Akt/protein kinase B pathways.

There was a clear theme throughout the whole study that baseline/day 0 cultures (10 days in culture) differed to day 2, 0 Dex cultures (12 days in culture). The only difference between the methods of culturing was the medium mixture used. All cultures were grown in a DMEM mix containing FBS, P/S and fungizone until 10 days in culture (baseline/day 0). Day 2 cultures then had DMEM added, containing either no dexamethasone or the two different concentrations. This is possibly the reason for the difference seen between the two groups, and as mentioned previously, it would be interesting in future studies to culture cardiomyocytes with and without FBS to identify if it affects proliferation rates and other parameters of interest.

Glucose transporter 4 (GLUT 4) expression was not affected by maternal protein restriction in the baseline cultures. Day 2 cultures were also not influenced by diet, but did respond to dexamethasone treatment. As the concentration of dexamethasone increased, the expression of GLUT 4 was decreased in both dietary groups. During early neonatal life GLUT 4 becomes the main glucose transporter, and is activated in response to insulin stimulation (Huang *et al.*, 2007). GLUT 4 expression is also known to be decreased during hypertrophy (Liao *et al.*, 2002, Santalucia *et al.*, 2004). Dexamethasone treatment resulted in the cultures expressing less GLUT 4, consistent with the idea that hypertrophy was occurring in these cultures. If cultures were grown for longer, increased expression may have been seen, because as cardiomyocytes mature, GLUT 4 may begin to be the main regulator of glucose uptake. The transition

from GLUT 1 to 4 becoming the main glucose transporter occurs during early postnatal life (Huang *et al.*, 2007), therefore in this current study cultures may not have reached the time of transition.

Looking at glucose transporter expression in relation to the insulin stimulated glucose uptake in day 2 cultures, GLUT 1 was unaffected by both diet and dexamethasone. GLUT 4 expression was decreased as dexamethasone concentration increased, but did not respond to maternal diet. It was expected that GLUT 4 expression would be increased with MLP diet and dexamethasone treatment, as it is the transporter which mediates uptake in response to insulin. Insulin signals lead to translocation of GLUT 4 to the plasma membrane permitting uptake of glucose into the cell (Shaohui *et al.*, 2007, Watson *et al.*, 2004). The reason for a decrease in expression could be because there was no insulin present in the cultures measured for GLUT 4. However, in the glucose uptake assay cells were incubated with insulin for 30 minutes. It would be interesting to investigate protein expression of the glucose transporters by western blot as there may be a difference in functional protein concentrations, which cannot be observed at the mRNA level.

From these findings glucose transporters may be ruled out as the mechanism underpinning the observed increase in insulin stimulated glucose uptake. Neither GLUT 1 nor 4 showed an increased sensitivity to dexamethasone due to maternal low protein diet. Future studies could investigate the effects of MLP diet *in utero* and glucocorticoids in culture on the insulin receptor or components of the insulin-signalling pathway, as insulin is the primary component that stimulates glucose uptake in the cell.

Ozanne *et al.* (1997, 2006) have investigated a panel of insulin signalling proteins in both rat and human adipose tissue. There are various components that make up the insulin signalling pathway and a few have been identified as important in determining

glucose transport and insulin resistance. These include insulin receptor substrate-1 (IRS-1) and protein-kinase C- ζ (PCK ζ also known as PRKCZ) thought to play an important role in mediating the action of insulin to stimulate glucose uptake. Two subunits of phosphatidylinositol (PI) 3-kinase, the regulatory subunit, P85 α , and the catalytic subunit, P110 β , are crucial in many metabolic activities of insulin, and are markers of insulin resistance. (Ozanne *et al.*, 1997, Fernandez-Twinn *et al.*, 2004, Ozanne *et al.*, 2006).

Ozanne *et al.* (2006) found that adult male humans of low birth weight had reduced GLUT 4, p85 α subunit, P110 β subunit and IRS-1 protein concentrations. There was no change in IR protein levels or mRNA levels. In rats fed MLP, adipocytes from three month old male offspring exhibited significantly higher basal and insulin-stimulated glucose uptake (Ozanne *et al.*, 1997). This was thought to be linked to a three-fold increase in insulin receptors, thereby altering glucose transport. They also showed an increase in IRS-1 associated and p85 associated PI3-kinase activity, all key components in the insulin signalling pathway (Ozanne *et al.*, 1997). In a similar study by Fernandez-Twinn *et al.* (2004), twenty-one month old rats displayed a decrease in PCK ζ expression in muscle, and p110 β associated p85 α expression in abdominal fat. These are downstream effects of IR in peripheral tissues and potentially markers for insulin resistance. Further investigation of the expression of these proteins in cardiomyocytes may be necessary to determine the mechanisms by which MLP and dexamethasone impact upon glucose uptake.

As the glucocorticoid receptor is the main receptor for glucocorticoids to bind to (Cole *et al.*, 1995), alteration in its expression could have lasting effects on GC regulation and sensitivity, and blood pressure (Lillicrop *et al.*, 2005). It was hypothesised in this study that GR expression would be programmed in offspring exposed to maternal protein restriction. Diet had no impact on the expression at either time point, which did not support the hypothesis. This also disagrees with evidence that suggests that

the MLP diet increases expression of GR in various tissues. Lillycrop *et al.* (2005) found that MLP diet fed during gestation increased GR methylation status in the offspring's liver at weaning. Langley-Evans *et al.* (1996) noted an increased GR binding capacity and receptor numbers in the hippocampus in offspring from protein deficient mothers, resulting in permanent alterations in the HPA axis. GR protein and mRNA expression was also increased in the peripheral tissues including kidney, liver and lung of MLP offspring (Bertram *et al.*, 2001). This was 2-fold higher in MLP compared to control in fetal and neonatal life, which increased in adult life to a 3-fold increase. Conversely, GR expression was decreased in the hypothalamus, and comparable to this study, no alteration of GR expression was found in the hearts of these offspring (Bertram *et al.*, 2001). The increase in peripheral tissue GR expression is likely to result in increased sensitivity of specific tissues to GC's, and decreased sensitivity in central tissues, which exhibits a decrease in expression.

Possible suggested mechanisms for altered GR and other glucocorticoid regulating receptors, such as MR, include the suggestion that MLP causes a decrease in the expression of 11 β -HSD-2. This enzyme, prominent in feto-placental tissue, converts active GC's to an inert form as they pass from mother to fetus (Benediktsson *et al.*, 1993). A decrease in 11 β -HSD-2 results in overexposure of the fetus to maternal GC's, which leads to increased sensitivity of the offspring to circulating glucocorticoids (Langley-Evans *et al.*, 1996). There are several reasons as to why MLP had no effect on the cardiomyocyte cultures in this study. As discussed, the heart is not a tissue where GR is influenced by MLP diet. It appears that GR is more abundantly expressed in tissues such as the liver and kidney than the heart. The heart is also not a major GC target tissue because of low expression of receptors. There is also a possibility that any *in vivo* effect of MLP has been lost after 10-12 days in culture.

Cultures treated with 100nM of dexamethasone had a higher expression of GR in comparison to untreated cells, while 10nM dexamethasone treated cells showed

intermediate expression. As GR is the main glucocorticoid receptor it was expected that its expression would be increased with dexamethasone treatment. This indicates that the cultures are responsive to glucocorticoids and are functioning as they would *in vivo*. Nyirenda *et al.* (1998) found administering dexamethasone to pregnant rats in the last week of pregnancy led to an increased expression of hepatic glucocorticoid receptor, but attenuated GR expression in hippocampal nuclei that mediate the HPA axis. Interestingly, when Dex was administered at any other time points in pregnancy it had no effect, demonstrating that GC's have specific effects at different time points of development. This suggests that GR expression is down-regulated in response to glucocorticoids in certain tissues and up-regulated in others.

In summary, the main findings from this study were that diet had no effect on maternal food intake, weight or litter size. Binucleation was considerably lower than mononucleated cardiomyocytes in both trials, indicating that all cultures were actively proliferating. Baseline binucleation was no different between dietary groups in either trial. Day 2 MLP cultures in trial one, however had an increased incidence of binucleated cardiomyocytes, suggesting that low protein diet increased the number of fully differentiated cardiomyocytes. The protein:live cell count ratio suggested that MLP diet decreased the size of cells in day 2 treated cultures, but increased in day 2 untreated, even though dexamethasone did not have a significant impact. High concentrations of dexamethasone increased insulin stimulated glucose uptake in neonatal cardiomyocytes, but glucose uptake transporters were not identified to be the mechanism behind this. Maternal low protein diet had no impact on GLUT 1, 4 or GR mRNA expression at the baseline level, but dexamethasone treatment decreased expression of GLUT 4 mRNA and increased expression of GR. These findings are consistent with *in vivo* work indicating that cultures show changes in structure and function of neonatal cardiomyocytes.

There were various limitations to this study, some discussed throughout this thesis. Cell counting was a crude method susceptible to human error. Future studies might want to explore different methods of cell counting that could give more replicates in order to gain the more accurate counts. De Vries *et al.* (2002) and Corstius *et al.* (2005), measured cell and nuclei number using histological techniques in heart tissue. It would also be interesting to measure neonatal ventricle wall and septum thickness, and chamber size to assess hypertrophy. As the baseline DNA content of the cardiomyocytes fell below the detection limit in the fluorescence method used in this study, using the bromodeoxyuridine (BrdU) cell proliferation method may be more sensitive to small amounts of DNA. One of the main lines of investigation for further study, as mentioned previously, would be to measure the expression of other genes and proteins involved in insulin stimulated glucose uptake, and to further explore MLP cultures sensitivity to administration of glucocorticoids neonatally.

It would also be interesting to grow cardiomyocytes for longer periods of time to investigate if hypertrophic-like cell characteristics continue in MLP cultures, and if there are windows of increased sensitivity to glucocorticoids. Also culturing cells for a shorter amount of time could highlight if effects of MLP that may be lost over time as cultures multiply. Cardiomyocytes could also be treated with other hormones, such as insulin, to monitor their effects and examine if MLP diet impacts on the cultures responses.

4.4 Conclusion

Cell proliferation and differentiation measures, glucose uptake and mRNA expression possibly indicate hypertrophic growth occurring prematurely in MLP cultures compared to control. Findings however, were not consistent enough across the two trials to

conclude this and will need further investigation to clarify. Findings from this study were varied so cannot fully support the work of Cheema *et al.* (2005), Tappia *et al.* (2005), Corstius *et al.* (2005) who concluded that MLP diet during gestation leads to remodelling of heart tissue, which is a precursor to the later onset of CVD. Clarification of the effects of MLP diet on heart remodelling is needed with further animal studies and optimisation of cardiomyocyte cultures. It is more likely that effects seen from MLP diet on the cardiomyocytes are due to glucocorticoid overexposure during fetal development.

The primary aim was to establish if cultured cells retain memory of fetal diet. To some extent cardiomyocytes replicated findings from *in vivo* studies, but it is difficult to interpret if cardiomyocytes retain memory of maternal undernutrition as findings were not consistent across trials. Many of the effects seen in offspring due to undernutrition during gestation are reliant on whole body integration of physiological and endocrine signals. In the case of hypertension and reduced nephron number it has been suggested that one impacts on the other causing a cycle to develop (Mackenzie *et al.* (1995), discussed in Harrison *et al.* (2008)). This effect would not be seen in culture.

The replacement, refinement and reduction of animals in biological research is a high priority from an ethical perspective. The development of alternatives to *in vivo* studies for the investigation of fetal programming was one of the hoped outcomes for this study, as cultures would allow for reduction of animal use. It is unlikely though that cell culture will become a viable alternative model to animals for the further investigation of fetal programming, but it is proving to be a useful tool for exploring focused mechanisms. This study has led to new paths of exploration into the mechanisms that underpin heart remodelling, and partially supported current findings, demonstrating the potential of using cell culture in the investigation of fetal programming.

5 References

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6 Appendix

Cell Culture Reagents

Medium Solution for growing Cardiomyocytes

500ml DMEM plus 50ml of fetal bovine serum, 5ml of Fungizone, and 5ml of penicillin-streptomycin. This was aliquoted and frozen at -20°C.

The following reagents were provided in the Worthington Neonatal Cardiomyocyte Isolation System kit.

Leibovitz L15 Medium

The sachet of medium was dissolved in 1 litre of autoclaved distilled water and filtered into sterile containers.

Trypsin (Vial #2)

Vial #2 was dissolved in 2ml CM-HBSS on ice.

Trypsin Inhibitor (Vial #3)

Vial #3 was dissolved in 1ml of CM-HBSS at room temperature.

Collegenase (Vial #4)

Vial #4 was dissolved in 5ml of filtered L15 medium at room temperature.

Dexamethasone

Stock Soutlion: 39.2mg dissolved in 1ml of ethanol (100mM).

Working Soutlions: 1µM: 5ml of 100µM solution dissolved in 500ml DMEM.

10nM: 1ml of 1µM solution dissolved in 100ml DMEM.

100nM: 50ul of 100µM solution dissolved in 50ml DMEM.

Endpoint Measurements Reagents

PBS

Stock (10x): 40g NaCl, 1g KCl, 7.2g Na₂HPO₄, 1.2g KH₂PO₄ in 500ml dH₂O.

Working (1x): 100ml of 10x PBS solution plus 900ml dH₂O.

Autoclaved.

Staining Cardiomyocytes

1mg/ml solution of Bisbenzamide H3385 (Hoechst stain) dissolved in dH₂O.

Cell Lysis Buffer for DNA and Bradford Assay

10mM Imidazole pH7.3, 0.5M NaCl, 1% Triton X-100, 2mM Sodium Azide, 0.2mM Sodium Ortho-vanadate, 0.2mM PMSF dissolved in 1 litre of distilled water, aliquoted and stored at -20°C.

Determination of Protein Content

Reagent: Dissolved 40mg of Coomassie Brilliant Blue (Sigma, UK) in 20ml Phosphoric Acid (FLUKA, UK) then added 40ml ethanol (Fisher Scientific, UK) made up to 500ml with distilled water (440ml) and mixed thoroughly. Solution was stored in the dark for 2 weeks to mature and remained stable for 2 months.

DNA Assay

10x TNE buffer: 10mM Tris HCl, 1mM EDTA, and 200mM NaCl in 100ml of dH₂O.

2x TNE buffer: 1:5 dilution of 10x TNE with dH₂O.

Bisbenzimidazole H33258 Dye

Stock: 1mg/ml with dH₂O

Working: 15µl of 1mg/ml stock dye plus 15ml of 2x TNE. Protect from the light.

Cell Death Kit

Reagent 1 Antihistone Biotin – Reconstituted in 450µl double distilled water for 10 minutes.

Reagent 2 Anti-DNA POD - Reconstituted in 450µl double distilled water for 10 minutes.

Reagent 3 Positive Control - Reconstituted in 450µl double distilled water for 10 minutes.

Immunoreagent For 10 tests – 720µl Incubation buffer, 40µl Reagent 1, 40µl Reagent 2.

Incubation buffer Background control.

Liver Homogenate for QC for Cell Death, DNA and Protein assays

DNA

Tissue (approximately 100mg) was homogenised in 1ml of cell lysis buffer, then aliquoted and frozen at -20°C.

Protein

Tissue (approximately 100mg) was homogenised in 1ml of cell lysis buffer, and diluted 1:20 with dH₂O, then aliquoted and frozen at -20°C.

Cell Death

Tissue (approximately 100mg) was homogenised in 1ml of lysis buffer provided in the kit, and diluted 1:20 with dH₂O, then aliquoted and frozen at -20°C.

Glucose Uptake Assay

Insulin

Stock: 10mg insulin dissolved in 1ml 1x PBS (1.73mM). Stored at 4°C.

Working: 1µl insulin stock in 9ml 1x PBS. Made fresh daily.

2-deoxyglucose (2-dG)

Stock: 1g dissolved in 60ml of 1x PBS. Stored at 4°C.

Working: 10µl stock 2dG in 10ml of 1x PBS (1:1000 dilution). Made fresh daily.

H3-2-DG Solution: 10µl H3-2-DG, 10µl working 2dG, 10ml 1x PBS. Made fresh daily.

Specific activity, 1.0TBq/mmol.

RNA extraction using the Qiagen RNeasy Mini Kit

70% ethanol was prepared using standard laboratory ethanol (Fisher Scientific, UK) and RNase free water (Sigma, UK).

10µl β-mercaptoethanol per 1ml Buffer RLT was prepared fresh daily.

Buffer RPE - Added 4 volumes of ethanol (Fisher Scientific, UK) on first use of the kit.

DNase Treatment Step

DNase I stock solution – Dissolved lyophilized DNase I in 550µl RNase free water (provided in the kit).

DNase incubation mix - 10µl of DNase I stock solution plus 70µl Buffer RDD per sample.