Cardiac birth defects caused by lifestyle and their potential prevention by nutritional molecules

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Abstract

Congenital heart defects are responsible for more neonatal deaths than any other birth defect. Although genetic and environmental factors play an important role, either separately or in combination (multifactorial), still the cause in most cases remains unknown.

Changing life styles, e.g. exposure of the mother to excessive alcohol, nicotine in tobacco smoke, easily available traditional and, over the counter medicines and environmental contaminants could be possible causes of congenital malformations. Maternal diseases like diabetes mellitus are also one of the etiological factors for developmental defects. Several developmental genes, for instance connexin 43; one of the key proteins involved in cardiovascular development, and endothelin 1; another important gene required in many developmental processes, could be responsible for developmental anomalies of the heart. Supplementation with micronutrients such as folic acid and Vitamin C during the periconceptional period has been shown to prevent some neural tube and congenital heart defects. This study was aimed at evaluating the adverse effects of ethanol, retinoic acid, nicotine, cadmium chloride, sodium fluoride, ginseng and diabetic conditions on chick cardiomyocytes cultured in the micromass system, and examining the potential protective effects of folic acid and vitamin C. Also teratogenic effects of some of the teratogens, ethanol, nicotine, retinoic acid and diabetic conditions, were examined using in ovo culture.

Hearts were dissected from 5 day old White Leghorn chick embryos and the cells were isolated and cultured. They were exposed to different

concentrations of test chemicals. Folic acid and vitamin C were added to see any protective effects. Cell viability was assessed using the resazurin reduction assay and the kenacid blue assay was performed for determining cell number. For in ovo culture, day 3 chick embryos were injected with ethanol, nicotine, retinoic acid or diabetic molecules or a combination of teratogenic chemicals and vitamins (folic acid and vitamin C). Immunohistochemistry and western blotting were employed to detect the expression of connexin 43 and endothelin 1. Results of micromass culture revealed that ethanol, retinoic acid, nicotine, cadmium chloride and diabetic conditions dramatically reduced cellular differentiation, cell viability and protein content in a dose dependant manner. However, vitamin C (100µM) and folic acid (1mM) administered concurrently with these chemicals, except for cadmium chloride, could significantly improve all parameters such that the values were comparable with the control. Nicotine had no effect on cell viability and protein content, but cell beating was significantly affected. This effect was reversed by the addition of Vitamin C and folic acid. Results of in ovo culture showed that ethanol and diabetic conditions caused gross and histological malformations in chick embryos. However their effects were abrogated with supplemental folic acid and vitamin C. Immunohistochemical and western blotting results demonstrated a decreased expression of Cx43 and endothelin 1 in ethanol, retinoic acid, nicotine and diabetic condition treated cells while addition of vitamins restored their expression so they were comparable to controls.

It may be that environmentally induced teratogenic effects on heart development could be prevented by supplementation with Vitamin C and folic acid during pregnancy.

Publications

Memon, S., and Pratten, M. K. (2009). Developmental toxicity of ethanol in chick heart in ovo and in micromass culture can be prevented by addition of vitamin C and folic acid. *Reproductive Toxicology* **28**, 262-269.

Memon, S., and Pratten, M. K. (2009). Cardiac Birth Defects Caused by Lifestyle and Their Potential Prevention by Nutritional Molecules. *FASEB J.* **23**, 642.641 (Poster presented at Experimental Biology Annual Meeting held in NewOrleans, USA. 18-22 April, 2009).

Memon, S., Pratten, MK. (2008) The potential of folic acid to prevent retinoic acid induced cardiac defects: an evaluation in chick cardiac micromass culture *J Anat*. 212(1): 80–96. (Poster presented at Annual Meeting of ASGBI, Durham, UK. 3-5 July, 2007).

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Dr Samreen Memon
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Dedication

This thesis is dedicated to my beloved daddy **Prof. Rasool Bux Memon,** who left me alone in this world when I was very young but his teachings and sayings made me promise that I would complete my studies no matter what the circumstances would be, and I have kept my promise. All of this work is for him. I love you and miss you, daddy.

Prof. Dr Rasool Bux Memon

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Abbreviations

APES	3-Aminopropyltreethxysilane
BSA	Bovine serum albumin
cAMP	Cyclic AMP
Cx	Connexin
Da	Daltons
DMEM	Dubeco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DAPI	4', 6-diamidino-2-phenylindole
ECE	Endothelin converting enzyme
ECE1	Endothelin converting enzyme 1
ECE 2	Endothelin converting enzyme 2
Ednra	Endothelin type A receptor
EDTA	Ethylene diamine tetra acetate
ES	Embryonic stem cells
EST	Embryonic stem cell test
ET-1	Endothelin 1
ET-2	Endothelin 2
ET-3	Endothelin 3
ET_A	Endothelin type A receptor
ET_B	Endothelin type B receptor
FITC	Fluorescein isothiocyanate
FSG	Fish skin gelatine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HB	Hindbrain

HBSS	Hank’s balanced salt solution
H & E	Haematoxylin and Eosin
HH	Hamburger and Hamilton
H	Heart
CH1	Anti tropomyosin antibody
DPX	Mounting medium
IgG	Immunoglobulin G
INVITOX	*In vitro* techniques in toxicology
LIF	Leukemia inhibitory factor
MB	Midbrain
MM	Micromass
NGS	Normal goat serum
NaCl	Sodium chloride
Na_2Co_3	Sodium bicarbonate
NaF	Sodium fluoride
NaOH	Sodium hydroxide
NaK tartrate	Sodium potassium tartrate
NTD	Neural tube defects
PBS	Phosphate buffered saline
PKA	Protein kinase A
PKC	Protein kinase C
RA	Retinoic acid
RARs	Retinoic acid A receptors
RXRs	Retinoic acid X receptors
RARE	Retinoic acid response element

SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBST	Tris Buffered Saline with Tween 20
TPA	12-O-tetradecanoylphorbol-13-acetate
TRITC	Tetramethylrhodamine isothiocyanate
VC	Vitamin C

Chapter 1

General Introduction

1. Introduction

This project is focused around the influence of environmental factors and maternal diseases on pregnancy outcome and also the protection of embryonic development, which may be brought about by factors such as proper nutrition and supplementation with vitamins.

Congenital anomalies, birth defects and congenital malformations are the terms currently used to describe developmental disorders present at birth (Moore & Persaud, 1998). These anomalies are the leading cause of infant mortality. An estimated 495,000 infants die every year worldwide due to congenital defects (Rosano *et al.*, 2000; Dolk, 2005).

Out of all the congenital defects 60-70% are of unknown cause, 20% are because of genetic transmission, 3-5% from chromosomal anomalies and the remaining 3-4% are due to chemicals and drugs (Giavini *et al.*, 1994). However, many common congenital anomalies are thought to be caused by genetic and environmental factors adding together as multifactorial inheritance (Moore & Persaud, 1998).

Environmental agents are only responsible for a small group of birth defects reported but are also the most amenable to control. For this reason much research has been focused on environmental agents as a cause of congenital defects (Steel & Copping, 1993).

Maternal nutrition (both micro and macro nutrients) plays a vital role in normal growth and development of the fetus. These micro and macro nutrients are critical for a woman to maintain a normal pregnancy with good pregnancy outcome. A woman's nutritional status is prejudiced by

several factors, including genetics, lifestyle habits, underlying diseases or exposure to toxic drugs (Keen CL *et al.*, 2003).

Various studies around the world show a direct impact of environmental insults on the nutritional status of pregnant women. Studies on the Dutch wartime famine showed an increased risk of early abortions and fetal miscarriages, intrauterine growth retardations, and congenital malformations (Smith, 1947). Several women of child-bearing age in both the developing and developed world do not maintain a healthy diet pre, peri and post conceptionally. There are many reasons for taking in less than the recommended diet during pregnancy, and these include lack of knowledge or lack of access to a high quality diet (Mehta, 2008). Moreover, a number of studies have also shown that most women of reproductive age are not attaining adequate vitamins A, C, B6, and E, folic acid, calcium, iron, zinc, and magnesium levels in their diet (de Weerd S *et al.*, 2003; Yang QH *et al.*, 2007). Although the precise mechanisms of how these micronutrients can influence pregnancy outcomes are not entirely understood, some of the likely mechanisms include an improvement in the mother's immune system, energy metabolism, anabolic processes, ability to respond to stress, and hemoglobin levels etc. All these effects in turn affect the incidence of preterm birth, fetal intrauterine growth restriction, congenital malformations and fetal growth retardation (Susser, 1991; Ladipo, 2000; Keen CL *et al.*, 2003; Fawzi *et al.*, 2007).

1.1. Teratogens and teratology

A teratogen is any substance, agent or process that induces developmental abnormalities in the embryo/fetus as defined by the Oxford Medical Dictionary. The effect produced by a teratogen depends on the stage at which the embryo/fetus is exposed to it, the most vulnerable period is the period of organogenesis (Brown & Fabro, 1983).

Teratology is the study of abnormal prenatal development and congenital malformations induced by exogenous chemicals or physical agents (Bailey *et al.*, 2005). Although only some 10% of all congenital defects are caused by teratogens (Brent, 1995), those teratogens compromise the quality of life for millions of people worldwide and cost billions of dollars in health care every year (Bailey *et al.*, 2005).

1.2. History and principles of teratology

It was in the 1920s and 1930s when teratology was born as a science, with the birth of malformed piglets, especially with a lack of eyes, from mothers fed an experimental diet low in vitamin A and high in fat (Bailey *et al.*, 2005). Subsequent evidence came to light over the next two decades with the recognition in 1941 of congenital defects in children born to mothers who had suffered from rubella virus infection, and a little later with environmental contamination with mercury in 1956 (Igata, 1993). Then in 1961, thalidomide, a sedative and anti-emetic, was identified as a potent teratogen to embryos which caused minimal or no toxicity to adults (Bailey *et al.*, 2005).

It was following the revelation of thalidomide as a teratogen, that the study of teratogens increased and more guidelines were introduced regarding environmental agents, considered safe for fetal exposure.

Since then many other teratogens including drugs, infectious diseases, radiation and maternal factors have been identified by various experimental studies (Moore & Persaud, 1998).

A number of basic principles and concepts applicable to teratology research have been formulated. Six main principles have been outlined by Wilson on which the study of teratogens should be based (Bailey *et al.*, 2005).

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with environmental factors.
2. Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure.
3. Teratogenic agents act in specific mechanisms on developing cells and tissues to initiate abnormal embryogenesis.
4. The final manifestations of abnormal development are malformation, growth retardation, death and functional disorder.
5. The access of adverse environmental influences to developing tissues depends on the nature of the influences.
6. Manifestations of deviant development increase in degree as dosage increases from the no effect to totally lethal.

1.3. Teratogenicity testing

Teratogenicity testing of different toxins, chemicals and drugs, which pregnant mothers come across in everyday life, is very essential. Screening of new chemicals with potential toxicity would allow medical experts to help pregnant women in avoiding direct contact with these potentially hazardous substances. Currently most teratogenicity assays utilize in vivo animal studies to attain the goal of detecting chemical hazards (Bailey *et al.*, 2005).

1.3.1. In vivo tests

In vivo studies give the preliminary guidelines on whether a drug or chemical causes any teratogenicity during pregnancy. A range of doses is usually administered through the most appropriate route, to pregnant animals during the period of organogenesis, and the results compared to untreated animals. The standard sample size in most of the cases is 20 or more pregnant females per dose, with a range of doses from highest to lowest; in such a way that the highest dose only causes some signs of maternal toxicity and lowest dose has no toxicity in mothers.

The most common species used are rats, mice and rabbits. Although the rodents are the species of choice in teratology research, one should consider many physiological and biochemical differences between the animal species used in teratology research and humans, and it is not surprising that no one species can be shown to be the perfect experimental animal for developmental toxicity studies. None of the species absorb, metabolise and eliminate test substances like humans and none of them

have the same placental transfer properties. Despite these liabilities, rodents have become the most commonly used species for evaluating potential human teratogens. Because of the intrinsic problems and inadequacies of teratology testing with the animals most commonly used (mouse, rat, rabbit, hamster, and monkey), scientists have tried to integrate more species into their experiments in order to find one that fulfils the criteria of an ideal animal. Dogs, cats, pigs and non-human primates were tested with a selection of known teratogens, but none of them proved to be the appropriate animal for developmental toxicity testing.

Several inter-species differences including; anatomical differences, metabolic variations, response to potential teratogens, sensitivity to environmental conditions, route of administration and vehicles used, must be considered, when designing animal developmental toxicology studies and extrapolating to humans (Pratt *et al.*, 1982).

1.3.2. *In vitro* alternatives to animal studies

In recent years, scientists started using *in vitro* methods to overcome the intrinsic problems and differences in animal teratology studies. These methods are now well established and invaluable for conducting these studies, and are very useful for the screening of chemicals (Brown *et al.*, 1995). The *in vitro* tests are less expensive, quicker, and much more reproducible. There is now an absolute need for alternatives to conventional animal-based methods due to the fact that every year hundreds of drugs are introduced to the market and pregnant women are exposed to thousands of toxic substances in everyday life (Bournias-

Vardiabasis & Teplitz, 1982). The European Union (EU) white paper published in 2001 suggested the organization of testing requirements for approximately 30,000 chemicals marketed before September 1981 (Registration, Evaluation and Authorization of Chemicals, 'REACH') (Hartung *et al.*, 2003). If animal-based methodology were applied, this attempt would require the use and sacrifice of an estimated 12.8 million animals at a cost of 8.68 billion Euros over a period of 45 years (Hartung *et al.*, 2003). Therefore, there can no longer be any justified resistance to the development and adoption of alternative teratology methods. A wide range of alternatives have been investigated, with a potential to follow the "Three Rs" (Reduction, Refinement and Replacement) principle in order to minimize and eventually replace animal models with non-animal alternatives without compromising high quality science (Russell & Burch, 1959). There are many available *in vitro* tests, e.g. hydra regeneration assay, the frog embryo teratogenesis assay (FETAX), drosophila assay, which detect the developmental and reproductive toxicity in mammals and other primates. Out of all the *in vitro* tests, three are validated as embryo toxicity assays by the ECVAM advisory committee (ESAC). These are the embryonic stem cell test, the micro mass test and the whole embryo culture test (Genschow *et al.*, 2000; Brown, 2002; Genschow *et al.*, 2002).

1.3.2.1. The Hydra Regeneration Assay

Johnson and his colleagues proposed an assay using lower organisms such as Hydra for the detection of teratogenic substances for the first time. Since then these organisms have been used by many scientists in a

modified manner to test for potential teratogenic effects (Johnson & Gabel, 1983). Hydra are usually 2 to 25 mm in height and live in fresh or salt water. The most common way of reproduction is vegetative, with the ability to totally regenerate the body from parts or cells. Hydra attenuata is readily available and can be maintained in fresh water in a laboratory. Their population doubles every 4 days under favourable temperature conditions. The artificial embryos generated from the budding of adult hydra exhibit the same features as mammalian embryos such as proliferation and regression of cells, creation of organ fields, initiation of competence, pattern configuration, metabolic changes, organogenesis, and well-designed ontogeny. This assay can detect the teratogenic potential of many chemicals except those which are water insoluble and contain copper. Also this should not be considered a reliable assay for the evaluation of toxicity risk of any compound, due to its single end point (Wilby & Tesh, 1990).

1.3.2.2. The Drosophila Test

The fruit flies or *Drosophila melanogaster*, had been employed by scientists for mutagenicity studies since many years. The *drosophila* is also used for detection of possible teratogens. Initial experiments were performed to detect the toxicity of vinblastine, thymidine, deoxycytidine and 5-bromodeoxyuridine in the 1970s. Later on several other chemicals were studied by Schular et al. The male and female adult fruit flies are anaesthetized and positioned in vials; containing distilled water and instant Drosophila medium. The chemicals to be tested are added to the distilled

water, and the medium. The flies are allowed to mate and lay eggs. The larvae of the eggs are exposed to the maximum tolerated dose of chemicals to be tested. The time required for the adults to hatch from larvae is about 9 or 10 days. The adults are then examined within 16 hr of coming out from the puparium. The adult flies and independent body parts are morphologically examined and scored in terms of shape, colour, body alignment, and growth and missing parts. At least 200 flies per dose level are scored.

The *Drosophila* system has shown a great degree of similarity not only with *in vivo* animal studies but also with human epidemiological data; furthermore, the vast and detailed knowledge of *Drosophila* molecular biology could potentially be of use in the derivation of teratogenic mechanisms (Bournias-Vardiabasis *et al.*, 1983). Although this test is not very expensive and time consuming, results of this test rely on morphological scoring only, which could be biased and depends on expertise (Bournias-Vardiabasis & Teplitz, 1982; Bournais-Vardiabasis, 1994).

1.3.2.3. The Frog Embryo Teratogenesis Assay (FETAX)

Amphibian embryos, for instance toads and frogs, have been utilized in developmental toxicity assays since the 1970s. A teratogenic screening assay (FETAX), using the South African clawed frog (*Xenopus laevis*), was developed by Dumont and colleagues (Dumont *et al.*, 1982). FETAX is a 96-hour whole embryo *in vitro* test which is based on the fact that the initial 96 hours of *Xenopus* development are equivalent to many of the

major developmental processes of human organogenesis. ECVAM approved this test as the only non-avian (non-mammalian) vertebrate system which was worth pursuing for teratogenicity screening. The inherent metabolic deficiencies of the frog are overcome by addition of an external metabolic activation system. This would further increase the specificity and accuracy of the FETAX system. The principal endpoints include mortality, malformations and growth inhibition. Using these endpoints to assess the teratogenic effects of up to 90 substances, FETAX proved to be more accurate, more specific, and more predictive in terms of results when compared to human teratology data than other animal teratology data. Although this method has been used for screening hazardous chemicals rather than directly replacing animal teratogenecity studies, this method has advantages of being time and cost effective (Dawson *et al.*, 1989). Dawson and his colleagues tested water samples from abandoned lead and zinc mines with FETAX. It was concluded that it was a good test for screening environmental substances that cause birth defects (Dawson *et al.*, 1989). FETAX has shown to be as reproducible and reliable an assay for screening of environmental toxins which cause birth defects, as many other bioassays (Bantle *et al.*, 1994). Further more in the ICCVAM held in May 2000 the FETAX system was particularly discussed. The results of this meeting concluded that as a teratogenesis assay, FETAX is not sufficiently validated to be used for regulatory applications (FETAX Expert Panel Meeting Summary Minutes, 2000).

1.3.2.4. Whole Embryo Culture (WEC)

Whole embryo culture is a teratogen screening assay, utilizing whole embryos (mouse, rat, and rabbit) for a short duration from fertilization to the end of period of organ formation (Sadler *et al.*, 1982; Schmid, 1985; Ninomiya *et al.*, 1993). Usually the test involves the dissection of embryos at the head-fold or early somite stage away from maternal tissue. The embryos are then cultured in an appropriate medium for 1 or 2 days. This test is difficult and lengthy as compared to other validated tests (micromass and embryonic stem cells) due to the number of factors involved, which need to be considered (Genschow *et al.*, 2002). This test is advantageous due to the fact that it determines potential toxicity of chemicals in embryos without any maternal factors and test chemicals can be applied at different times during the culture period. However there are some obstacles to undertaking this system; first, at least ten to twelve pregnant mothers are required for each time; second, it is also complex and needs technical expertise to get reproducible results; third, it is very costly.

1.3.2.5. Embryonic Stem Cell Test (EST)

In the Embryonic stem cell test (EST), the teratogenic potential of different chemicals is assessed in two permanent murine cell lines embryonic stem (ES) cells known as D3 cells represent embryonic tissue, and 3T3 fibroblast cells represent adult tissue (Spielmann *et al.*, 1997; Scholz *et al.*, 1998; Genschow *et al.*, 2000). D3 Embryonic stem cells can differentiate into a variety of cell types when cultured in vitro. The embryonic stem cells are maintained in an undifferentiated stage in the presence of

leukaemia inhibiting factor (LIF), and in order to differentiate into cardiomyocytes these cells must be cultured without LIF. The cultured cells are then exposed to a series of different concentrations of the potentially embryotoxic substance. The endpoints are assessed after 10 days of culture: the inhibition of cell differentiation, the inhibition of cell growth. The results of the values derived from endpoints, at which there is inhibition of 50% of differentiation and growth, are then used to classify substances as not embryotoxic, weakly embryotoxic, and strongly embryotoxic. This test when applied to detect potential toxicity of substances in a validation study proved to be accurate, precise and predictable when compared to *in vivo* studies. The EST seems to be promising, as a pre-screening method and also a useful replacement for animal-based techniques, as it has achieved a '3Rs' success by greatly reducing the numbers of animals involved and bypassing a need for the use of pregnant animals to provide tissue for *in vitro* culture, and will still provide useful and applicable information for human teratology assessments (Spielmann *et al.*, 1997).

1.3.2.6. The Micro-mass test (MM)

The Micromass system involves the culture of primary cells, isolated either from the mesencephalon, heart or the limb buds of developing embryos, plated at high density. The most important mechanisms of development including cell–cell communication, cell proliferation, cell–matrix interactions and differentiation, are found in micromass cultures. Due to these reasons, this system has been proposed and prevalidated as a

potential screening assay for teratogen detection (Spielmann *et al.*, 2001). The basic principle of assay is the potential of teratogens to disrupt normal differentiation of primary embryonic cells in vitro. Formation of neuronal tissues, chondrocytes or contractile cardiomyocytes is the basis of the endpoints used along with cell viability measurements. Several species have been used for micromass cultures: rat (Flint & Orton, 1984; Parsons *et al.*, 1990; Tsuchiya *et al.*, 1991); mouse (Tsuchiya *et al.*, 1991; Doyle & Kapron, 2002), and chick (Wiger *et al.*, 1988; Meyer *et al.*, 2001; L'Huillier *et al.*, 2002; Hurst *et al.*, 2007). The MM rat limb bud test has been subjected to extensive validation studies. The outcome of the validation study conducting on rat limb bud cells (INVITTOX protocol 114) demonstrated that results were reproducible and the embryotoxicity potential could easily be categorised into non-embryotoxic, weakly embryotoxic and strongly embryotoxic (Genschow *et al.*, 2002).

1.3.2.7. Chick micromass assay

The chick MM assay utilizes primary cells obtained from midbrain, limbs or heart of chick embryos. This assay is superior to mammalian assays because mammalian assays are time consuming, require animal housing and maintenance and mating to get the embryos at the required age. Also for the mammalian micromass assay, mothers have to be sacrificed in order to remove the embryos and therefore this requires a Home office licence in UK. On the other hand with chick embryos there is no need to sacrifice the mother. Moreover the development of embryos can be postponed by storing eggs at 12^{o}C. In order to assess early developmental defects, and to

get the maximum tissue from each embryo, cells could be collected from three different systems, limb bud, mid brain and heart at embryonic day 5. The organs at day 5 have still the ability to differentiate and can be clearly identified (L'Huillier *et al.*, 2002; Hurst *et al.*, 2007). Once the cells are cultured in high density they can then be exposed to the test chemicals in replicates and observed for cytotoxic effects by the use of relevant endpoints, i.e. differentiation, and cytotoxicity assays (Atterwill *et al.*, 1992).

1.4. Development of the Human heart

The heart is the first organ in the human embryo to develop and function as a circulating system (Sakabe *et al.*, 2005). During the third week of development, the heart forming mesodermal cells become apparent (Moorman *et al.*, 2003). The heart begins to function at the start of 4th week (Moore & Persaud, 1998).

1.4.1. Origin of Heart forming cells

The ovoid human embryonic plate is formed at the union of the yolk sac and amniotic cavity. A streak called the primitive streak, with a node on its cranial end, is present in the midline on the dorsal aspect of embryo. Through this streak, cells migrate from the outermost layer by the process of gastrulation to form the three germ layers of the embryo proper (the ectoderm, the mesoderm, and the endoderm). It is the mesodermal layer which gives rise to cardiac cells (Moorman *et al.*, 2003), as shown in fig 1.1.

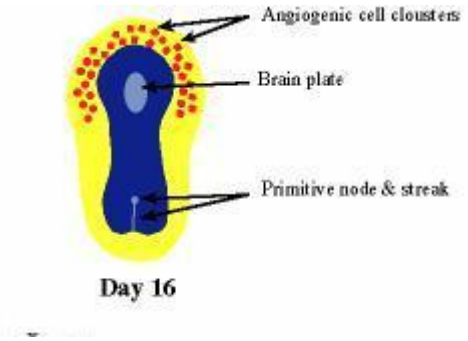

Fig.1.1. Horseshoe-shaped cluster of angiogenic cells lying lateral and anterior to the brain plate
(http://www.luc.edu/faculty/wwasser/dev/heart.htm).

1.4.2. Establishment of cardiac fields

Several studies have shown that the heart forms from two separate heart fields, which segregate from a common progenitor at gastrulation. The earliest cardiac progenitors, called the primary heart field, originate in the anterior splanchnic mesoderm and give rise to the cardiac crescent that ultimately contributes to the left ventricle and atria. An additional source of cardiac precursors, the secondary or anterior heart field, is derived from pharyngeal mesoderm, located medial to the primary heart field. Cells from the secondary heart field form the right ventricle and outflow tract of the heart (Garry & Olson, 2006).

1.4.3. Formation of Heart tube

The heart primordium first becomes evident at day 18 of development (Moore & Persaud, 1998). On day 19, paired vascular elements called endocardial tubes begin to develop from splanchnic mesoderm in the cardiogenic region, which is located cranial and lateral to the neural plate. Later the lateral folding of the embryo brings these tubes into the midline, where they fuse to form a single heart tube (Larsen, 1997) as shown in fig

1.2. At this stage the heart tube consists of a thin endothelial tube, surrounded by cardiac jelly which separates it from the primitive myocardium.

The endothelial tube becomes the future endocardium, whereas primordial myocardium becomes myocardium and the epicardium is derived from mesothelial cells of the external surface of the sinus venosus. Beating starts at the 22nd-23rd day and blood flows through it during the 4th week of development. With craniocaudal folding of embryo, the heart tube moves to lie anterior to the foregut and caudal to the oropharyngeal membrane (Moore & Persaud, 1998).

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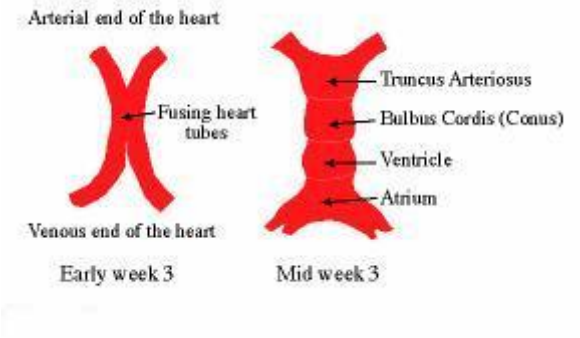


Fig.1.2. Showing the **two endocardial tubes** fuse along the embryonic midline, and a single tubular heart, develops many constrictions outlining future structures. (http://www.luc.edu/faculty/wwasser/dev/heart.htm).

1.4.4. Looping of heart tube

With the addition of new cells to the cranial and caudal poles of the primitive heart tube from the secondary heart field and with the proliferation of myocardial cells, the heart tube begins to elongate. Concurrent to this change the dorsal myocardium, which initially joined the left ventricle to the dorsal mediastinum undergoes degeneration and liberates a large portion of the tube (Moorman *et al.*, 2003; Dunwoodie,

2007). Once released, the dorsal heart tube bends towards the right side to form a d shaped heart. At this moment the presumptive heart segments begin to appear as truncus arteriosus, bulbus cordis, ventricle, atrium and sinus venosus (Moore & Persaud, 1998; Sakabe *et al.*, 2005).

Once the heart segments become specified as shown in fig 1.3, some of the endothelial cells in the region of the atrioventricular and outflow tract change their phenotype to mesenchymal cells and migrate into the adjacent cardiac jelly, where they form the endocardial cushion tissue, primordia of valves and septa of the adult heart. Along with these events, another population of mesenchymal cells from the cardiac neural crest cells move towards the outflow tract via the pharyngeal arches, where they, along with endocardial derived mesenchyme, contribute to outflow tract septation, including formation of the conus septum, aorticopulmonary septum and semilunar valves (Sakabe *et al.*, 2005).

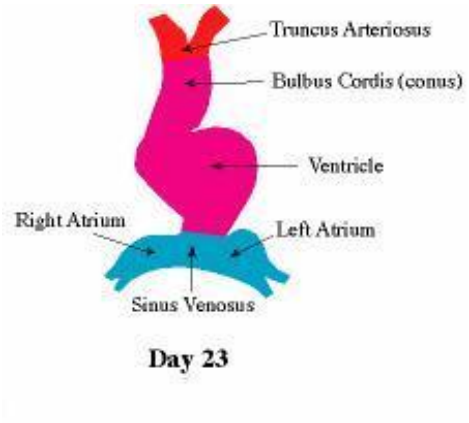


Fig.1.3. The heart tube will bend ventrally, caudally and slightly to the right and show various parts (http://www.luc.edu/faculty/wwasser/dev/heart.htm).

1.4.5. Formation and separation of the cardiac chambers.

Chamber formation is first evident during looping of the heart tube, as they balloon out as discrete portions of the heart tube (Dunwoodie, 2007). Partitioning of the primitive atrium, ventricle and atrioventricular (AV) canal begins in the middle of the third week and is completed by end of the fifth week of development.

Division of the primary AV canal is facilitated by fusion of the endocardial cushions which act as AV valves, while the atrium is divided in to two halves by means of two septa, the septum primum and septum secundum. Similarly the primordial ventricle is separated into two halves by the interventricular septum, which consists of a muscular part, which starts near the apex of the ventricle and later is completed by fusion of the enlarged medial walls of the ventricle, and a membranous part, which is derived from the right side of the endocardial tubes and merges with the muscular part. Until the 7th week of development a crescent shaped defect is present between the IV septum and endocardial cushions, which is closed by fusion of the right bulbar ridge, left bulbar ridge and endocardial cushions.

With further development of the heart, the sinus venosus which initially acts as a separate chamber and opens in to the right atrium, shows enormous changes. Its left horn becomes smaller and forms the coronary sinus and its right horn undergoes enormous enlargement and receives all the blood from the superior and inferior vena- cavae and is incorporated in the wall of the right atrium as mentioned in fig 1.4 and 1.5.

1. Venous valves
2. Septum primum
3. Ostium secundum
4. Septum secundum
5. Pulmonary veins
6. Endocardial cushions
7. Interventricular foramen
8. Muscular interventricular septum
9. Foramen ovale

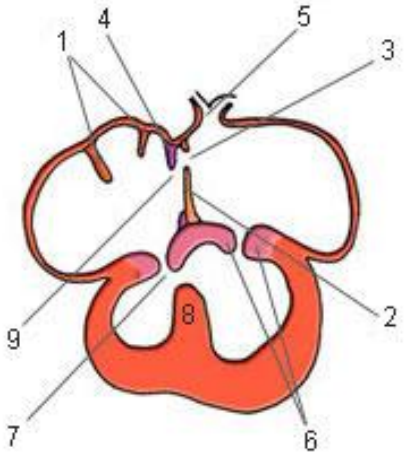


Fig.1.4: http://embryo.chronolab.com/

The primordial pulmonary vein, which develops as an outgrowth from the dorsal wall of the left atrium, forms most of smooth wall, of the left atrium. With more development of the atria, the primordial pulmonary vein and its main branches are incorporated into the wall of the left atrium, which results in the formation of four pulmonary veins (Larsen, 1997; Moore & Persaud, 1998).

1. Superior vena cava
2. Pulmonary veins
3. Left atrium
4. Right atrium
5. Septum primum
6. Septum secundum
7. Primitive left atrium
8. Primitive right atrium
9. Valve of inferior vena cava
10. Valve of coronary sinus
11. Sinus venarum

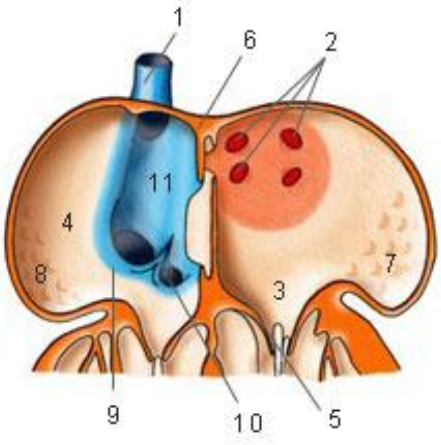


Fig.1.5:http://embryo.chronolabc

1.4.6. Conduction system of the heart

The cardiac conduction system (CCS) is responsible for the generation and timely transmission of electrical impulses, necessary for a coordinated

contraction produced by the myocardium. This system consists of several functional subunits and includes, the sinoatrial node (SA node), which is responsible for generating electrical impulses, the AV node, causing a delay in the propagation of generated impulses at the atrioventricular junction, and the bundle of His (Purkinje system), by which the impulses rapidly spread throughout the ventricles.

When the primitive heart starts beating, all the myocytes have the ability to generate impulses which are of the same velocity and transmitted amongst them by means of gap junctions. These impulses propagate through the entire heart in a very well organized way, started at posterior wall of right atrium and reaching to the outflow tract.

As the development of the heart progresses, especially when looping begins, three main changes become apparent in cardiac electrical activity.

1. The velocity of the generated impulse becomes significantly slowed at the atrioventricular junction.

2. Any direct electrical coupling through myocyte-myocyte gap junctions is disassociated with the formation of the ventricular septum.

3. During separation of the ventricles, an obvious shift in the direction of impulse is observed; the impulse which previously ran from apex to base now runs in an opposite direction.

The above mentioned changes are necessary for effective pumping by the heart. It is evident from several studies that CCS develops from myocardium and it has no neurogenic contributions (Gourdie *et al.*, 2003).

Several signalling and transcriptional factors have been implicated during the development of CCS. Signalling factors include endothelins,

neuregulins, Notch and Wnt, while MSX, NKX, Hop, Tbx and GATA gene family have been detected as transcriptional factors important during the development of CCS (Mikawa & Hurtado, 2007).

1.4.7. Molecular cascades during development of heart.

The fate of cardiac cells depends upon a number of positive and negative regulatory signals that are released either from surrounding tissues or from the heart forming region itself. As the cardiac progenitors lie between the endoderm anteriorly and the epidermis posterior to it, signals from these regions, in conjunction with signals from the anterior lateral mesoderm itself, promote cardiac specification, while signals from axial tissues and the neural plate repress heart formation in the head region.

The signalling systems implicated in mammals for cardiac induction include, Hedgehog, BMP, FGF, Wnt-JNK as positive influences, while inhibitory signals include Wnt ligands expressed in the dorsal neural tube, (Wnt-1, Wnt-3a) and anti-BMPs expressed in axial tissues as shown in fig 1.6.

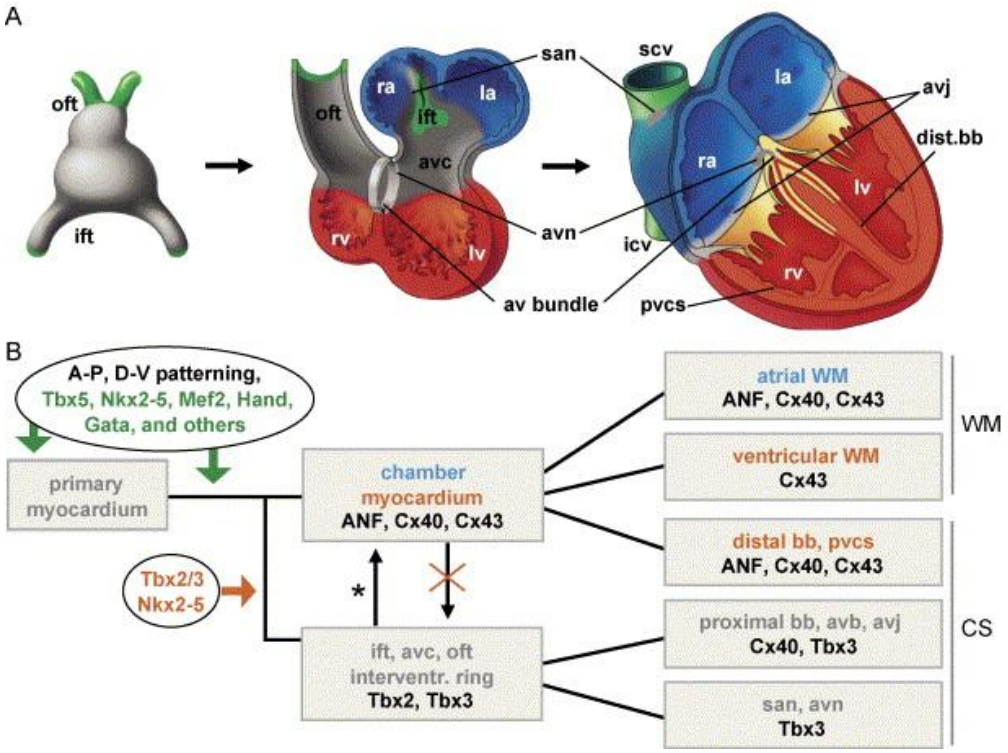


Figure1.6: Development of the components of the heart. (**A**) The primary myocardium/nodal myocardium is in *gray*, and the chambers and ventricular conduction system are in *red* and *blue*, respectively. The mesenchyme at the connections between the heart and the body is shown in *green*. (**B**) Scheme showing the developmental relationships between components of the heart, many transcription factors are required to form and pattern the primary myocardium and, subsequently, to effect the localized formation of ventricles (cranioventral) and atria (dorsocaudal). A subpopulation of the primary myocardium retains its phenotype by the action of Tbx2/3/Nkx2-5. Cells of this pool may subsequently differentiate to chamber myocardium (*). The nodal components of the conduction system are formed from the primary myocardium, whereas the working myocardial chambers and ventricular conduction system are formed from the early chamber myocardium. Marker genes expressed in each of the components are indicated. avb, atrioventricular bundle; avj, atrioventricular junction; avn, atrioventricular node; bb, bundle branches; pvcs, periventricular conduction system (Purkinje); san, sinoatrial node; s(i)cv, superior (inferior) caval vein (Christoffels *et al.*, 2004), ***reproduced with permission***).

The specification of cardiac chambers is expressed using transcriptional regulators like Hand1, Cited1, and Irx1/Irx3/Irx5, hormones like atrial naturetic factor, conduction proteins of the connexin family and the muscle specific cytoskeletal protein, Chisel. These markers are expressed at the outer curvature of the heart tube. Other factors in chamber specification include Tbx5, Tbx20, which promote specification of the chamber myocardium, while Tbx2 and Tbx3 act to inhibit these factors in order to maintain the non chamber character of the inner curvature of the heart as shown in fig 1.7.

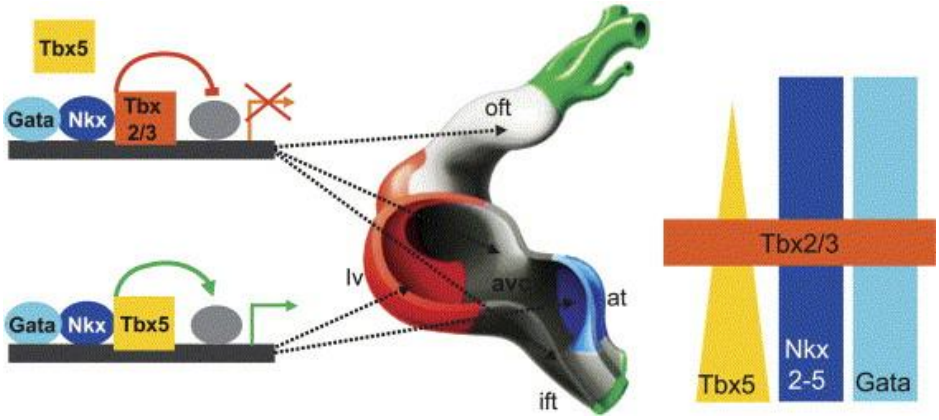


Fig.1.7: Model for the mechanism of transcriptional control of chamber and non-chamber genes. Broadly expressed transcription factors Nkx2-5, Tbx5 and Gata4, cooperatively activate gene expression in the chambers. Locally expressed Tbx2 and Tbx3 compete with Tbx5 for DNA binding, and for Nkx2-5. Complexes containing Tbx2/3 will repress gene expression, chamber myocardium will not form in these regions, and rather inflow tract, atrioventricular canal, and outflow tract will from. Figure from Architectural plan for the heart: early patterning and delineation of the chambers and the nodes.(Christoffels *et al.*, 2004; Dunwoodie, 2007) ***(reproduced with permission).***

Despite current advances in the detection of molecular factors responsible for the development of the heart, a comprehensive understanding of the complex molecular interactions is still lacking (Dunwoodie, 2007).

1.5. Comparison between human and chick heart development

The heart development in humans	The heart development in Chicks	Developmental events
Days	**Hours/Days**	
16/17	**12-22 hrs**	Angiogenic cells align themselves in cardiogenic plate
18-20	**23-25 hrs**	Endocardial tubes are formed and start to move towards each other.
21-22	**26-32 hrs**	Both endocardial tubes fuse with each other and move towards thoracic region due to folding of embryo (both lateral and cephalic).
22	**33-44 hrs**	Endocardial tubes completely fuse with each other and form a single heart tube. The Heart begins to beat and splanchnic mesoderm invests the newly formed heart tube to form pericardial sac.
23	**45-46 hrs**	The heart tube grows rapidly and forcing it to fold upon itself
24	**47-48 hrs**	The shifting of centrally located sinoatrial node is obvious.
25-28	**49-56 hrs**	There is formation of bulboventricular loop. The septum primum appears to begin.
28	**52-69 hrs**	The ventricular septum appears at the floor of common ventricles. The endocardial cushions begin to form. Single pulmonary begins to arise from dorsal wall of left ventricle.
29	**70-71 hrs**	Appearance of truncal swellings is obvious.
30/31	**Day 3-3.5**	Perforations appear in septum primum
32/33	**Day 4**	The ostium secundum begins to appear near the edge of septum primum. The bulboventricular flange begins to shrink.
35	**Day 4**	The sinoatrial junction has shifted completely towards right side.
37	**Day 4**	The free edge of septum secundum forms foraman ovale.
42	**Day 5-6**	Both superior and inferior endocardial cushions fuse completely.
46	**Day 7**	The coronary sinus is formed and ventricular septum stops to grow further.
End of 7th	**Day 8**	The interventricular canal is completely closed.
8th week	**Day 8**	The outflow tracts are completely separated from each other.

http://www.meddean.luc.edu/lumen/MedEd/GrossAnatomy/thorax0/heartdev/main_fra.html

(Martinsen, 2005).

1.6. Environmental teratogens

1.6.1. Alcohol (Ethanol)

Ethanol, or alcohol, one of the major environmental toxins, when consumed in excess during pregnancy creates wide range of fetal dysmorphogenesis (Riley & McGee, 2005). The fetal alcohol syndrome (FAS) is the most familiar form of ethanol teratogenesis and is characterized by growth retardation, central nervous system disorders particularly mental retardation, and a distinguishing pattern of cardiovascular, facial and limb defects. Data from animal studies show features of fetal alcohol syndrome in embryos when treated with high doses of ethanol. There are many possible mechanisms involved in the pathogenesis of ethanol toxicity and these include increased oxidative stress, mitochondrial damage, interference with growth factor activity, effects on cell adhesion, lack of blood supply, oxidative damage and nutritional imbalance. But still the exact molecular pathways which cause the FAS are yet to be discovered (Cudd, 2005; Sulik, 2005).

1.6.2. Nicotine

Smoking during the early stages of pregnancy, results in abnormal development of many vital systems including the central nervous system, respiratory system and cardiovascular system (Bauman *et al.*, 1991). Vulnerability has been correlated to cardiovascular defects, neural tube defects, orofacial clefts, limb defects and kidney malformations (Bertolini *et al.*, 1982; DiFranza & Lew, 1995). The majority of data show the

relation between combustion products in smoke and congenital defects. However there are many other substances mainly nicotine, present in cigarettes which proved to cause congenital defects in animal studies. Nicotine exerts its effects by acting on acetylcholine receptors, which are present in even very early brains and might contribute to developmental defects. Also nicotine causes an altered cell function and cell death due to generation of many reactive oxygen species with resultant oxidative stress (Atluri *et al.*, 2001).

1.6.3. Cadmium chloride

Heavy metals, like cadmium, have no biological role and are rather toxic even at very low concentrations (Foulkes, 2000). These metals once they enter into cells, cause damage to cellular activities with resultant injury or death (Sunda & Huntsman, 1998). The occurrence of this toxic metal in the environment has been increased in recent years due to its large benefits in some industrial and agricultural activities (Rule *et al.*, 2006). Cadmium has well known toxic effects to living creatures: e.g. it shown to have teratogenic effects in Xenopus embryos (Mouchet *et al.*, 2006; Mouchet *et al.*, 2007) and has also proved to be carcinogenic in hamsters (Waalkes & Rehm, 1998). It exerts its toxic effects by different cellular and molecular mechanisms. Studies on animals suggest that cadmium-induced damage is associated with blockage of oxidative phosphorylation, glutathione depletion and antioxidant enzymatic activity inhibition, production of ROS, DNA damage, and inhibition of relative repair mechanisms, a general reduction of protein synthesis coupled to an increase in stress proteins

(Ercal *et al.*, 2001; Schröder *et al.*, 2005; Lin *et al.*, 2007). Also cadmium exposure results in apoptotic events in several cell types (Risso-de Faverney *et al.*, 2001; Kim *et al.*, 2002; Agnello *et al.*, 2007).

1.6.4. Sodium fluoride

Fluoride has been used for the treatment of dental caries for many centuries (Sognnaes, 1979). Water fluoridation is a common practice in many countries and nearly 200 million people drink water which is fluoridated with hydrofluorosilicic acid, sodium silicofluoride, or sodium fluoride. Also an additional 100 million people reside in areas of high natural fluoride in water (Sognnaes, 1979). Apart from used in fluoridation of water, sodium fluoride is also utilized in many pesticides, in glass and vitreous enamel processing, in frosting glass, as a wood preservative, as a steel degassing agent, and in electroplating (Heindel *et al.*, 1996). The data regarding its potential toxic effects in developing embryo are scarce. Some animal studies show its toxic effects on sperms when fluoride is administered intraperitoneally to male Swiss mice (Pati & Bhunya, 1987).

1.6.5. Ginseng

Ginseng is one of the most popular herbal remedies taken by many women in the western world as a treatment of various diseases, and for daily nourishment and health building. It has been shown to act as an anti-diabetic, anti-hypertensive, anti stress, antioxidant and anti-tumour agent (Jeon *et al.*, 2000; Kaneko & Nakanishi, 2004). Also it is used for increasing memory and as a mood stabilizer (Scholey & Kennedy, 2002; Seely *et al.*, 2008). The active components of ginseng are ginsenosides.

Ginsenosides comprise two major groups' panaxadiol and panaxatriol. Ginsenoside Rb1 (GRb1) is representative of panaxadiol (Attele *et al.*, 1999). Rb1 was shown to have teratogenic effects on rat embryos (Chan *et al.*, 2003). Congenital defects were also observed in the mouse when embryos were exposed to ginsenoside 1(Rb1) (Liu *et al.*, 2005).

1.7. Maternal diseases particularly Diabetes Mellitus

Mothers with Diabetes Mellitus have a high incidence of pregnancy complications and fetuses with developmental disorders of the cardio-vascular system, brain, skeleton and other anomalies compared to non-diabetic women (Eriksson & Borg, 1993). The rate of maternal and fetal complications is inversely proportional to the degree of diabetic control (Eriksson *et al.*, 2003). The precise mechanism behind the high rate of congenital defects in diabetic pregnancies is presently unknown. It has been proposed that both maternal factors such as hyperglycemia, hyperketonemia, disturbed prostaglandin metabolism, as well as the embryonic genetic background may be involved. One of the possible mechanisms involved is higher generation of ROS (Ornoy, 2007). Glucose at higher concentrations results in non-enzymatic protein glycosylation, increased mitochondrial electron transport chain flow, glucose auto-oxidation and changes in the redox potential. Also degradation of high glucose levels leads to high production of ketone bodies and a further increase in the production of ROS. The resultant oxidative stress in the embryonic environment causes massive cell damage, an increase in

apoptotic events and defective embryonic development (Zangen *et al.*, 2002).

1.8. Vitamins

1.8.1. Folic acid

Folic acid is a water soluble vitamin, and it is recommended by many countries for all women of child bearing age to take at least 400µg of folic acid per day to prevent neural tube defects in their offspring (Botto *et al.*, 2003). It is now well established that folic acid when consumed in the periconceptional period protects against neural tube defects. Apart from protecting against neural tube defects folic acid has also been shown to prevent many birth defects including those of the heart (Shaw *et al.*, 1995; Botto *et al.*, 1996) and genito-urinary system (Czeizel *et al.*, 1998), as well as cleft lip and palate, and limb reduction defects (Werler *et al.*, 1999). The role of folic acid in avoiding other unfavourable reproductive outcomes e.g. early abortions, still births and premature delivery is also obvious in many studies. The other beneficial effects of folic acid in the general population include protection against cereberovascular and cardiovascular diseases (Lucock, 2000).

1.8.2. Vitamin C & E

Vitamin C (ascorbic acid) is a water soluble molecule with the ability to scavenge several free radicals. It is an important micronutrient required for normal metabolic functioning of the body (Jaffe, 1984). It is not synthesized in humans and other animals due to the deficiency an enzyme required for its biosynthesis (Woodall & Ames, 1997). Therefore it must

be consumed in the diet. The foods which are rich sources of vitamin C include fresh fruits, particularly citrus fruits, and vegetables (Levine, 1986). Deficiency of vitamin C in the diet causes a disease called scurvy. Scurvy is characterized by weakening of collagenous structures, causing tooth loss, joint pains, bone and connective tissue disorders, and poor wound healing. Apart from these symptoms scurvy is also characterized by mood swings and depression due to the fact that vitamin C also functions as a cofactor for catecholamine biosynthesis, in particular the conversion of dopamine to norepinephrine, catalyzed by dopamine b-monooxygenase. Vitamin C is also implicated in various other metabolic activities of the body (Weber *et al.*, 1996).

Vitamin E' is a common term used for all compounds that show the biological activity of α-tocopherol (Bramley *et al.*, 2000). The RRR-α-tocopherol is the most active form of vitamin E (formerly D-α-tocopherol), which accounts for approximately 90% of the vitamin E found in animal tissues (Bjorneboe *et al.*, 1990; Debier & Larondelle, 2005). The eight naturally occurring vitamin E compounds are α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols, Vitamin E compounds are water insoluble and are soluble in alcohol and other organic solvents (diethyl ether, acetone and chloroform) as well as in oils. Rich sources of vitamin E are polyunsaturated vegetable oils, cereal seeds, fish, nuts, eggs, liver, dairy products and green vegetables (Bramley *et al.*, 2000).

1.8.3. Retinoic acid

Vitamin A comprises all compounds that demonstrate the same biological activities. These compounds include retinol, retinal, and retinoic acid. Vitamin A and its analogues are essential dietary elements required for many biological functions including growth, reproduction, vision and cellular differentiation etc. Both deficiency and excess of this vitamin result in many pathological conditions in the body. Many animal studies proved it to be essential in reproduction and embryonic development but on the other hand its excess dose can be teratogenic. Pregnant women can be exposed to high doses of retinoic acid in several different ways: excess intake of those foods which contain high amount of retinoids (e.g. carrots, liver, eggs, green leafy vegetables, milk etc.); use of retinoids to treat reluctant acne or other skin disorders as well as different kinds of cancers; excessive use as nutrient supplement (Soprano & Soprano, 1995).

1.9. Connexins

Connexins are highly regulated integral membrane proteins that form vertebrate gap junctions. Connexins regulate coordinated contraction of excitable cells, tissue homoeostasis and controlled cell growth and differentiation and are also thought to regulate embryonic development. Disruptions in their expression have been related to numerous diseases (Gong *et al.*, 1997; Paznekas *et al.*, 2003), including oculodentodigital dysplasia, atrial septal defects and arrhythmias. Many connexins (i.e. Cx31, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx46, Cx50 and Cx56) have been recognized. Cx43 is the most extensively expressed connexin, being

present in at least 34 tissues and 46 cell types (Laird, 2006), and is the predominant connexin expressed in most cell lines. The connexins assist in direct contact between adjacent cells by allowing passage of ions and small metabolites (Gong *et al.*, 1997; White & Paul, 1999; Saez *et al.*, 2003; Sohl & Willecke, 2004). Because connexins also regulate embryonic development and help control cell growth and differentiation, disruption or mutations of the connexin gene has effects on development. Extensive evidence also indicates that gap junctions play important roles in the control of cell growth and inhibit tumour formation (Cronier *et al.*, 2009).

1.10. Aims and objectives

The main aims and objectives of the current study are

- To evaluate chick heart micromass culture for use in teratogenicity assays.
- To develop and assess in ovo culture to be used in detection of potential developmental toxicity of various environmental agents.
- To determine the efficacy of chick heart micromass in predicting potential toxicity of different chemicals.
- To detect possible preventive effects of multi-vitamins on ethanol and nicotine (social drugs) induced toxicity in micromass and in ovo cultures.
- To assess possible teratogenic effects of diabetic conditions and their possible prevention by use of multivitamins utilizing both in vitro (micromass) and in ovo cultures.
- To evaluate the effects of different teratogens on connexion 43 and endothelin 1 expression.

Chapter 2

Materials and methods

2.1. Materials and Methods for micromass culture

2.1.1 Materials

2.1.1.1. Eggs

White Leghorn Fertile chicken eggs	Henry Stewart & Co. UK

2.1.1.2. Tissues and cell culture reagents

Bovine serum albumin	Sigma-Aldrich, UK
Carbon dioxide	British Oxygen Company
Dimethylsulphoxide (DMSO)	Sigma-Aldrich, UK
Dulbecos modified Eagles medium (1x)	Cambrex Biosciences,UK
Glacial acetic acid	BDH, UK
Horse serum	Life technologies, UK
Hanks Balanced salt solution	Sigma-Aldrich, UK
Heat inactivated fetal bovine serum	Autogen Bioclear, UK
Kenacid blue	Sigma-Aldrich, UK
ES®7X liquid detergent	SLS, UK
L-glutamineCambrex	Biosciences, UK
Neutral red	Sigma-Aldrich, UK
Paraformaldehyde	Sigma-Aldrich, UK
Penicillin/streptomycin	Sigma-Aldrich, UK
Potassium acetate	Fisher scientific, UK
Resazurin	Sigma-Aldrich, UK
Resorufin (95% dye colour)	Sigma-Aldrich, UK
Trigene	Medichem International, UK

Trypsin (0.05%) /EDTA (0.02%)	Life technologies, UK

2.1.1.3. Plastic and Glass ware

Bijoux bottles(7ml)	Bibby Sterilin Ltd, UK
Cell star tubes (50ml)	Greiner Lab technik, France
Chamber slides (8 well)	SLS, UK
Cover slips	SLS, UK
Disposable serological pipettes	Corning Incorpated, UK
Eppendorf tubes (0.5 & 1.5ml)	Sterilin, UK
Pipette tips	Starstedt, Germany
100mm round petridishes	Sterilin, UK
Transfer pipette (3.5ml)	Sterilin, UK
Transfer pipette (3.5ml)	Sterilin, UK
Universal bottles (30ml)	Bibby Sterilin Ltd, UK
Universal tubes, 25ml and 50ml	Sterilin, UK
24 well plates	Nunclon, UK
96 well plates	Nunclon, UK

2.1.1.4. Consumables

Aero disc filter (0.2µM)	Sartorius, Germany
Autoclave bags	NHS supplies
Pipettes (0.5-1000µl)	SLS, UK
Pipette tips (200µl and 1000µl)	Starstedt, UK
Reservoir liners (8 channel)	Corning Incorpated, UK
Scalpels	SLS, UK

Universal graduated glass bottles	SLS, UK
Vinyl gloves	Labsales Co.,UK
Yellow biohazard bags	NHS supplies

2.1.1.5. Equipment used

ASYS HITEC Expert 96	SLS, UK
Assorted dissection instruments	A. Durmount, Switzerland
Autoclave	Denley, UK
Automatic egg turner	MFG, USA
Balance D 40T	Stanton, UK
Calibrated pipettes	Gilson, France
Centrifuge (centaur2)	Fisons, UK
Class 1 sterile laminar flow cabinet	Glossop, UK
Class 2 sterile laminar flow cabinet	Rossandale, UK
CO_2 incubator	Fisons, UK
Cooled egg incubator	Gallenkamp, UK
FLUOROSTAR Galaxy	BMJ Cambridge, UK
Freezer	New Brunswick, UK
Grant Water bath	Cambridge, UK
Haemocytometer	Hawksley, UK
Ice machine	Ziegra, UK
Incubator	LEEC, UK
Laminar flow hood (Gelaire)	ICN, UK
Inverted Phase Microscope	Nikon, Japan
Light Microscope	Vickers, UK

Plate shaker	Luckham Ltd, UK
Stirrer hot plate	Gallenkamp, UK
Warming block	Camlab, UK
Water purifier	Egga, UK

2.1.1.6. Software used

Prism statistical software, version 5.0	Graph pad, USA

2.1.1.7. Test Chemicals

All-trans-Retinoic acid	Sigma-Aldrich, UK
Folic acid	Sigma-Aldrich, UK
Ethanol	BDH, UK
Vitamin C	Sigma-Aldrich, UK
Glucose	Sigma-Aldrich, UK
B hydroxybutyric acid	Sigma-Aldrich, UK
Nicotine	Sigma-Aldrich, UK
Sodium fluoride	Sigma-Aldrich, UK
Cadmium chloride	Sigma-Aldrich, UK
Superoxide dismutase	Sigma-Aldrich, UK

2.1.2. Preparation of culture Medium

2.1.2.1. Dulbeco's Modified Eagle's Medium (DMEM) culture medium for chick heart cells

Dulbeco's Modified Eagle's Medium was supplemented with 10% fetal calf serum (heat inactivated), 2mM L-glutamine and 50U/ml penicillin with 50µgml^{-1} streptomycin. Once prepared Medium was stored at 4^{o}C

until use, and was warmed to 37^{o}C prior to use. The prepared medium was used within 2 weeks.

2.1.2.2. Culture media quality control

In bijoux bottles, 5 ml of prepared culture medium (section 2.1.2.1) was incubated in for 2-3 days at 37°c, 5% (v/v) CO_2 in air and inspected microscopically for micro-organism contamination. If contamination was present, the medium was discarded.

2.1.3. Preparation of stock solutions

2.1.3.1. Resazurin solution

Resazurin	5mg
HBSS	50ml

Resazurin stock (5ml, 1mg/ml in HBSS) was filter sterilized in to a 50ml universal container. HBSS (45ml) with calcium and magnesium was added to create a working dilution of 100µg/ml. This was subsequently diluted 1:10 in HBSS and the tube wrapped in aluminium foil to avoid photo reduction. Resazurin solution (10mg/ml) was stored at 4^{o}C and used within 2 weeks. Resazurin solution was warmed to 37^{o}C prior to exposure to cells.

2.1.3.2. Kenacid blue protein detection

2.1.3.2.1. Kenacid blue fixative

Fixative was prepared by adding 10ml glacial acetic acid to 500ml ethanol and 490ml distilled water. It was stored at room temperature until required.

2.1.3.2.2. Kenacid blue stock solution

Stock solution was prepared by dissolving 0.4g kenacid blue to 250ml ethanol and 630ml distilled water. The solution was stored at room temperature.

2.1.3.2.3. Kenacid blue working solution

Working solution was formed by adding 6ml acetic acid to 44ml stock solution. It was formed immediately prior to use.

2.1.3.2.4. Kenacid blue washing solution

Washing solution was formed by adding 50ml glacial acetic acid to 100ml ethanol and 850ml distilled water, and was stored at room temperature.

2.1.3.2.5. Kenacid blue desorb solution

Desorb solution was formed by dissolving 98.15g potassium acetate in 700ml ethanol and 300ml distilled water. The solution was stored at room temperature.

2.1.4. Methods

2.1.4.1 Sterilization

2.1.4.1.1. Sterilization of solutions by Autoclaving

Sterilization by autoclaving was carried out in an autoclave at 15lb/sq inch for 20 minutes at 120°C.

All purchased media were supplied sterile. The culture media were prepared using sterile stock solutions. All non heat-labile stock solutions

were sterilised by autoclaving, while heat labile solutions were filter sterilized using irradiated cellulose acetate membrane filters.

2.1.4.1.2. Sterilization of glassware by Autoclaving

All glass bottles and beakers were first washed in ES®7X liquid detergent and then rinsed in distilled water to remove the detergent, air dried and sterilised by autoclaving.

2.1.4.1.3. Sterilisation by Oven

Oven sterilization was performed by placing all pasteur pipettes in a metal canister, which was then secured with autoclave tape and sterilised at 180°C overnight. Alternatively disposable sterile plastic pipettes were employed.

2.1.4.1.4. Sterilization of dissecting instruments

Dissecting instruments were soaked overnight in trigene (2% v/v with water) after use. The instruments were then washed in tap water and with alcohol and dried before use.

2.1.4.2. Microscopy

2.1.4.2.1. Light Microscopy

All microscopic observations were carried out using an inverted microscope using x 40, x10 and/or x4 objective lenses with x10 eye piece.

2.1.4.2.2. Confocal Microscopy

Fluorescein isothiocyanate (FITC), Tetramethylrhodamine isothiocyanate (TRITC), and Alexa 488 were detected using a Leica confocal microscope

with excitation filter of 485nm, and an emission of 530nm for FITC or 545nm excitation filter and emission of 590nm for TRITC staining.

2.1.4.3. Preparation of test chemicals

All chemicals were made up at twice the concentration required as 500µl of test solution was added to the 500µl culture medium already present in the 24 well plates, which gave the required concentration. All the test chemicals were made up the day to be used and applied to the MM cultures 24 hours after they were seeded from their stock solutions as shown in table 2.1.

Table 2.1. Solvents and stock solutions of chemicals

Chemicals	Solvent	Stock Concentrations
All-trans retinoic acid	Ethanol (100%)	50mM
Ascorbic acid	Medium	50mM
B hydroxybutyric acid	Medium	50mM
Cadmium chloride	Medium	50mM
Folic acid	Medium	50mM
Ethanol	Medium	Immediately prepared before use
Ginsenoside (Rb1)	Medium	50mM
Glucose	Medium	50mM
Nicotine	DMSO	5mM
Sodium fluoride	Medium	50mM
Trolox	Medium	50mM

2.1.4.4. Calculation of cell number

A haemocytometer was used to count cell number under light microscope. Cells that crossed or touched at the right side and bottom of the square were excluded from the count. The volume of cell suspension needed to give the required seeding cell density was then calculated by following equation

$$V_1 = \frac{(C_2)\,(V_2)}{(C_1)}$$

V_1 denotes total ml of cell stock required, C_1 denotes total number of cells in stock, V_2 denotes final volume of cell suspension needed and C_2 is the final number of cells.

2.1.4.5. Micromass cell culture

2.1.4.5.1. Embryo culture

Fertile White Leghorn chicken eggs were labelled with the date of delivery and stored in a cooled incubator at 12-14^{o}C until required, and used within two weeks of being laid. The eggs were placed onto the automated egg turner and incubated at 38^{o}C with relative humidity of 100% for 5 days, day zero being defined as the day when the eggs were set in the incubator. A minimum of 24 eggs were incubated for each micromass system to ensure the availability of enough viable embryos. All the embryos were killed by decapitation after being removed from incubator.

2.1.4.5.2. Micromass culture

On the day of dissection, the dissecting tissue culture laminar flow hood was swabbed down with 2% trigene^{TM}, followed by 70% alcohol

immediately prior to use. All the dissection tools (curved and straight no toothed forceps) were cleaned with 70% ethanol before being placed in to the sterile culture hood in order to avoid contamination. Sterile gloves were worn throughout the procedure.

Eggs were removed from the incubator, six at a time and transferred to the culture hood. Using the blunt end of the curved forceps the broader end of the eggs (near air sac region) was struck until the broken shell could be gently removed and discarded. The shell and all the membranes including vitelline membranes were removed with the help of sterile forceps to expose the embryo. The embryo was lifted from the egg with curved forceps and placed into a petri dish containing Hank's balanced salt solution (HBSS). After removing the allantois and remaining amnion, under the dissecting microscope, the heart was taken out from the embryo and transferred using sterile forceps to a sterile universal tube containing horse serum (50% v/v in HBSS) and stored on ice. Once all the hearts had been collected, the solution of horse serum (50% v/v in HBSS) was removed and tissues were washed twice with HBSS. Heart tissue was incubated in 4ml of trypsin/EDTA (for 24 eggs) at 37^{o}C in 5 % (v/v) CO_2 in air for 20 minutes, agitating every 5 minutes for further disassociation of tissue. The solution was triturated numerous times in order to break the tissues as much as possible. To inhibit further actions of trypsin 6ml of DMEM culture medium was added. Samples were centrifuged at 1500rpm for 5 minutes, the supernatant was removed and the pellet resuspended in 1ml of pre warmed DMEM culture medium. Cell density was established

using a haemocytometer and the cell suspension diluted to $3x10^6$ cells ml^{-1}. A 20µl aliquot of the cell suspension was pipetted on to the centre of the well within a 24 well plate for about two and half hours, to allow them to attach, before being flooded with 500µl of prewarmed culture medium (DMEM), and returned to the incubator for 24 hours to recover.

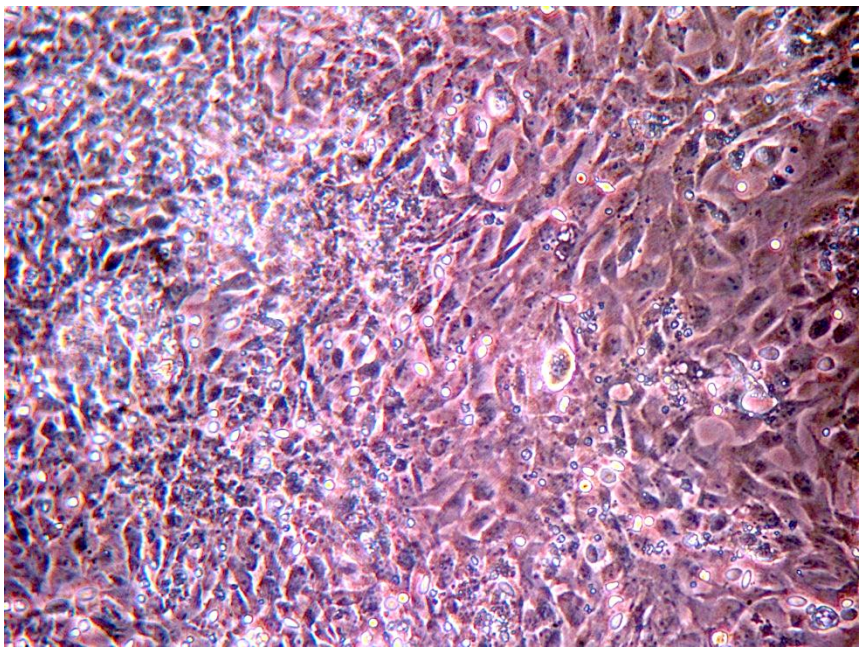


Figure 2.1. Light microscopy of micromass. Original magnification x200

2.1.4.5.3. Scoring method for cardiomyocyte activity

A numerical morphological scoring system was constructed to determine the amount of contractile activity observed for heart micromass (MM). Cardiac foci that were observed to be contracting throughout entire plate were scored a total of 3, whereas no contractile activity was scored as zero. Table 2.2 illustrates the scoring criteria for determination of contractile activity of cardiomyocytes.

Table 2.2. Morphological scoring system to determine contractile activity of cardiomyocytes

Numerical morphological score	Contractile activity
0	No contractile activity
1	Few contracting foci
2	Numerous contracting foci
3	Entire plate contracting

2.1.4.5.4. Chemical dose testing

The micromass cells were prepared as in section (2.1.4.5.2) and cultured for 24 hours. The media were aspirated before performing a resazurin assay. The cells were cultured within a 24 well plate in media containing the varying concentrations of different chemicals. MM cultures were observed at 24 hours intervals up to 144 hours post exposure. The arrangement of a typical 24 well plate in respect to the chemical concentrations can be seen in fig 2.2.

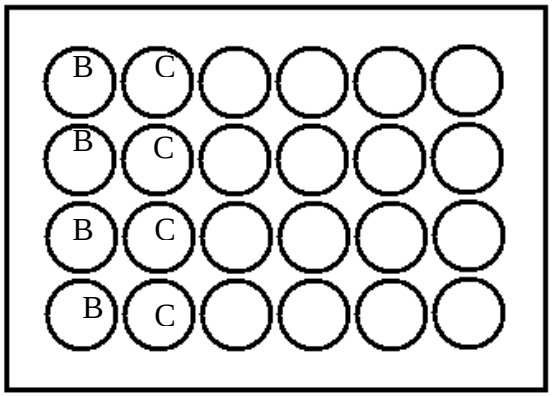


Figure 2.2. The arrangement of a 24 well plate for micromass culture. **B indicates for blank, C for control and other wells for chemicals used, with increasing concentrations from left to right.**

2.1.5. Cell viability and cytotoxicity assays

2.1.5.1. Resazurin conversion assay

The resazurin assay was performed on day 6 following explantation. The resazurin stock (100µg/ml) solution was diluted 1:10 in sterile HBSS and warmed to 37^{o}C in a water bath prior to use. The medium was removed from the 24 well plates and replaced with 500µl resazurin solution. The plates were then incubated for one hour at 37^{o}C and 5% (v/v) CO_2 in air. The optical density was read using a FLUOR star plate reader, excitation wavelength of -530±12.5nm, with a gain of 10. The data was expressed as the increase in optical density above the non-cell blank as a percentage of the untreated cultures. Analyses of data, for statistically significant differences, were performed on the raw data. If repeat assays were performed, once the plate had been read in the spectrofluorimeter, the

resazurin solution was removed and the cells were fixed with 300µl of kenacid blue fixative. The plates were then kept in refrigerator until the kenacid blue assay was performed.

2.1.5.1.1. Resazurin standard curve

Interpretation of resazurin results was made possible by the creation of a standard curve. The optical density could be converted into resorufin production by plotting the resorufin standard curve. Resorufin solutions of different concentrations 4000ng/ml, 2000ng/ml, 1000ng/ml, 500ng/ml, 250ng/ml and blank consisting of just HBSS solution were prepared. Aliquots of 500µl of each concentration were transferred in to a 24 well plate. The plate was then read in the cytofluorimeter with excitation wavelength of -530±10nm, and emission of 590±12.5nm, with a gain of 10. Results were plotted on a scatter graph and a line of best fit added in order to form an equation to allow interpretation of raw data of the assay from plate reader (Fig. 2.3).

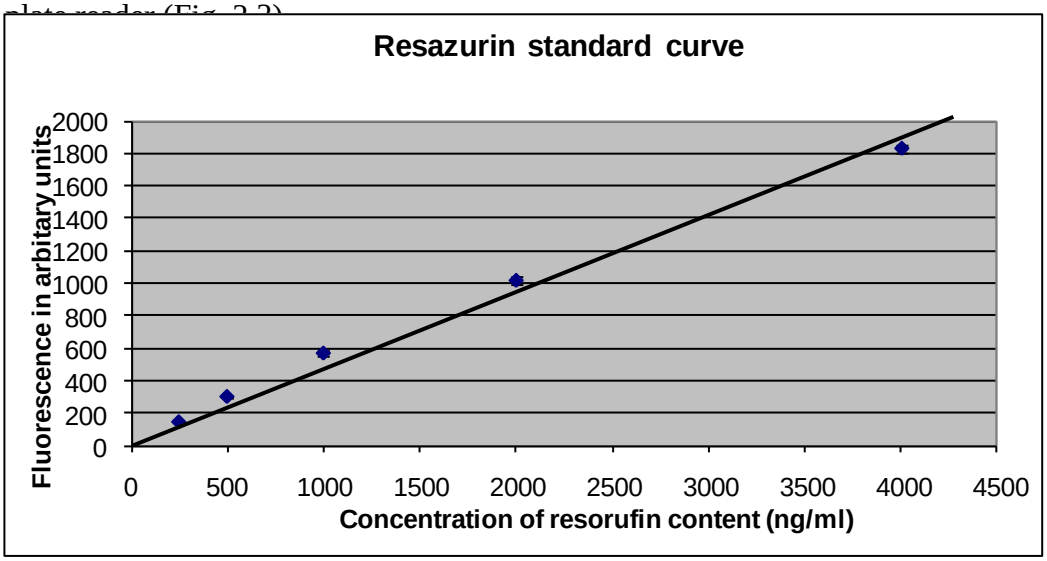


Fig. 2.3. Resazurin reduction assay standard curves (±S.D. n=3).

2.1.5.2. Kenacid blue total protein assay

Wells were aspirated and 300µl kenacid blue fixative was added and allowed to evaporate overnight at 4^{o}C. Kenacid blue working solution (400µl) (Knox et al, 1986) was added to each well and the plate placed on a plate shaker for at least 2 hours. Excess stain was removed and cells were quickly rinsed in 400µl of washing solution before being washed for 20 minutes with agitation. The washing solution was replaced with 400µl of desorb and gently agitated on the plate shaker for one hour. The optical density was read on an ASYS HITEC Expert 96 plate reader with a reference filter of 405nm, and a reading filter of 570nm. The amount of protein per well was calculated from standard curves.

2.1.5.2.1. Protein standard curve

For plotting a standard curve bovine serum albumin was used. 2.4 mg BSA was dissolved in 3 ml of distilled water. 7 ml of 100% ethanol was added to produce a concentration of 2.4mg/10ml 70% ethanol or 0.24 mg BSA/ml 70% ethanol. 3 ml was added to 6 ml of 70% ethanol, to give a concentration of 80µg/ml BSA, the highest concentration. From this maximum concentration (80µg), all other concentrations (40µg/ml, 20µg/ml, and 10µg/ml) were serially diluted and prepared. The 70% ethanol was allowed to evaporate overnight enabling the BSA to adhere to the bottom of each well. Plates were then fixed with kenacid blue fixative and left overnight at 4^{o}C. The Kenacid blue assay was performed as described above. Results were plotted on a scatter graph with the equation of the best fit line used (Fig. 2.4). The optical density could then be

converted in to the amount of protein present in each test well using the protein standard curves and following formula

Protein content (µg)=Optical density(OD)/0.016

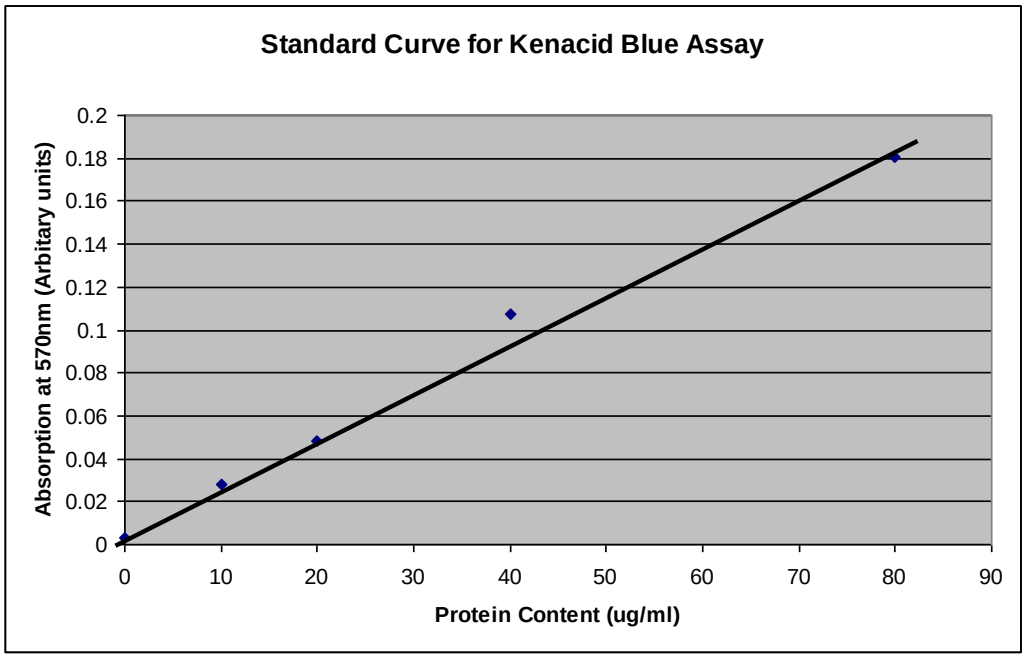


Figure. 2.4. Standard curve for kenacid blue assay.

2.2. Materials and Methods for in ovo chick

2.2.1 Materials

2.2.1.1. Eggs

White Leghorn chicken Eggs	Henry Stewart & Co. UK.

2.2.1.2. Tissues and cell culture reagents

Ethanol	BDH, UK
Formaldehyde	Sigma-Aldrich, UK
HBSS	Sigma-Aldrich, UK
Neutral red dye	Sigma-Aldrich, UK
Phosphate buffered saline tablets	Oxoid, UK

2.2.1.3. Plastic and Glass ware

Adhesive tap	Comply™ , UK
Disposable syringe	B.D. Republic of Ireland
Disposable needles (25guage)	B.D. Republic of Ireland
Para film	Pechiney plastic, UK 100mm
rounded petri dish	Sterilin, UK
Universal containers (50ml)	Sterilin, UK

2.2.1.4. Sectioning and H&E staining

Eosin 1% aqueous	Raymond A lamb limited, UK
Haemotoxylin	Raymond A lamb limited, UK
Histology moulds	Fisher scientific, UK
Lamb's cassettes (Blue)	Raymond A Lamb-limited UK

Lambs cassette lids	Raymond A Lamb-limited UK
Leica automatic tissue processor (TP1020)	Leica, Germany
Microtome	Sleemainz, UK
Paraffin section mounting bath	Electrothermal,UK
Wax dispensor	Leica, Germany
Whatman filter paper	Whatman international , UK

2.2.1.5. Masson's trichrome staining

Ethanol	BDH, UK
0.2% light green in 0.2% acetic acid	Raymond A lamb limited, UK
Mayers Haematoxylin	Raymond A lamb limited, UK
Ponceau Acid Fuschin	Raymond A lamb limited, UK
1% Phosphomolybdic acid	Raymond A lamb limited, UK

2.2.2. Preparation of solutions

2.2.2.1. Preparation of 10% formalin solution

10% formalin solution was made by adding 10 ml of formalin to 90ml distilled water. This solution was prepared inside the fume cupboard as formaldehyde is highly toxic and carcinogenic.

2.2.2.2. Preparation of PBS

PBS was prepared by adding 5 tablets PBS to 500ml distilled water.

2.2.3. Methods

2.2.3.1. Chick embryos and treatment groups

White leghorn fertilized chicken eggs were obtained from Henry Stewart and Co. Ltd. Eggs were labelled with the date, received and kept in a cooled incubator until use. Ideally eggs should be used within 15 days of being laid. Eggs were randomly selected and divided in to 4 groups immediately before incubation. The first group was incubated as the non-treated group (n = 12). The rest were labelled according to different treatment modes. The day on which the eggs were incubated was counted as day zero. On day 3 of incubation the eggs were taken out of the incubator 3 at a time, after being swabbed down with 70% ethanol and under sterile conditions the blunt ends of eggs was struck with forceps to make a small hole. Eggs were injected with either test chemical or PBS alone, using a 25- gauge needle attached to a 1 ml disposable syringe. All the injections were under the vitelline membrane through air sac region. After being injected the holes were sealed with parafilm and taped with insulating tape in order to avoid drying out of the embryos. Only viable embryos were injected in each group. All the eggs were placed blunt end up into the automated egg turner and incubated at 37.5 °C and relative humidity of 55% with 5% CO_2 in air till day 9 of incubation.

2.2.3.2. Sample collection and measurements

On day 9 eggs were cracked under sterile hoods and embryos were examined in terms of their viability. After removing all the membranes

viable embryos were examined for any growth retardation by measuring crown rump length, and any malformations or gross abnormalities observed including limb deformities, facial defects, and brain vesicle development. Hearts were taken out and fixed in 10% formaldehyde. All the hearts were kept at 4^{o}C, until used for sectioning.

2.2.3.3. Paraffin tissue processing for sectioning

Fixed hearts went through a series of processes starting from dehydration and clearing to wax impregnation before being sectioned.

2.2.3.3.1. Dehydration

Dehydration was carried out by placing the tissues in increasing concentrations of alcohol ranging from 70% to 100%.

2.2.3.3.2. Clearing

After removing all the water from tissue samples, they were cleared of alcohol by means of xylene in order to mix wax into them.

2.2.3.3.3. Wax impregnation

Following all the clearing the samples were transferred to two changes of molten wax till thoroughly impregnated. At this point temperature was strictly controlled.

2.2.3.3.4. Automatic tissue processing

An automatic tissue processor, (Leica TP1020) was used to perform all the above mentioned processes.

The tissues were sectioned at 25µm by microtome and slides were prepared using APES coated glass slides.

2.2.3.4. Haematoxylin and Eosin staining of tissue slides

Once all the slides were prepared, they were hydrated with water and then placed into the Haematoxylin solution for 5 minutes. The haematoxylin was removed and tissues were washed with tap water. Tissues were differentiated in acid alcohol to remove excess stain before bluing the nuclei in a saturated solution of lithium carbonate. The tissues were again washed in tap water then placed in to 1% eosin for approximately 1-2 minutes. Excess stain was removed and slides were washed in tap water. Finally the tissues were dehydrated by passing through increasing concentrations of alcohol and allowed to dry before being covered with cover slips.

2.3. Materials and Methods for immunohistochemistry

2.3.1. Immunostaining reagents

Bovine serum albumin (BSA)	Sigma-Aldrich, UK
Frozen tissue matrix (OCT)	Lamb, UK
2-methylebutane (isopentane)	Fisher Scientific, UK
Normal goat serum (NGS)	Sigma-Aldrich, UK
Paraformaldehyde Phosphate Buffered Saline	Sigma-Aldrich, UK
Triton X100	Sigma-Aldrich, UK
Vectashield™ (containing DAPI)	Vector Laboratories, UK

2.3.2. Plastic and glass ware

APES coated slides	Menzel Glaser, UK
Chamber slides	Nunc, UK
Glass containers	Storopack, Germany
Glass coverslips	VWR international, UK
Plastic moulds	VWR international, UK

2.3.3. Antibodies

2.3.3.1. Primary antibodies

Table 2.3. Primary antibodies used

Antigen Specificity	Host	Form	Reactivity	Dilution	Source
Skeletal muscle	Mouse	Monoclonal	Rabbit, Rat, Chicken, Mouse, Xenopus	25µg/ml	DSHB, lowa, USA
Cardiac troponin T	Mouse	Monoclonal	Broad species specificity	25µg/ml	DSHB, lowa, USA
Striated muscle (Sarcomere	Mouse	Monoclonal	Mammalian, Avian, Amphibian	25µg/ml	DSHB, lowa, USA
Connexin 43	Rabbit	Polyclonal	Hu, Ms, Rat, Chk, Cow, Hm,	1:500	Abcam, UK
Endothelin 1	Rabbit	Polyclonal	Hu, Ms, Por, canine, Rat, Bovine	1:500	Phoenix pharmace u-ticals, UK

Hu (Human); Ms (Mouse); Por (Porcine)

2.3.3.2. Secondary antibodies

Table 2.4. Secondary antibodies used

Antigen Specificit	Host	Form	Reactivity	Label	Dilutio	Source
Rabbit IgG	Goat	Polyclonal	Rabbit	Alexa 488	25µg/ml	Sigma-Aldrich, UK
Rabbit IgG	Goat	Monoclonal	Rabbit Fc	FITC	25µg/ml	Sigma-Aldrich, UK
Mouse IgG	Goat	Monoclonal	Mouse Fc	FITC	25µg/ml	Sigma-Aldrich, UK

Mouse IgG	Goat	Monoclonal	Mouse Fc	TRITC	25µg/ml	Sigma-Aldrich, UK

2.3.4. Stock solutions

2.3.4.1. Normal goat serum (NGS) (0.5% v/v)

5% NGS was prepared by adding 500µl NGS to 9.5ml PBS immediately before use.

2.3.4.2. Paraformaldehyde solution (4% w/v)

4% paraformaldehyde solution was made by adding 4g of it to 100ml PBS, solution was then heated to 60^{o}C with continuous agitation until completely dissolved. All this procedure was done in a fume cupboard (paraformaldehyde is a carcinogen). The solution was further diluted to 2% by adding to PBS and stored at -20^{o}C until required.

2.3.4.3. Triton X 100 (0.2% v/v)

Triton X 100 (20µl) was mixed with 9.98ml PBS just prior to use.

2.3.5. Methods

2.3.5.1. Indirect immunohistochemistry

The cultures were fixed in paraformaldehyde solution (2% w/v in PBS) for 1 hour at room temperature. This was aspirated and the cells were washed thrice with PBS each for 5 minutes, before being permeabilised with 0.2% Triton X-100 in PBS (v/v) for 10 minutes. Once permeabilised, the cells were incubated in 5% goat serum in PBS for 1 hour to block nonspecific binding of secondary antibody. The goat serum was removed and the cells were incubated with the primary antibody (200µl) (Table2.3) overnight at 4^{o}C. The primary antibody was removed and the cells were washed 3 times

in 0.1% BSA in PBS (w/v). The secondary antibody (Table 2.4) diluted in 0.1% BSA in PBS and incubated for 1 hour in the dark. Cells were washed in PBS (3 x 5 minutes) to remove excess unbound secondary antibody before mounting with Vectorshield™ containing DAPI. Sections were viewed under a confocal microscope.

2.3.5.2. Double immunofluorescence

For double immunostaining micromass cultures were treated in a similar way as described in previous section. Here two monoclonal antibodies were used to assess the composition of the cardiomyocyte MM cultures. The first primary (table 2.3) antibody was applied and incubated overnight at 4°C. The MM cultures were rinsed with 0.1% BSA in PBS (w/v). The secondary antibody FITC (table 2.4) was applied and incubated at room temperature for 1 hour in the dark. Slides were rinsed and then washed in fresh BSA solution (0.1% w/v in PBS). The process was repeated for the second primary antibody (table 2.3) using the secondary antibody TRITC (table2.4). Slides were washed in distilled water and mounted using Vectashield™ containing DAPI ($50\mu g ml^{-1}$). The slides were viewed under a confocal Microscope.

2.4. Materials and methods for Western blotting

2.4.1. Plastic and glass ware

Cassettes	Millipore, UK
Centrifuge (cold)	Sigma, UK
Centrifuge (room temperature)	Sanyo, UK
Electrophoresis module	Bio-Rad laboratories, UK
Filter paper	Whatman's Ltd, UK
Fish skin gelatine	Sigma-Aldrich, UK
Loading marker	Lonza, UK
Nitorcellulose membrane	Shleicher & Schuell, Germany
Oddyssey scanner	Licor, UK
Plate reader	Dynax, UK
Polytron homogenizer	Heildolph, UK
Precast gels (10% 1mm)	Lonza, UK
Snap i.d.	Millipore, UK
Transfer module	Bio-Rad laboratories, UK
Tanks	Bio-Rad laboratories, UK
Transfer cassettes	Bio-Rad laboratories, UK

2.4.2. Antibodies

2.4.2.1. Primary antibodies

Table 2.5. Primary antibodies used in western blotting

Antigen specificity	Host	Form	Source	Working dilution
Cx 43	Rabbit	Polyclonal	Abcam (UK)	**1:500**
GAPDH	Mouse	Monoclonal	Sigma-Aldrich, UK	**1:3000**

2.4.2.2. Secondary antibodies

Table 2.6. Secondary antibodies used in western blotting

Secondary antibodies	Host	Form	Source	Working dilution
Green	Rabbit	Polyclonal	Licor, UK	**1:9000**
Green	Mouse	Monoclonal	Licor, UK	**1:9000**
Red	Rabbit	Polyclonal	Licor, UK	**1:9000**
Red	Rabbit	Monoclonal	Licor, UK	**1:9000**

2.4.3. Solutions and Reagents

Lysis Buffer pH 7.6

20mM Tris	Invitrogen, UK
1mM EGTA	Sigma-Aldrich, UK
320mM Sucrose	BDH, UK
0.1% Triton X100	Sigma-Aldrich, UK
1mM NaF	Sigma-Aldrich, UK

10mM Beta glycerophosphate	Sigma-Aldrich, UK
2X solubilisation Buffer	
0.5M Tris	Invitrogen, UK
Glycerol	Sigma-Aldrich, UK
10% SDS	Fisher Scientific, UK
β- mercaptoethanol	Fisher Scientific, UK
2.5% Bromophenol Blue	Fisher Scientific, UK
TBST 0.1% tween	
25mM tris	Invitrogen, UK
125 mM NaCL	Sigma-Aldrich, UK
Lowry A	
NaOH	Fisher Scientific, UK
SDS	Sigma-Aldrich, UK
Na_2Co_3	Fisher Scientific, UK
Lowry B	
$CuSo_4$	Sigma-Aldrich, UK
Na K tartarate	Fisher Scientific, UK
Folin's Reagent	Sigma-Aldrich, UK

2.4.4. Sample preparation

2.4.3.1. Preparing cell lysates

Chick micromass cardiomyocyte cultures were prepared, grown and treated with teratogens in 24 well plates as described in section 2.1.4.5.2. Cell lysates for western blotting were prepared by following method.

Cells in each well of 24 well plates were washed with ice cold PBS on ice and then cells were scraped off from the bottom of the well. 100µl of freshly prepared lysis buffer with protease inhibitor was added to each well. Plates were then kept on a plate shaker for 1 hour at 4^{o}C. Then 100µl of 2X solubilisation buffer was added to the wells.

2.4.3.2. Preparing tissue lysates

Chick hearts were collected in 1.5 ml Eppendorfs on ice. 1ml of lysis buffer with protease inhibitor was added to each 100mg of tissue, such that the final concentration of tissue solution would be 100mg/ml. Tissues were then homogenized with polytron homogenizer until all tissues were homogenized properly. During homogenization, tissues were placed on ice at all times. Once homogenized, Eppendorf tubes were kept on the wheel in the cold room for 1 hour followed by centrifugation for 5 minutes at 13000rmp at 4^{o}C. After centrifugation, supernatant was removed and collected in a separate Eppendorf tube. 10µl of supernatant was taken for the Lowry test. To the remainder of supernatant 2X solubilisation buffer was added in 1:1 ratio. After the protein concentration had been determined by the Lowry assay, 1:1 solution of lysis buffer and 2X solubilisation buffer was added to the samples to normalize them.

2.4.3.2.1. Lowry test

For the Lowry test, prepared tissue samples were diluted in 1:20 dilutions in distilled water. BSA standards were prepared in 1mg/ml concentrations. Then 1 ml of Lowry AB solution (Lowry A+ B; 1:1) was added to all samples and standards. Samples were incubated for 10 minutes. 100µl of

1:1 the Folin reagent to water was added to all samples and standards. After leaving the samples and standards for more than 45 minutes at room temperature, 200µl of each sample and standard were pipetted in to a clear 96 well plate in triplicate as shown in figure 2.5. The plate was read at 750 nm. A standard curve was plotted and proteins were normalized to 1mg/ml.

←	B1	→	←	S8	→	←	T8	→			
←	S1	→	←	T1	→						
←	S2	→	←	T2	→						
←	S3	→	←	T3	→						
←	S4	→	←	T4	→						
←	S5	→	←	T5	→						
←	S6	→	←	T6	→						
←	S7	→	←	T7	→						

Figure 2.5. Arrangement of standards and samples in 96 well plate

2.4.5. Western blotting

The proteins were denatured by heating the samples to 95^{o}C for 5 minutes in a heating block. The samples were then vortexed and centrifuged at 13000rmp for 1 minute. Then 20µl of each sample was loaded on to the precast gels, along with 1µl of marker. The gel was run in 1X electrode buffer for approximately 45 minutes at 200V. When the gel had finished running, it was removed from the electrophoresis unit. The gel was then transferred to nitrocellulose membrane. The transfer was performed in cold transfer buffer for 60 minutes at 100V. The transfer was checked by

adding few drops of Ponceaus solution on the blot. Once good transfer of protein was confirmed, the blot was cut into appropriate sections.

The rest of the procedures including blocking and adding primary and secondary antibodies were carried out in Snap i.d, using Snap i.d cassettes.

2.4.5.1. Blocking

The nitrocellulose blot was placed in a pre-soaked Snap i.d cassette. The cassette was then put in to Snap i.d and blot was blocked by 1.5% fish skin gelatine (FSG) in TBST (v/v) by turning on the Snap i.d.

2.4.5.2. Adding Primary antibody

Primary antibody (Cx 43+ GAPDH) was made in 1.5% FSG. It was added to the blot by turning off the Snap i.d. The blot was covered in primary antibody for at least 10 minutes. The blot was then washed three times with TBST by turning on the Snap i.d.

2.4.5.3. Adding Secondary antibody

Secondary antibody was made at a dilution of 1:9000 in 1.5% FSG. It was added to the blot by turning off the Snap i.d. Blot was covered in secondary antibody for at least 10 minutes in dark as secondary antibody is light sensitive. The blot was then washed three times with TBST by turning on the Snap id.

The blot was scanned by the Odyssey scanner at resolution of 84µm and was statistically analysed using Prism 5.

2.5. Statistical analyses

The raw data was calculated using Microsoft Excel and analysed by one way ANOVA with the post hoc Dunnett's test for parametric data. For nonparametric data (scoring of cells), a Kruskal-Wallis test was performed and a Dunn's post hoc test was used to test the significance. In all cases p value < 0.05 was considered significant. For all statistical analysis Prism 5 software was used.

Chapter 3

Evaluation of chick cardiomyocyte micromass culture

3.1. Introduction

In order to obtain reproducible results with chick cardiomyocyte cultures, it was necessary to establish a protocol. This required determining the optimum day of explantation and cell density which were necessary to form cultures with identifiable contractile foci. Once a standardised protocol had been established, teratogenicity of different chemicals was measured by observation of contractile activity. The cytotoxic effects were measured by the use of the resazurin reduction and kenacid blue assays (Knox *et al.*, 1986; Clothier, 1992).

3.1.1. Chick embryonic development

Some embryonic development has already occurred before the egg is laid, and usually stops until proper cell environmental conditions are established by incubation under appropriate conditions for development to restart. At first, all the cells are alike, but as the embryo develops, cell differences are observed. Some cells may become vital organs; others become a wing or leg. Soon after incubation begins, the primitive streak appears as a pointed thickened layer of cells in caudal or tail end of the embryo. The formation of the primitive streak starts the process of gastrulation. Gastrulation is the process of development of three germ cell layers by mass migration of cells, so that they come to lie in appropriate areas and are ready to begin differentiation. The development of gut and neural tube follows gastrulation and starts from the first day of development. On day 2 of incubation, small blood islands form and begin linking and form a vascular system (vitelline circulation), at the same time the heart is being joined by

these blood islands and beginning to beat. The neural groove starts to close and forms three distinct brain regions (forebrain, mid brain and hind brain). By the end of the third day of incubation, the beak begins to develop and limb buds for future wings and legs become visible. The mouth, tongue, and nasal pits develop as parts of the digestive and respiratory systems. At this time the head and tail regions come close together so the embryo forms a "C" shape. The heart continues to enlarge even though it has not been enclosed within the body. At the end of the fourth day, the embryo develops all organs needed to sustain life after hatching, and most of the embryo's parts can be identified as shown in figure 3.1. However the chick embryo can hardly, be distinguished from mammalian embryos at this time point. The growth and development of the embryo becomes more rapid beyond this time up until 21 days of incubation when the chick finally begins its escape from the shell (Bellairs & Osmond, 2005).

Fig 3.1 Developmental stages of chick embryo from day 4 to day 6

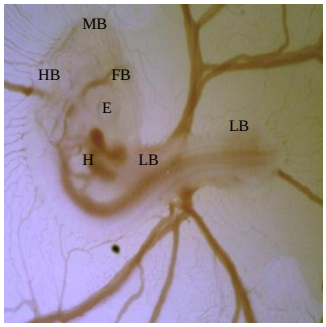
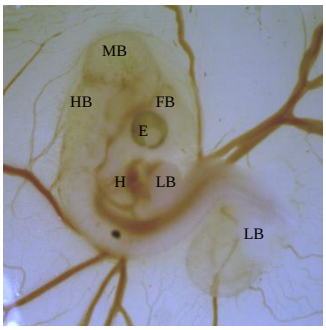
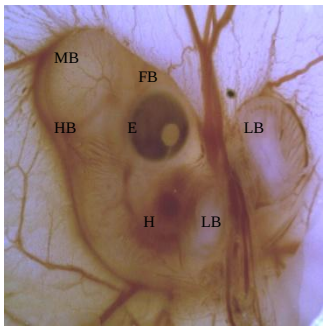
Days of incubation	Photographs of chicks	HH stage	HH stage description (Hamburger & Hamilton, 1951)
Day 4 (A)		HH 24	Limb buds and wings are much longer then are wide. The toe plate in leg bud is distinct but toes are not demarcated.
Day 5 (B)		HH 26	Limb buds are considerably longer and contour of digital plate is round. Demarcation of first three toes is distinct. Beak is barely prominent.
Day 6 (C)		HH 29	Wings are bent at elbow and second digit is larger than others. Grooves between the digits are prominent. Beak is more prominent but no egg tooth is visible.

Fig.3.1: Shows external features of day 4 (A), day 5 (B), and day 6 (C) chick embryos. Original magnification 200x. MB midbrain; FB forebrain; HB hind brain; E eye; H heart; LB limb bud.

3.1.2. Development of chick heart

The heart is one of the earliest organs to develop during embryonic life. The chick heart primordial cells develop few hours after incubation in the epiblast layer at the time of its separation from the hypoblast. These cardiac progenitor cells moved towards the rostral end of the primitive streak at around 12-13 hours of incubation. These then begin to extend caudally in the primitive streak. These cardiac progenitor cells give rise to endocardium, myocardium, and parietal pericardium. By 18-22 hours these cardiac progenitor cells move toward the lateral plate mesoderm and align themselves in to the splanchnic layer on both sides of the primitive streak and form the crescent shaped primary heart field. These heart forming cells ultimately differentiate to form different segments of the heart upon inducing signals from the head organizer (Henson's node in chick), whereas the definitive endoderm provides some signals later that reinforce the initial induction of the precardiac mesoderm (Martinsen, 2005). Once differentiated, these presumptive endocardial cells begin to separate from the splanchnic mesoderm by the process of epithelial to mesenchymal transformation and migrate to start the formation of the bilateral heart tubes at around 23 hours. The two heart tubes begin to fuse and form a straight heart at 26-29 hours at HH stage 8+ or 9 (Manner, 2000). The newly formed heart begins to beat and starts its function even before the process of looping at nearly around 42 hours of gestation. The initial contractions by myocytes are peristaltic, but with maturation of the

heart, and once the conduction system is fully functional, the contractions become more synchronous. Although beating and contractile activity is an intrinsic property of cardiomyocytes, yet there must be some signals to initiate and propagate these contractions within different chambers of the heart. Cardiomyocytes contain some contractile proteins such as actin and myosin, which are assembled at the sarcomere of cardiac muscles and help in the generation and propagation of contractions and beating. Cardiomyocytes also have some specialized communications at cellular junctions known as gap junctions which allow them to contract synchronously (Bellairs and Osmond, 2005; Fishman *et al.*, 1997).

3.2. Effects of days of development on explantation of chick heart micromass culture systems

3.2.1. Introduction

Micromass culture systems have been used to study development and to evaluate the potential teratogenicity of chemicals (Flint, 1980). This chapter will focus on the heart micromass culture technique as an embryotoxicity testing system. The methods for cardiomyocyte micromass culture were evaluated to be used for all micromass experimentations carried out in this project. The chick was chosen because of the ease of storage and handling of the eggs.

3.2.2. Materials and Methods

Chick cardiomyocytes were seeded into 24 well plates at a cell density of 3.0×10^6 cell ml^{-1} and cultured in DMEM supplemented growth media

(chapter 2, section 2.1.4.5.2). For each time point triplicate plates were established. All micromass cultures were maintained at 37ºC, 5% CO_2 (v/v) in air. To determine the optimal day of explantation, isolations were taken from day 4, 5 and 6. The resazurin reduction, the kenacid blue (chapter 2, section2.1.5.2) assays and microscopic observations were employed to detect the optimal day, suitable for chick micromass cultures used in this project.

3.2.3. Results

The microscopic observations revealed that micromass cultures of cardiomyocytes from days 4, 5 and 6 appeared to attach successfully to the bottom of tissue culture treated plastic plates with majority of foci having good contractile activity (Figure 3.4). However, it has been observed that those micromass cultures taken from day 4 have a tendency to detach during the total protein (kenacid blue) assay, resulting in a lower absorption reading than would be otherwise expected as shown in figure 3.2. The reason for this detachment has yet to be investigated, but it may well be related to the development of the capacity of the cells to adhere to other cells and the tissue culture plate. The cells in the early stages of development are likely to be more mobile within the embryo until the tissues are developed. Resorufin production by cells was consistent throughout all explantation days (figure 3.3).

3.2.4. Conclusions

Heart micromass cultures generated from days 4, 5 and 6 showed a good tendency to differentiate in to contractile foci with beating. However

cultures from day 4 had poorer attachment capacity than embryos from other days. Day 5 embryos were also chosen in this study due to the fact that they still have differentiating cells within developing organs, particularly the heart, brain and limb buds. The time of embryonic organ development is an important factor, due to the fact that differentiating cells have the maximum potential to be affected by teratogens (Moore & Persaud, 1988).

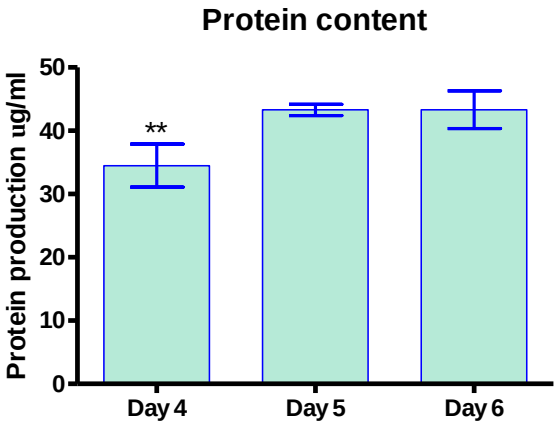


Figure 3.2

Effects of explantation day on protein content (µgml^{-1}) of heart micromass cultures 144 hours post seeding grown in DMEM. The protein content was significantly lower at day 4 compared to cultures explanted at day 5 and day 6 (mean±S.D, n=3). {** ($p<0.01$) for Dunnett's post hoc test}.

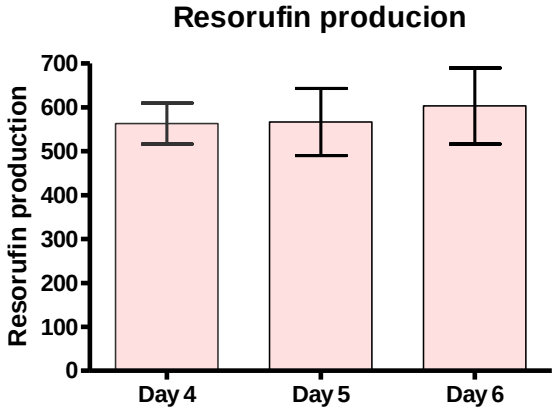


Figure 3.3

Effect of explantation day on resorufin production of heart micromass cultures 144 hours post seeding grown in DMEM. The resorufin production was consistent throughout all explantation days. (mean± S.D, n=3).

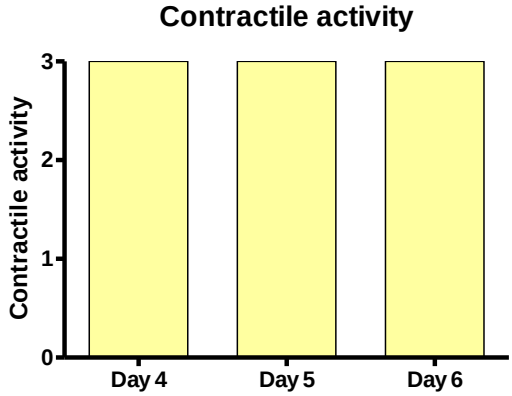


Figure 3.4

Effects of days of explantation on contractile activity of chick cardiomyocytes cultured in DMEM supplemented medium. The contractile activity was consistent for all 3 days.
(mean± S.D, n=3).

3.3. Effects of seeding density on optimal chick heart micromass culture systems

3.3.1. Introduction

Preliminary studies were carried out to determine a suitable seeding density whereby the heart micromass would have optimal culture conditions. In addition, the resazurin reduction, and kenacid blue assays for cell activity and total protein respectively, were optimized for use with the micromass culture when utilizing 24 well plates.

3.3.2. Material and Methods

The 24 embryonic hearts from day 5 embryos generate approximately 5 x 10^6 cells sufficient for 8 replicates of micromass cultures. A larger number of embryos could be dissected but the tissue extraction step can become too long with the resultant risk of lower overall viability. Micromass cultures were seeded at 1 x 10^6cells ml^{-1}, 2 x 10^6cells ml^{-1}, 3 x 10^6cells ml^{-1}, 4 x 10^6cells ml^{-1}, 5 x 10^6cells $ml^{-1}$and 6 x 10^6cells ml^{-1}. Twenty microlitre droplets of cell suspension were seeded per well into 24 well plates and incubated at 37^{o}C, 5% CO_2 in air with a relative humidity of 100% for 2 hours, after which DMEM medium was added as described in (chapter2, section 2.1.4.5.2). The resazurin reduction and kenacid blue assays (chapter2, section 2.1.5.1 and 2.1.5.2 respectively) were performed sequentially the on the same cultures. The cells were also morphologically inspected under a light microscope for intrinsic beating and contractile activity of cardiomyocytes.

3.3.3. Results

In addition to protein measurements, microscopic observations revealed that a seeding density of $3.0x10^6$ cells ml^{-1} resulted in optimal culture. At densities 1.0×10^6 cells ml^{-1} to 4.0×10^6 cells ml^{-1}, cellular protein measurement increased in direct proportion to cell densities which reached to a plateau at 5.0×10^6 cells ml^{-1} and 6.0×10^6 cells ml^{-1} (figure 3.5). This apparent inconsistency is associated with the increased cellular packing, within the micromass that occurs at higher seeding densities.

At seeding densities ranging from 1.0×10^6 cells ml^{-1} to 4.0×10^6 cells ml^{-1} resorufin production also increased proportionally with increasing density. A plateau of resorufin production was achieved at seeding densities between 5.0×10^6 cells ml^{-1} and 6.0×10^6 cells ml^{-1} which was consistent with the total well protein content reaching a maximum at these higher seeding densities (figure 3.6).

When the cells were plated at 1.0×10^6cells ml^{-1}, the micromass cultures generated were very small. At 2.0×10^6 cells ml^{-1} cultures were satisfactory in terms of attachment and cardiomyocyte beating could be observed, as found for the 3.0×10^6 cells ml^{-1} recommended cell density, but at 2.0×10^6 cells ml^{-1} cells did not show any joining with one another and intrinsic synchronous beating was not observed in all areas of the micromass cultures at this seeding density. At 5.0×10^6 cells ml^{-1} and above cultures attached sufficiently but were too densely packed and it was very difficult to observe individual beating of the cardiomyocytes.

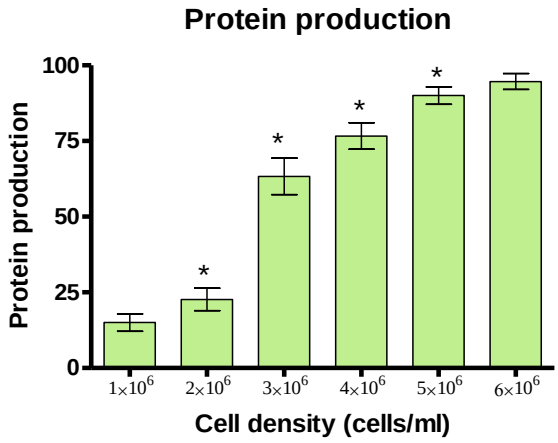


Figure 3.5

Effect of seeding density protein content of heart micromass cultures 144 hours post seeding grown in DMEM supplemented media. The protein content increased significantly with respect to previous seeding densities when cultured up to and including $5.0x10^6$ cells ml^{-1}.(mean± S.D, n=3). {* ($p<0.05$) for Dunnett's post hoc test}.

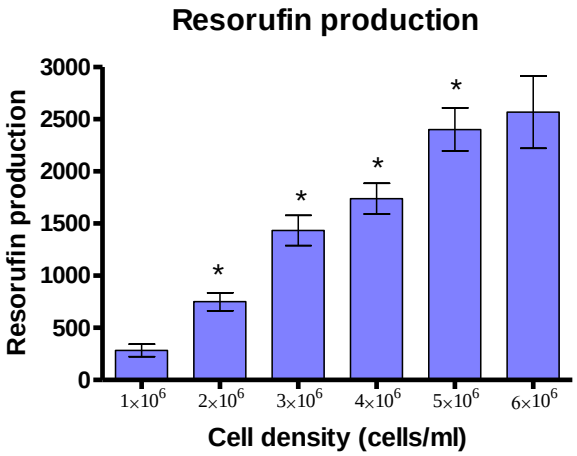


Figure 3.6

Effect of seeding density on resorufin production of cultures 144 hours post seeding grown in DMEM media. The resorufin production increased significantly with respect to previous seeding densities when cultured up to and including $5.0x10^6$ cells ml^{-1}.(mean± S.D, n=4). {* ($p<0.05$) for Dunnett's post hoc test}.

3.3.4. Conclusions

The results show that $3x10^6$ cells ml^{-1} was the optimal cell density to be used in further experiments. Concentrations below this level were shown to have no synchronous beating, while concentrations above $3x10^6$ cells ml^{-1} were too densely packed and were difficult to observe.

3.4. Identification of cell types present in chick heart micromass cultures

Micromass (MM) cell cultures are derived from primary cells, and involve the dissection of relevant organ or tissue to be used, with subsequent enzymatic dissociation of tissues in to cells, before plating the cell cultures into a suitable growth medium. Although the tissues can be accurately dissected under the microscope, it is questionable whether there are any other cell types present within the MM cultures apart from the cells which are required i.e. cardiomyocytes. The purpose of this investigation was to observe whether the heart MM cells differentiated into cardiomyocytes or other cell types were also present. To determine the cell types present within the cardiomyocyte cultures, cells were stained with either histological or immunohistochemical staining.

3.4.1. Histological staining

In order to detect particular cell types within MM cultures, traditional histological staining techniques can be used. For example, the MM cultures derived from embryonic hearts can be stained to detect cardiomyocytes by using stains that are specific to muscle these include

Van Gieson's and Masson's Trichrome (Carleton, 1967). The heart is composed of myocytes that are joined together in fibres by intercalated discs (Kerr, 1999). The histology stain for muscle cells is the Masson's trichrome stain. The rationale for using this technique is that the components of trichrome stains select specific tissue due to the dye molecule size and tissue permeability (Bradbury *et al.*, 1977). When staining with Masson's Trichrome, red blood cells take up the dye consisting of the smallest molecular size; muscle is stained by an intermediate molecular size and collagen by the largest molecular size (Bradbury *et al.*, 1977).

3.4.2. Heart/cardiomyocyte immunohistochemistry

The MM cultures were stained with an antibody specific to muscle cells, to make sure that most of the cells present in the culture are cardiomyocytes. Kolker and his colleagues used an anti-tropomyosin antibody to detect cardiac cells in cultures (Kolker *et al.*, 2000). Here in this study an anti tropomyosin antibody (CH1) was used to detect muscle cells in culture. As tropomyosin is a protein which is found in the contractile apparatus of all muscle cells (Gartner *et al.*, 1994), it was necessary to double stain the micromass cultures with other antibodies specific to cardiac muscles. For these reasons an antibody specific to the myosin (sarcomere) (MF20) was double stained with an antibody marker for skeletal muscle only (12/101).

3.4.3. Materials and Methods

3.4.3.1. Histochemical staining

The following histology staining methods were performed on micromass cultures, cultured in chambers slides.

3.4.3.1.1 Haematoxylin and Eosin staining (H & E)

The following staining technique is an adapted method from Stevens, 1977. The cells were hydrated with tap water and then placed into the haematoxylin solution for 5 minutes. The haematoxylin was removed and the cells were washed several times in tap water. Then they were differentiated in acid alcohol to remove excess stain before blueing the nuclei in a saturated solution of lithium carbonate. Once stained in lithium carbonate the cells were washed in tap water and then placed into the 1% eosin for approximately 1 minute. Excess eosin stain was removed and the cells were again washed in tap water. All slides containing cells were then dehydrated through graded alcohol and then placed into xylene before mounting with DPX.

3.4.3.1.2. Masson's Trichrome

This technique is adapted from the methods described by Carleton, 1967. Cells were incubated in Weigert's haematoxylin for 10 minutes at room temperature (RT). Excess stain was removed with several washes in tap water and the nuclei 'blued' in lithium carbonate. Cells were then incubated in Ponceau-acid fuchsin solution for 5 minutes, rinsed in tap water to remove excess stain and incubated for 5 minutes in 1% phosphomolybdic acid. The cells were quickly washed before incubation

for 5 minutes in the light green solution, followed by a rinse in tap water. All slides were then dehydrated through graded alcohol and then placed into xylene before mounting with DPX.

3.4.3.1.3. Immunohistochemistry

The cardiomyocytes were seeded in 20µl droplets at $3x10^6$cells ml^{-1} in the DMEM culture medium as described in chapter 2 (section 2.3.5.1), but instead of using 24 well plates they were cultured in 8 well chamber slides. The cultures were fixed in paraformaldehyde solution (2% w/v in PBS) for 1 hour. This was aspirated and the cells were washed thrice with PBS each for 5 minutes, before being permeabilised with 0.2% Triton X-100 in PBS (v/v) for 10 minutes. Once permeabilised, the cells were incubated in 5% goat serum in PBS for 1 hour to block nonspecific bindings of secondary antibody. The GS was removed and the cells were incubated with the undiluted primary antibody (CH1) overnight at 4^oC. The primary antibody was removed and the cells were washed 3 times in 0.1% BSA in PBS (w/v). The secondary antibody, FITC goat anti-mouse, was diluted 1:300 in 0.1% BSA in PBS and incubated for 1 hour in the dark. Cells were washed in PBS (3 x 5 minutes) to remove excess unbound secondary antibody before mounting with Vectorshield™ containing DAPI. Sections were viewed under a confocal microscope.

3.4.3.1.4. Double immunofluorescence

For double immunostaining micromass cultures were treated in a similar way as described in previous section. Here two monoclonal antibodies were used to assess the composition of the cardiomyocyte MM cultures.

The primary antibody 12/101 identified specifically skeletal muscle and MF20 identified striated muscle, but also reacts with all sarcomere myosin both present in skeletal and cardiac muscle. These antibodies cross react with chick skeletal only or skeletal and cardiomyocytes respectively. The primary antibody was applied and incubated overnight, at 4°C. The MM cultures were rinsed with 0.1% BSA in PBS (w/v). The secondary antibody, fluorescein isothiocyanate (FITC) labeled goat anti-mouse was applied at a concentration of $0.1\mu gml^{-1}$ in (0.1% BSA w/v in PBS) and incubated at room temperature for 1 hour in the dark. Slides were rinsed and then washed in fresh BSA solution (0.1% w/v in PBS). The process was repeated for the second primary antibody MF20 using the secondary antibody tetramethylrhodamine isothiocyanate (TRITC) label. Slides were washed in distilled water and mounted using Vectashield™ containing DAPI ($50\mu gml^{-1}$). The slides were viewed under a confocal Microscope.

3.4.4. Results

3.4.4.1. H & E and Masson's trichrome staining

H & E staining of chick cardiomyocytes shows that cardiomyocytes have an irregular shape and many other cells apart from cardiomyocytes are visible as well. Also cells in the culture form connections with each other and it's very hard to distinguish individual cells in contractile units (fig 3.7). Masson's trichrome clearly reveals the presence of other cells (fibroblasts and epithelial cells) surrounding cardiomyocytes (fig 3.8).

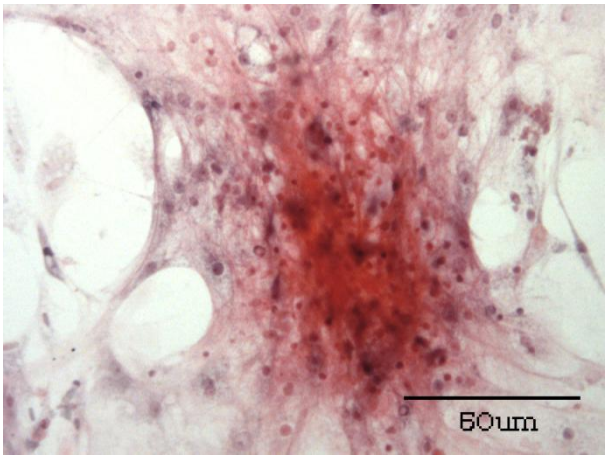


Fig. 3.7. H & E staining of cardiomyocytes Original magnification 200x.

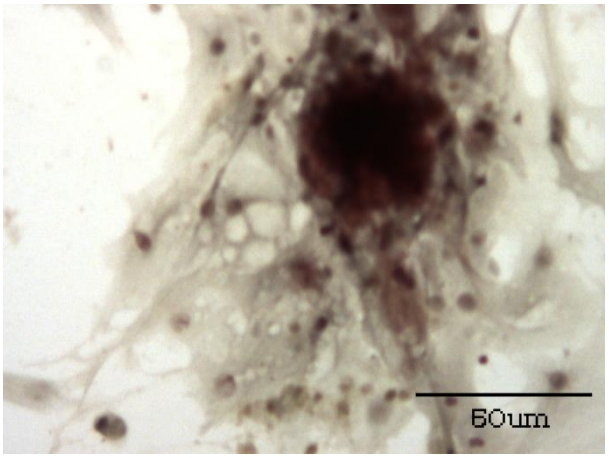


Fig. 3.8. Masson's trichrome staining of cardiomyocytes staining of cardiomyocytes. Original magnification 200x.

3.4.4.2. Immunohistochemistry

The chick heart micromass cultures were stained with antitropomyosin (CH1) as shown in figure 3.9 (A). Positive stain (green) can be clearly seen in culture, indicating that there were predominately muscle cells in culture. The staining with DAPI indicates the presence of active viable cells prior to fixation in micromass culture fig 3.9 (B & C).

To identify the presence of cardiac muscle cells in the cultures double immunostaining was done. The positive labeling with MF20 (reacts with sarcomere myosin of all muscles) for striated muscle indicates that most of the muscle cells present in the culture were striated fig 3.10 (A). While labeling with skeletal muscle specific antibody (12/101) shows negative staining fig 3.10 (B), indicating that there were no skeletal muscle cells present. The nuclear labelling with DAPI show that nuclei were all bound to MF20, while 12/101 shows negative stain fig 3.10 (C & D). These results confirm the presence of cardiomyocytes in micromass cultures.

3.4.5. Conclusions

The histological staining results show some epithelial cells as well apart from cardiomyocytes. The antibodies used to identify the cardiomyocytes in chicks were all anti mammalian antibodies, but these antibodies reacted with chick cardiomyocytes confirmed that most of the cells present in the micromass cultures were cardiomyocytes. No skeletal muscle or epithelial tissue had overgrown the cardiomyocytes.

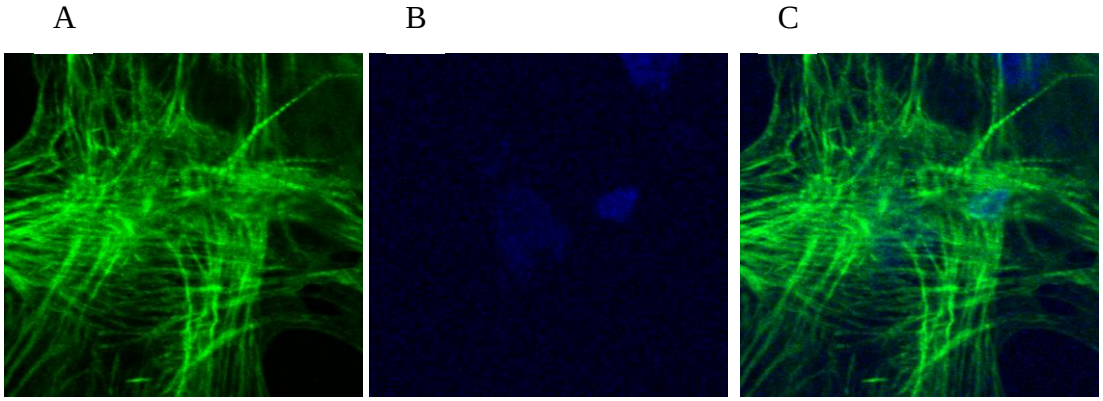


Figure 3.9.{A}Immunofluoresence staining of tropomyosin (CH1) in chick heart micromass cultured in DMEM supplemented media. {B} the same cultures with DAPI staining, {C} both staining for tropomyosin and nuclei. Original magnification 200x.

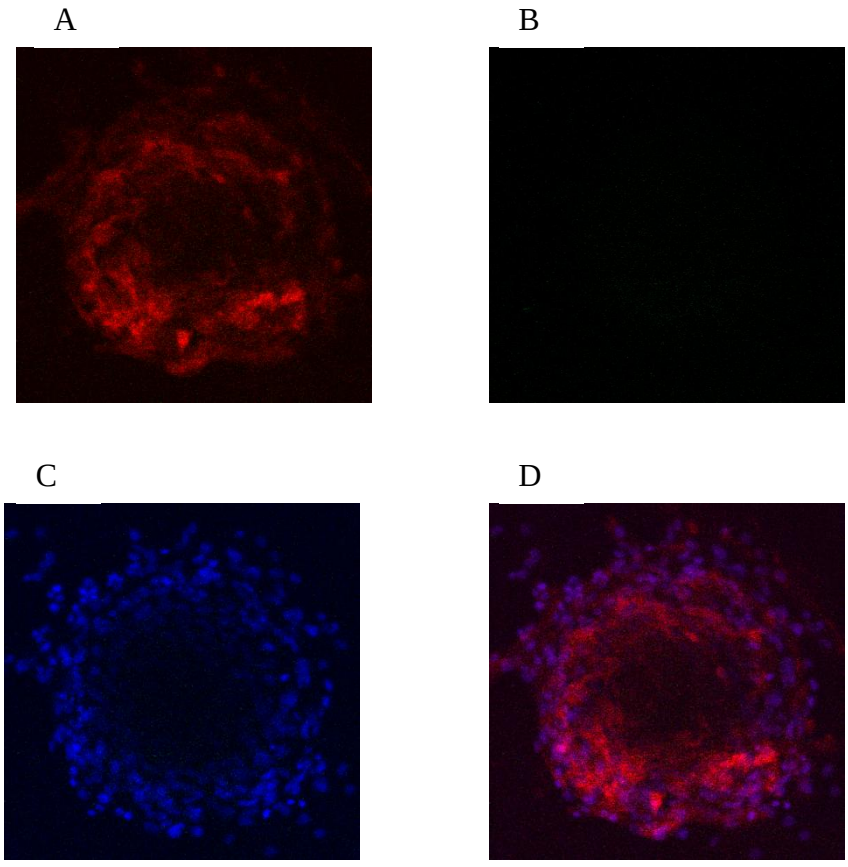


Figure 3.10. Shows Double immunofluorescence for {A} myosin (MF20 stained with TRITC) and staining for {B} skeletal muscle (12/101 with FITC) in chick cardiomyocyte micromass cultures. {C and D} shows DAPI nuclear staining. Original magnification 200x.

3.5. Conclusions

The results in this chapter demonstrate that, from all the days examined it was clear that for both day 5 and day 6 cells, the cellular differentiation, protein level and cell viability were similar while day 4 cells showed less protein content. The day 5 embryos still have the ability to differentiate, and for teratogenicity testing it is necessary to test the chemicals during the embryogenesis period. As far as cell density is concerned 3×10^6 cells/ml were chosen due to the fact that cell densities below this showed no intrinsic synchronous beating while at higher densities cells were highly packed and it was very difficult to analyze them individually.

The histological staining showed apart from cardiac cells some epithelial cells were present as well. However double immunofluoresence revealed that the majority of cells in micromass cultures were cardiomyocytes and not endothelial cells or skeletal muscle cells.

Chapter 4

Evaluation of chick embryos in ovo culture

4.1. Introduction

For in ovo culture, preliminary experiments were conducted to ensure that the injection volume, PBS (vehicle control), neutral red dye (used as a guide for injections) and Para film (to seal the eggs) had no teratogenic or toxic effects on chick embryos. Also ethanol a known teratogen was used in two different concentrations to check the effectiveness of the technique. Furthermore a scoring system was also developed with reference to Hamburger and Hamilton (Hamburger & Hamilton, 1951) developmental series to compare the results of different treatments.

4.2. Materials and methods

White leghorn fertilized chicken eggs were obtained from Henry Stewart and Co.Ltd as described in chapter 2 section 2.1.1.1. Eggs were randomly selected and divided in to 5 groups immediately before incubation. The first group was incubated as the non-treated group (n = 12). The rest were labelled according to different treatment modes, either PBS (vehicle control, n = 12), Neutral red dye, or EtOH solutions at different doses (10%, n = 12, and 20%, n = 12 v/v in PBS) (chapter 2, section 2.2.3.1).

The day on which the eggs were incubated was counted as day zero. On day 4 of incubation the eggs were taken out of the incubator 3 at a time, after being swabbed down with 70% ethanol and under sterile conditions the blunt end of eggs was struck with forceps to make a small hole. Eggs were microinjected with 100µl of either neutral red dye dissolved in PBS, 10% or 20% ethanol in PBS, or PBS alone, some eggs were also injected with 50 µl of PBS using a 25- gauge needle attached to a 1 ml disposable

syringe. All injections were under the vitelline membrane through the air sac region. After being injected the holes were sealed with parafilm and taped with insulating tape in order to avoid drying out of the embryos. Only viable embryos were injected in each group. All the eggs were placed blunt end up into the automated egg turner and incubated at 37.5 °C and relative humidity of 60% with 5% CO_2 in air until day 9 of incubation. Non treated embryos were just struck and sealed with parafilm and adhesive tape without any injection (chapter 2, section 2.2.3.1).

4.2.1. Sample collection and measurements

On day 9 eggs were cracked under the sterile hood and embryos were examined in terms of their viability. After removing all the membranes viable embryos were examined for any growth retardation by measuring crown rump length, and any malformations or gross abnormalities observed including limb deformities, facial defects, and brain vesicle development according to the criteria shown in table 4.1 (chapter 2, section 2.2.3.2).

Table 4.1. Morphological scoring system based on Hamburger and Hamilton staging of chick development (Hamburger & Hamilton, 1951).

EMBRYONIC FEATURE	0	1	2	3	4
VITELLINE CIRCULATION			5-6 large vitelline vessels	Extensive network of vessels	Entire vitelline membrane well supplied
FLEXION	None	Cranial flexure visible	Trunk rotation in addition to cranial flexion visible	Trunk rotation and tail bud rotation visible	Cranial flexure, trunk rotation and tail bud bending visible
HEART	No beating	Paired heart primordia	Beating heart bent to right	Atrioventricular canal and ventricular loop	Four-chambered appearance
BRAIN	Primitive streak	Neural folds visible	Brain vesicle, Anterior neuropore closed	Telencephalon enlarged, Forebrain forms right angle with hindbrain	Enlarged and transparent, Forebrain parallel to hindbrain
GROSS FACIAL DEFORMITIES	None	Primary optic vesicles	Optic vesicles constricted at base	Optic cup completely formed on one side	Optic cup completely formed on both sides + beak is fully formed
LIMBS	None	None	None	Digits and toes visible	Digits and toes visible on both sides

Hearts were then taken out and fixed in 10% formaldehyde. All the hearts were kept at 4^{o}C, until used for sectioning.

4.2.2. Paraffin tissue processing for sectioning

Fixed hearts were taken through a series of processes starting from dehydration and clearing to wax impregnation before being sectioned as shown in chapter 2, section 2.2.3.3).

4.2.3. Automatic tissue processing

An automatic tissue processor, (Leica TP1020) was used to perform all the above mentioned processes. The tissues were sectioned at 25µm by microtome and slides were prepared using glass slides.

4.2.4. Haematoxylin and Eosin staining

Once all the slides were prepared, they were hydrated with water and then placed into the Haematoxylin solution for 5 minutes. The haematoxylin was removed and tissues were washed with tap water. Tissues were differentiated in acid alcohol to remove excess stain before bluing the nuclei in a saturated solution of lithium carbonate. The tissues were again washed in tap water then placed in to 1% eosin for approximately 1-2 minutes. Excess stain was removed and slides were washed in tap water. Finally the tissues were dehydrated by passing through increasing concentrations of alcohol and allowed to dry before being covered with cover slips.

4.3. Results

This investigation was done as a pilot study to check the reliability of in ovo culture, which would be used in our future studies. Ethanol was used as a test chemical, with 2 different concentrations (10% and 20%). Embryos were scored according to the criteria shown in table 4.1. Embryos which were either kept untouched or only treated with PBS and neutral red dye showed no gross abnormalities. Statistical analysis showed that the embryos treated with 10 and 20% ethanol were significantly different to other all groups in terms of forelimb developmental

abnormalities Fig 4.3 (E), brain vesicle defects figure 4.3 (D). Also ethanol treated embryos had heart defects figure 4.3 (C) and gross facial malformations figure 4.3 (F) as compared to controls. Moreover it was also observed that the embryos with ethanol treatment were smaller in length figure 4.2 (B) with less flexion, and also had a less vitelline circulation fig 4.3 (A).

Histological examination of hearts showed dilated ventricles and few papillary muscles in both groups of ethanol treated embryos (fig4.1 C, D), while all other groups in this study showed no histological abnormalities of the hearts (figure 4.1 A, B, E).

4.4. Conclusions

The results of this chapter show that in ovo culture is a good model to detect developmental defects caused by teratogens. In preliminary studies ethanol was used to check the reliability of this system, as ethanol is a known teratogen and proved to have toxic effects in many animal models. The chick embryos also show spectrum of malformations when treated with ethanol. Also effects of vehicle control, dye and adhesive tapes were examined and none of them showed any defects in developing embryos. On the basis of these preliminary experiments, this system was chosen for further experiments with different teratogens.

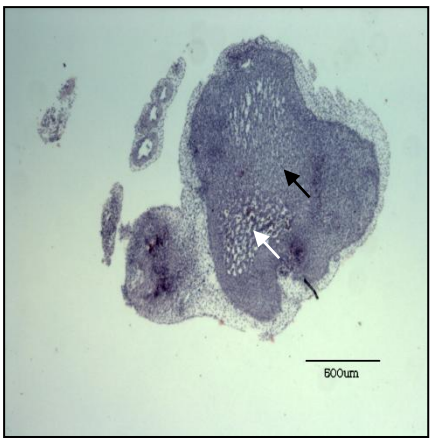


Fig A: Heart without any treatment

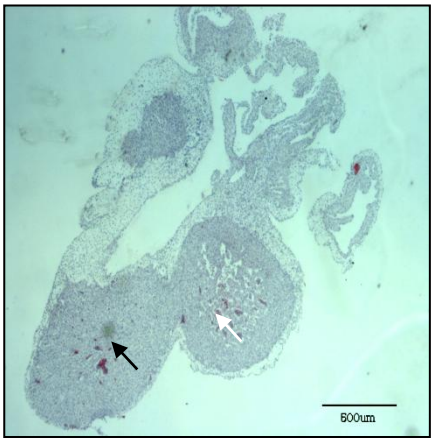


Fig B: Hearts treated with PBS

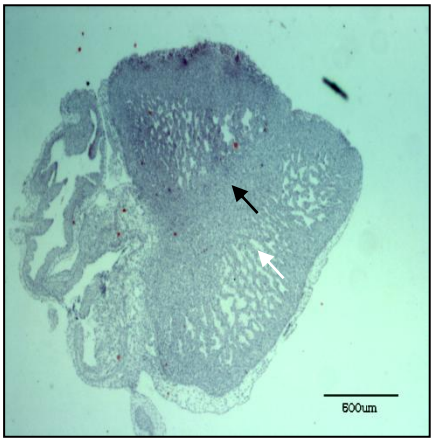


Fig C: Hearts treated with 10% Ethanol

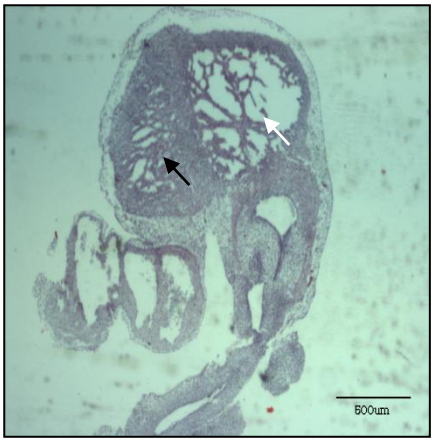


Fig D: Hearts treated with 20% Ethanol

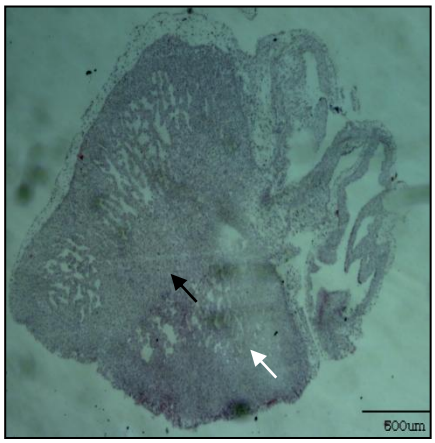


Fig E: Hearts treated with Neutral red

Fig. 4.1 Showing 5 days old Chick Hearts without any treatment A, treated with PBS B, 10% Ethanol C, 20% ethanol D and Neutral red dye E.

Original magnification x200). Black arrows indicate interventricular septum and white arrow indicates ventricular muscular wall.

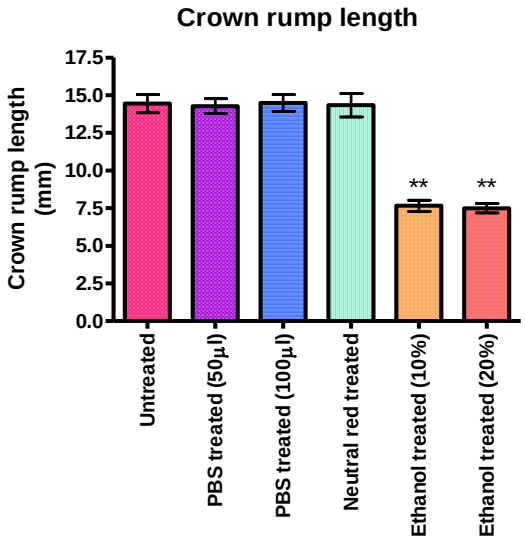


Figure 4.2 Shows crown rump length of different groups of day 9 old chick embryos {** ($p<0.01$) for Dunnett's post hoc test}.

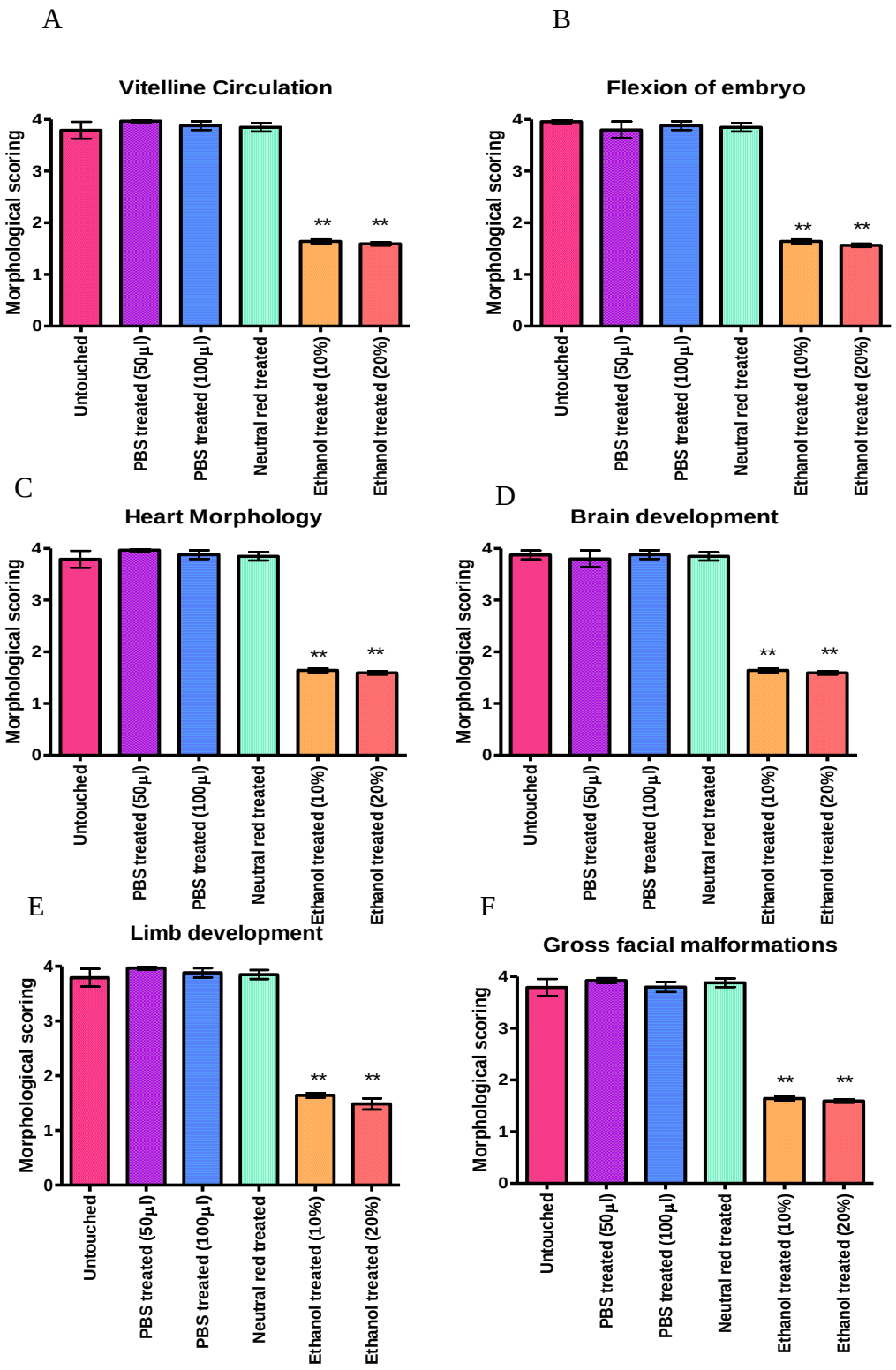


Fig 4.3: Showing vitelline circulation (A), flexion of embryo (B), heart morphology (C), brain development (D), limb development (E), gross facial malformation (F), in day 9 chick embryos with different treatments. {** ($p<0.01$) for Dunnett's post hoc test}.

Chapter 5

Evaluation of known teratogens on cardiomyocyte micromass culture and potential protective effects of vitamin supplementation

5.1. Introduction

Proper nutrition during pregnancy plays an important role for normal growth and development of the fetus. A healthy diet with both macro- and micronutrients is essential for a woman to sustain pregnancy, and also for appropriate growth of the fetus (Shah & Ohlsson, 2009). This chapter aims to evaluate the effects of certain nutritional elements particularly vitamins and other drugs which might have some impacts on pregnancy and embryonic heart development.

In order to investigate the effects, chick heart cells were cultured by micromass culture technique (chapter 2 section 2.1.4.5.2), a useful techniques for developmental toxicity studies. Micromass is a culture of primary cells (heart or the brain and limb buds) at high density, obtained from chick, mouse or rat embryos (Flint & Orton, 1984; Wiger *et al.*, 1988; Atterwill *et al.*, 1992).

5.1.1. Folate

The term folate represents all forms of the B vitamin series with same structure and function, including many derivatives found in biological systems (Tamura & Picciano, 2006). The primary ingested forms of folate include folic acid consumed in supplements and added to foods as the mono glutamyl form and the main naturally occurring folates that exist in various vegetables and fruits, such as tetra hydro folate species, are often in the poly glutamyl form (Gregory, 2001).

5.1.1.1. Structure of Folate

Folic acid is composed of a pterin core ring, which is conjugated to para-aminobenzoic acid by a methylene bridge to form pteroic acid as shown in fig 5.1. The carboxyl group of the para-amino benzoic acid is bound via a peptide to the alpha amino groups of glutamate to form folic acid. All three components must be present for biological activity (Moat *et al.*, 2004).

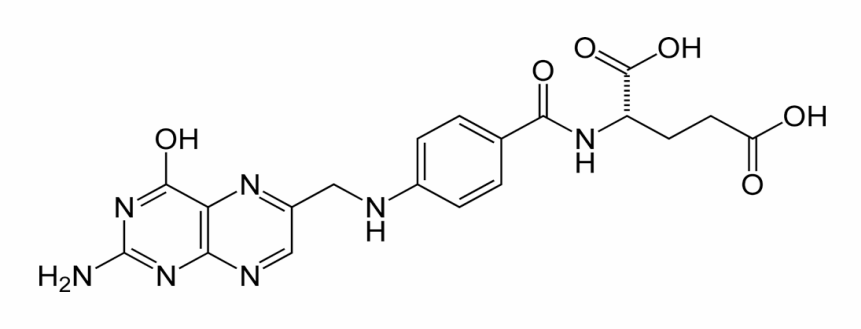


Fig.5.1. Structure of folic acid

5.1.1.2. Sources of Folate

All mammals lack the ability to synthesise folate de novo, as there are no folate forming enzymes present in them and therefore they depend entirely on preformed folates in the diet (Moat *et al.*, 2004). Folates which are consumed in the diet are either present in their natural form, found in green leafy vegetables e.g. broccoli, cauliflower, parsnips, fruits like oranges and pulses etc. In addition liver and yeast extracts are also good sources of natural folates. In the synthetic form (folic acid), they are found in many breakfast cereals, flour and some bread as a fortificant. There are also several multivitamin supplements available in the market which contain folic acids (Hughes & Buttris, 2000; Williumson, 2006). Food folates (natural) exist primarily as polyglutamate forms with bioavailability of

50% compared to that of folic acid (synthetic form) which is present as monoglutamate (Moat *et al.*, 2004).

5.1.1.3. Dietary recommendations

It is advised by the expert advisory group on folic acid (1992) in UK that all women of child bearing age, who may become pregnant, should take a supplement of 400µg of folic acid/day until the 12^{th} week of pregnancy and also try to consume foods which are rich sources of naturally occurring folates to prevent the occurrence of NTDs for the first time, while they should take 5mg/day to prevent recurrence of NTD (Williumson, 2006).

5.1.1.4. Uses/Biochemical functions

Folates play a role in the biosynthesis of pyrimidines and purines and thus in the synthesis of DNA and cell division. High intake of folates reduces the risk of certain cancers, such as colorectal cancers and breast cancers, as well as cardiovascular diseases (Hughes & Buttris, 2000). Folate has several important functions as a co enzyme including redox processing, DNA synthesis, protein metabolism and serving as a methyl donor (Dyke *et al.*, 2002). All these processes are essential for normal metabolism and homeostasis of the body; therefore any deficiency of folates leads to defective methylation and accumulation of cell metabolites and eventually disturbs normal homeostasis (Power *et al.*, 2000).

5.1.1.5. Absorption

Absorption of dietary folate is a two step process which starts in the proximal small intestine. Enzymatic deconjugation in the intestine is catalysed by a pteroyl polyglutamate hydrolase associated with the jejunal

brush border membrane, with possibly some contribution by hydrolase activity from pancreatic secretions, whereas pure folic acid is absorbed unmodified or in the reduced or methylated form. The optimum pH for folate transport is ~5 while enzymatic deconjugation is ~6-7. Physiological conditions or medications that alter the pH of small intestine could impair folate absorption. The liver is the main storage organ of folates (Gregory, 2001; Dyke *et al.*, 2002).

5.1.1.6. Role in embryogenesis

An adequate supply of dietary folate in pregnancy is essential for normal embryonic development, including that of cardiovascular development, and its supplementation lowers the incidence of congenital defects (Tang *et al.*, 2004; Torrens *et al.*, 2006). Much of the birth defect data focus on the well substantiated relationship between folic acid and prevention of neural tube defects (Green, 2002). It has been shown in several studies that supplemental folic acid can prevent >70% NTDs and spina bifida. Multivitamins that contain folic acid, have also been shown to prevent cardiovascular abnormalities, while a higher risk of abruptio placenta has been found in those mothers that are folate deficient (Burgoon *et al.*, 2002). Several epidemiological studies showed an association between folic acid deficiency and the occurrence of congenital heart disease and a significant reduction of conotruncal heart defects with the daily intake of folic acid fortified cereals (Rosenquist *et al.*, 1996; Hagroot *et al.*, 2006).

Moreover, it is also proved by experimental studies that additional supplementation with folic acid could prevent neural tube, orofacial and

conotruncal defects (Boot *et al.*, 2003). However the mechanisms underlying this effect remain unidentified (Brauer & Rosenquist, 2002; Hobbs *et al.*, 2005), but it is also thought that folate is intimately involved in the metabolism of homocystine and hyperhomocystinuria in maternal urine and a raised homocystine level in amniotic fluids is observed in the fetuses born with NTDs and cardiac defects (Brauer & Rosenquist, 2002).

5.1.2. Vitamin C

Vitamin C (ascorbic acid) is a water soluble micronutrient which is required for normal metabolic functioning of the body (Jaffe, 1984). Vitamin C acts as an antioxidant by removing oxygen derived free radicals, and thus protects the cells and tissues from damage by oxidation (Ames *et al.*, 1993). Ascorbic acid acts an electron donor for different enzymes, and it also participates in the biosynthesis of nor-epinephrine from dopamine. In addition, L-ascorbic acid helps in the maintenance of integrity of connective tissue, and plays a preventive and therapeutic role in many diseases including cancer and atherosclerosis (Chen *et al.*, 2005; Das *et al.*, 2006).

5.1.2.1. Sources of vitamin C

Humans and other primates have lost the ability to synthesise vitamin C. Vitamin C or ascorbic acid is synthesised by liver cells in many animals (Nishikimi & Yagi, 1991) but not in humans due to a mutation in the gene coding for L-gulonolactone oxidase, an enzyme required for the biosynthesis of vitamin C via the glucoronic acid pathway. So humans rely totally on their diet to get enough vitamin C. Many citrus fruits and

vegetables e.g. broccoli, brussel sprouts, cauliflower, cabbage, green leafy vegetables, red peppers, chillies, watercress, parsley, blackcurrants, strawberries, kiwi fruit, guavas, citrus fruits are a rich source of vitamin C and it is also commercially available in tablet forms (Asard *et al.*, 2004).

5.1.2.2. Recommended dose

The current recommended dose during pregnancy in the western world is 60-80 mg/day. The Tolerable Upper Level (UL) for adults is 2 g/day; the adverse effects upon which the UL is based are osmotic diarrhea and gastrointestinal disturbances (Rumbold & Crowther, 2005).

5.1.2.3. Absorbtion, metabolism and excretion

Once ingested, it is absorbed in the intestine through a sodium-dependent active transport process (Tsao, 1997; Rumsey & Levine, 1998). Nearly 70 to 90 percent of ingested dietary ascorbic acid (30 to 180 mg/day) is usually absorbed; however, absorption falls to about 50 percent or less with increasing doses at or above 1 g/day (Kallner *et al.*, 1979). With large intakes of the vitamin, unabsorbed ascorbate is degraded in the intestine, which causes diarrhea and intestinal discomfort. Cellular transport of ascorbic acid is mediated by transporters that vary by cell type (Jacob *et al.*, 1992; Tsao, 1997). Once taken up by the cells, it is localized mostly in the cytoplasm and exists predominately in the free reduced form as the ascorbate mono anion. Apart from its dose dependant absorption, its levels are maintained in the body by renal excretion as well. These two regulatory mechanisms allow conservation of ascorbic acid by the body

during low intakes and limitation of plasma levels at higher concentrations (Tsao, 1997).

5.1.3. Vitamin E

Vitamin E is a lipid soluble vitamin and includes a group of compounds with similar antioxidant activity. It was in 1922 when Bishop and Evon discovered the indispensible role of vitamin E in reproduction of rats (Evans & Bishop, 1922). Vitamin E was subsequently placed in the context of cellular antioxidant system and proved to be effective in preventing peroxidation of lipids and other free radical derived events in the body. Epidemiological studies have reported a direct relation of vitamin E intake and reduced risks of cardiovascular diseases in dose dependent manner. In 1966 the Cambridge Heart Antioxidant Study (CHAOS) reported that daily intake of vitamin E in patients with angiographically proved coronary atherosclerosis significantly reduced the incidence of myocardial infarction and death (Losonczy *et al.*, 1996). This could be due to decrease lipid peroxidation of low density lipoproteins (Wolf, 2005). Apart from its antioxidant functions, vitamin E, in particular α-tocopherol, also plays a significant role in cell signalling, it inhibits smooth muscle cell proliferation, increases phosphoprotein phosphatase 2A activity, and decreases protein kinase C activity. Its effects on protein kinase C inhibition have been reported in human platelets and monocytes. Vitamin E also plays a critical role in the male reproductive system (Traber & Atkinson, 2007). It prevents loss of spermatogenesis and thus helps in prevention of male infertility. The deficiency of vitamin E in humans

causes abnormalities in neuromuscular systems and is characterized by ataxia, peripheral neuropathies and myopathies (Bieri *et al.*, 1983). These are likely to occur due to free radical damage to these tissues. Patients with familial vitamin E deficiency present with many neurological symptoms such as cerebellar ataxia, absence of deep tendon reflexes, vibratory and proprioceptive sensory loss, dysarthria, and +ve Babinski's sign. These symptoms can be ameliorated with up to 2000 mg of vitamin E per day (Ouahchi *et al.*, 1995).

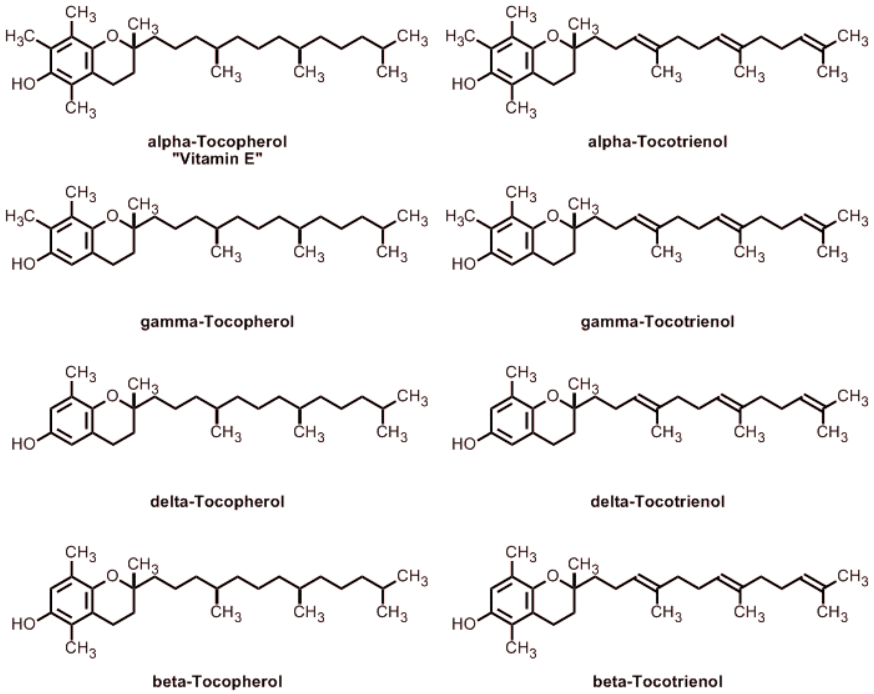
5.1.3.1. Sources of Vitamin E

Vegetable oils are the richest source of vitamin E. The α-tocopherol is also found in nuts like almonds and walnuts and its synthetic analogues are present in tablet form as well.

5.1.3.2. Structure of vitamin E

Vitamin E family comprises of eight different forms of molecules of related structure, each consists of a chromanol ring and an aliphatic side chain. These molecules are synthesized by plants and are consisting of α-, β-, γ-, and δ-tocopherols and the corresponding four tocotrienols depending on the saturation or unsaturation of their side chain respectively (Mustacich *et al.*, 2007) as shown in figure 5.2. All these molecules possess vitamin E activity and act as antioxidants. Apart from their antioxidant function they might have some other properties independent of their antioxidant activity (Schneider, 2005). Out of all naturally occurring vitamin E analogues α-, and γ- tocopherols are the most abundant in the human diet (Roy, 1999). α- tocopherol is the only one which is maintained in human plasma and

tissues due to the function of hepatic transfer protein. Therefore α-tocopherol is the most biologically active form of vitamin E and is present in the membranes of cells and organelles, where it plays an important role



in the suppression of lipid peroxidation (Traber *et al.*, 2005).

Fig.5.2. Structure of different forms of vitamin E
www.vita-dose.com/structure-of-vitamin-e.html

5.1.3.3. Metabolism of vitamin E

Once ingested all forms of vitamin E (natural and synthetic) are absorbed in the intestine together with lipids. They are then packed in to chylomicrons and transported to the liver via chylomicron remnants (Traber & Sies, 1996). There is no discrimination between all forms of vitamin E until they are taken up by the liver. Once taken up by the liver, regulation of the forms and concentrations appear to take place. The liver selectively sorts out α-tocopherol from all incoming forms for

incorporation in to VLDL. This is probably due to the presence of a specific protein called α tocopherol transfer protein (α-TTP). All other forms are excreted via bile or urine (Traber, 2007).

5.1.4. Retinoic acid

Vitamin A (retinoic acid), a fat soluble vitamin, and its active metabolites play an essential role in various life processes including cell proliferation, differentiation, vision, reproduction and immune functions (Vliet *et al.*, 2001). These compounds are also used in chemoprevention of some cancers e.g. acute promyelocytic leukemia. Isotretinoin, a vitamin A analogue, is effective in a wide variety of dermatologic disorders, including the treatment of severe, reluctant nodular acne refractory to conventional therapy (Robertson *et al.*, 2005).

Apart from its many effects on adults, Vitamin A also plays an important role during development, where it influences pattern formation of several organs including hind brain, heart, spinal cord, kidneys, and limb buds (Mic *et al.*, 2003).

Although these compounds are essential for normal development, both excess and deficiency results in developmental defects. A constellation of birth defects called retinoic acid embryopathy which includes craniofacial, cardiac and thymic abnormalities result from excessive intake of retinoids (Mulder *et al.*, 2000), while its deficiency or absence causes embryonic segmentation, growth failure and eventual resorption. These considerations make it necessary to maintain retinoid homeostasis during developmental processes (Ross *et al.*, 2000).

5.1.4.1. Sources of Retinoids

No animal has the capability for de novo synthesises of Vitamin A and therefore it must be consumed in the diet (Tzimas & Nau, 2001). The main sources of the preformed vitamin include animal foods (dairy products, liver, eggs etc.), fortified foods and pharmaceutical supplements, while several fruits and green leafy vegetables constitute rich sources for the provitamin form or carotenoids (Debier & Larondelle, 2005; Penniston & Tanumihardjo, 2006).

In Western countries, 25-75% of the total dietary intake of vitamin A includes the preformed form while the rest is being taken as carotenoids (Blomhoff & Blomhoff, 2006).

5.1.4.2. Structure of Retinoids

The retinoids are composed of three distinct structural domains, a B ionone ring, an isoprenoid tail, and a polar end group with an oxidation state (Ross *et al.*, 2000) as shown in fig 5.3.

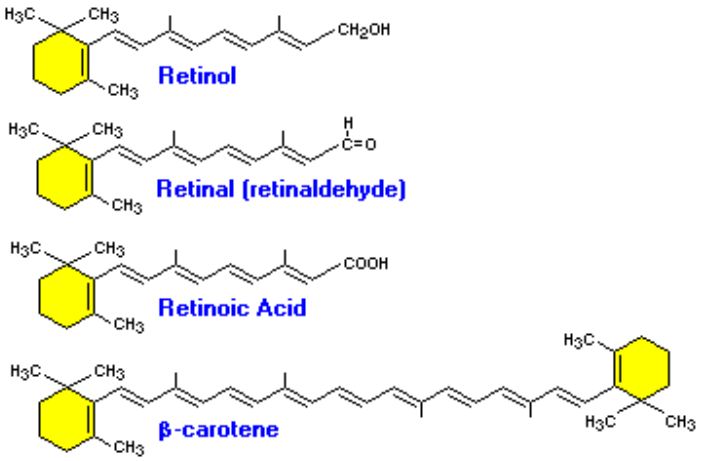


Fig.5.3:http://www.vivo.colostate.edu/hbooks/pathphys/misc_topics/vitamina.html

5.1.4.3. Metabolism of Retinoids

As mentioned previously, vitamin A is either ingested in the form of retinyl esters or β carotenes. These retinyl esters and β carotenes are hydrolysed to retinol and retinaldehyde in the intestine. Both of them bind to CRBP 11 protein. The CRBP 11-retinol complex serves as a substrate for retinol acyltransferase enzymes which reconvert the retinol to retinyl esters (Collins & Mao, 1999; Petkovich, 2001).

Once absorbed in enterocytes, these retinyl esters are packed in to chylomicrons, which are then taken up by hepatocytes. In the liver these esters are again hydrolysed into retinol. In satisfactory conditions, the newly formed retinol is transferred in the form of retinyl esters to hepatic stellate or Ito cells, where they can be stored in large quantities in the form of lipid droplets. If the vitamin A status in the body is insufficient, the retinol is bound to retinoid binding protein (RBP), and secreted in the blood, and taken up by the cells either by specific receptors or by the particular lipid composition of the cell membrane. Once inside the cell, the retinol is bound to cytoplasmic retinoid binding protein (CRBP) and oxidized to retinal and then retinoic acid. Vitamin A can be metabolized in 4 different ways, esterification, conjugation, isomerisation and oxidation. Each route will give rise to different metabolites. Retinyl esters are formed from esterification, while conjugation produces retinyl –β-glucoronide. During isomerisation there is interconversion between all-trans-retinal and 11-cis-retinal. Retinol can be reversibly oxidized to retinal, while retinal once formed is oxidized to retinoic acid in an irreversible manner.

Excess vitamin A is either excreted in urine or by the faecal route (Clagett-Dame & Deluca, 2002).

5.1.4.4. Role of retinoids in embryogenesis

Retinoic acid is an important signalling molecule during development, when administered in excess, RA produces striking teratogenic effects on early heart development. Adverse effects include either lack or fusion of the paired cardiac primordia, impaired or reversed heart looping and truncation of the posterior portion of the heart tube with abnormal expansion of anterior structures. All undesired effects depend on species, mode of administration and stage of development (Niederreither *et al.*, 2001). However maternal insufficiency of vitamin A also results in abnormal heart development and leads to abnormalities in the ventricular chambers, sometimes causing premature differentiation of ventricular cardiomyocytes and defects in the outflow tract (Zile, 2001; Niederreither *et al.*, 2003).

5.1.5. Cadmium chloride

Cadmium chloride is a non essential heavy metal with no known biological role in humans. Environmental sources of cadmium include combustion of fossil fuel, certain agriculture fertilizers, mining residues etc (Vahter *et al.*, 1992). It is also found in tobacco which is a significant source. One cigarette contains about 1-2µg of cadmium (Lewis *et al.*, 1972). Cadmium has been implicated in various deleterious effects on different species due to its very long half life. Cadmium is a well known developmental toxin. Its teratogenicity in many vertebrates is well

documented, depending on the species and the stage of treatment. Cadmium exposure gives rise to many developmental defects in chick including limb and anterior body wall defects (Gilani & Alibhai, 1990; Thompson *et al.*, 2005). Environmental exposure to cadmium also produces developmental defects in other species including rats (Chen & Hales, 1994), mouse (Jeong *et al.*, 2000), frog (Sunderman *et al.*, 1992) and zebra fish (Chan & Cheng, 2003b; Hen Chow & Cheng, 2003).

5.1.6. Sodium fluoride

Different forms of fluoride, including sodium fluoride have been added to drinking water by many countries to protect against dental caries. Other sources of exposure are through toothpastes, mouth washes, dietary supplements and food (Mullenix *et al.*, 1995). Although it is an essential component for humans at low concentrations but if consumed more than 1ppm, it causes fluorosis (Verma & Guna Sherlin, 2002), even if it is taken in lower concentrations but for prolonged duration it accumulates in different body tissues particularly bones and muscles and produce skeletal complications and locomotor impairment (Guna Sherlin & Verma, 2001; Verma & Sherlin, 2001; Verma & Guna Sherlin, 2002). Studies also reveal an association of fluorosis and increased birth defects and low birth rate in humans. Epidemiological data suggest that occupational exposure to fluorine causes abnormalities in menstrual cycles, and increases the frequency of miscarriages and abortions in female workers (Al-Hiyasat *et al.*, 2000). Animal studies also show an association between excess fluoride consumption in water and diet by the mother and abnormal

embryonic development, particularly skeletal and brain abnormalities (Goh & Neff, 2003). Furthermore excess fluoride has a toxic effect on the male reproductive system, resulting in abnormal spermatogenesis and infertility (Heindel *et al.*, 1996; Pushpalatha *et al.*, 2005; Izquierdo-Vega *et al.*, 2008).

5.1.7. Ginseng

Ginseng, a herbal medicine, has long been used as a tonic for prolonging life span, and is available without prescription (Poindexter *et al.*, 2006). It is employed by different people, including pregnant women (Gibson *et al.*, 2001). In a recent survey, 9.1% of pregnant women reported use of herbal supplements (Gibson *et al.*, 2001), including ginseng. In Asian countries, up to 10% of women had taken ginseng during their pregnancy (Chin, 1991). Although it is a general belief that herbal medicines are much better and safer than conventional medicines, in fact many herbal medicines are actually associated with serious developmental toxic effects (DeSmet, 1995). Ginseng is a common term used for Asian ginseng (Panax ginseng), prepared from a different plant and Siberian ginseng. Asian ginseng is commonly used as an antioxidant, anti-inflammatory agent, and an anticancer remedy (Kiefer & Pantuso, 2003). It also believed to have cardioprotective effects. Panax ginseng was reported to be effective in treating angina pectoris (Yuan *et al.*, 1997). It has also been used for its sedative, hypnotic, antidepressant, and diuretic activity. People usually take it to improve stamina, concentration, vigilance, and well-being (Ernst, 2002).

The active components in Asian ginseng are called ginsenosides (Kiefer & Pantuso, 2003; Ma *et al.*, 2005). These ginsenosides are divided into two major groups, panaxadiol (Rb1and Rc) and panaxatriol (Rg1 and Re) (Attele *et al.*, 1995). One study conducted on rat embryos showed embryotoxic effects of Rb1 on embryonic development (Chan *et al.*, 2003). Another study conducted by Chan et al on rat embryos revealed the embryotoxic effects of Rc and Re as well (Chan *et al.*, 2004). Developmental defects were also observed in mouse whole embryo cultures by Liu and his colleagues (Liu *et al.*, 2005a; Liu *et al.*, 2006). Although teratogenicity of different ginsenosides is confirmed in different animal models, there is very little knowledge available on human data to date.

5.2. Materials and Methods

Chick cardiomyocytes were seeded in 24 well plates at a concentration of 3 $\times 10^6$ in each well in the form of 20µl droplets. They were incubated for 2 hours to allow cellular attachment. Then 500µl of DMEM growth medium was added and the cultures were left for 24 hours (chapter 2 section 2.1.4.5.2). The DMEM medium was replaced by different concentrations of test chemicals after 24 hours.

5.2.1. Test chemical preparation

The test chemicals were serially diluted from their stock solutions in DMEM supplemented culture medium to give a desired concentration (chapter 2 table 2.1). All chemicals were prepared and applied to micromass cultures within 1 hour of preparation.

5.2.2. End points

The differentiation assay, resazurin reduction assay and kenacid blue assay were performed as described in chapter 2 (section 2.1.5.1 and 2.1.5.2 respectively). The cultures were inspected morphologically for cardiomyocyte contractile activity at 24, 48 and 144 hours, while resazurin and kenacid blue assays was performed at 144 hours.

5.2.3. Statistical analysis

All statistical analysis for this chapter was performed using Prism 5 (Graph pad Software Inc. San Diego, USA). All results with different dose concentrations were compared using one way ANOVA with Dunnet's multiple comparison post hoc test, with $p < 0.05$ was considered statistically significant.

5.3. Results

5.3.1. Folic acid

Exposure of chick cardiomyocytes to various concentrations of folic acid ranged from 1µM to 1mM tested over a period of 144 hours did not cause a reduction in cellular differentiation, cell number or resorufin production. The graphs for resorufin production show that the resorufin produced by cardiomyocytes treated with folic acid is not significantly different to controls at any concentrations used (fig 5.4 a and 5.4 b). The protein content of cardiomyocytes treated with folic acid is also not significantly different from controls at all concentrations used, as shown in fig. 5.4 c and 5.4 d. Statistical analyses of 24 hours post culture scores were not

significant due to the fact that no drug was added prior to their scoring. Moreover statistical analyses of day 24 and 120 hours post exposure also showed no significant difference to control for folic acid treated cells (1µM-1mM) (fig 5.4 e and f).

5.3.1.1. Conclusions

The concentrations of folic acid tested did not show any effect on cell viability or protein content. The cellular differentiation and rate of beating were also not effected at all concentration of folic acid.

Fig: 5.4 a

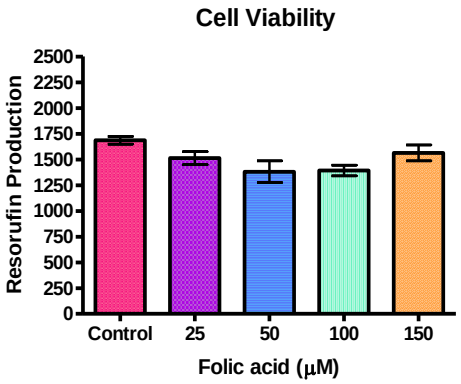


Fig: 5.4 b

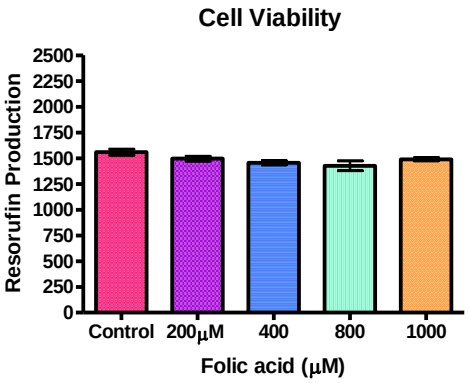


Fig: 5.4 c

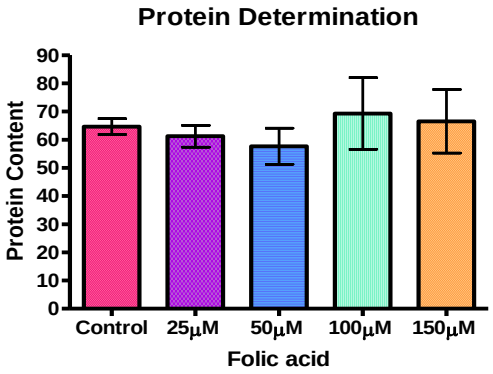


Fig: 5.4 d

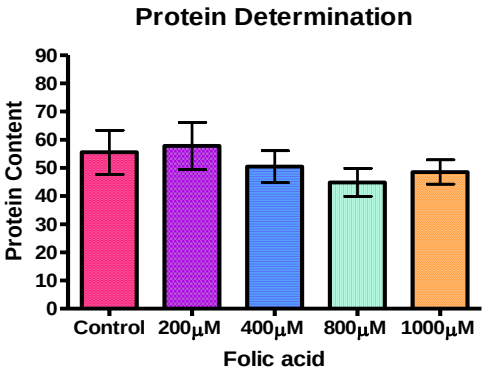


Fig: 5.4 e

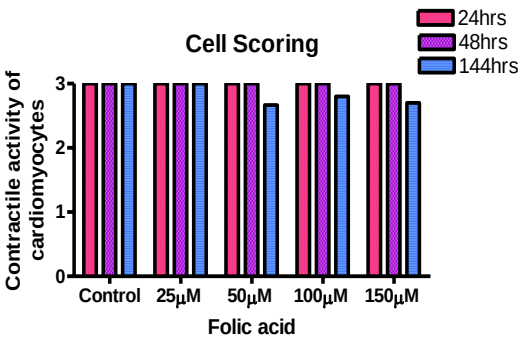


Fig: 5.4 f

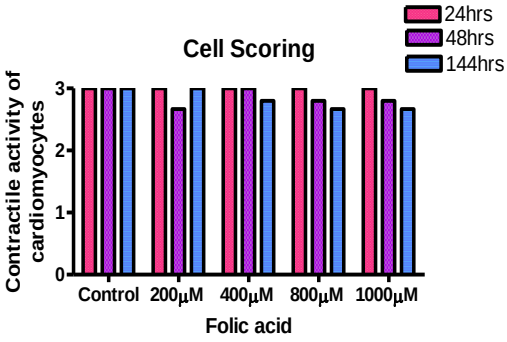


Fig 5.4. Shows resorufin production (a & b), protein content (c & d), and contractile activity of cardiomyocytes (d & e) with different concentrations of folic acid

5.3.2. Vitamin E

The effect of exposure of chick cardiomyocytes to various concentrations of vitamin E (trolox) ranging from 10µM to 100µM were tested over a period of 144 hours. All of these concentrations did not affect cellular proteins or cell viability. The graph for resorufin production shows that the resorufin produced by cardiomyocytes treated with vitamin E is not significantly different to controls at any concentrations used (fig5.5 a). The protein content of cardiomyocytes is also not significantly different from controls at all concentrations used as shown in fig. 5.5 b. Statistical analyses of 24 hours post culture scores were not significant because of the reason mentioned earlier. However statistical analyses of day 24 and 120 hours post exposure also showed no significant difference to control for vitamin E treated cells (fig 5.5 c).

5.3.2.1. Conclusions

The doses of vitamin E tested did not show any effect on cell viability or protein content. The cellular differentiation and rate of beating were also not effected at all concentration.

Fig: 5.5 a

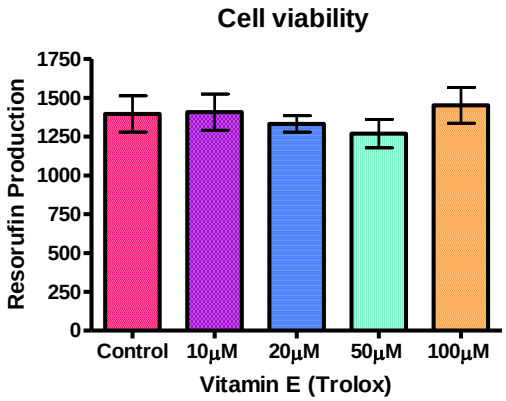


Fig: 5.5 b

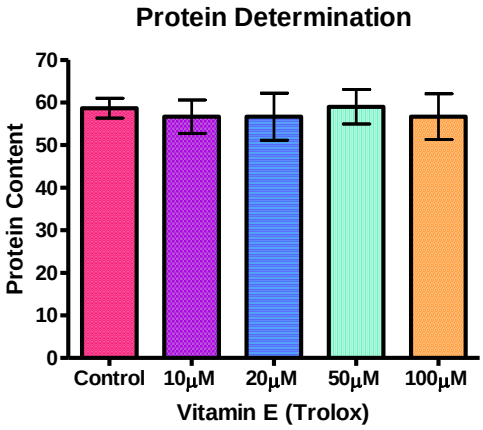


Fig: 5.5 c

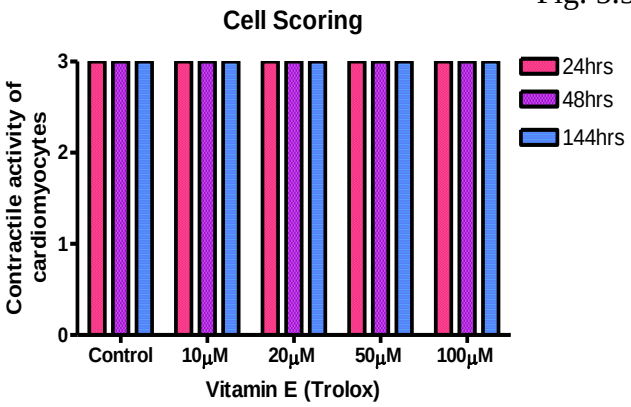


Fig 5.5. Shows resorufin production (a), protein content (b), and contractile activity of cardiomyocytes (c) with different concentrations of vitamin E.

5.3.3. Vitamin C

Exposure of chick cardiomyocytes to different concentrations of vitamin C (10µM-100µM), over a period of 144 hours did not show any reduction in cellular differentiation, cell viability, or cellular protein level. The graphs for resorufin production show that the resorufin produced by cardiomyocytes treated with vitamin C is not significantly different to controls at any concentrations used (fig 5.6 a). These concentrations of vitamin C did not produce any reduction in cell protein (fig 5.6 b). Statistical analyses of 24, 48 and 144 hours also showed no significant difference to the control for vitamin C treated cells (fig 5.6 c).

5.3.3.1. Conclusions

The concentrations of vitamin C tested did not show any effect on cell viability or protein content. The cellular differentiation and rate of beating were also not affected at all concentration of vitamin C.

Fig: 5.6 a

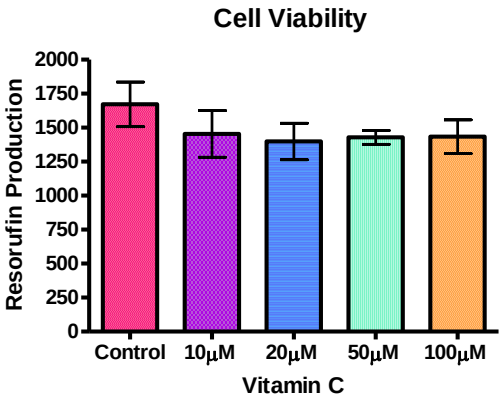


Fig: 5.6 b

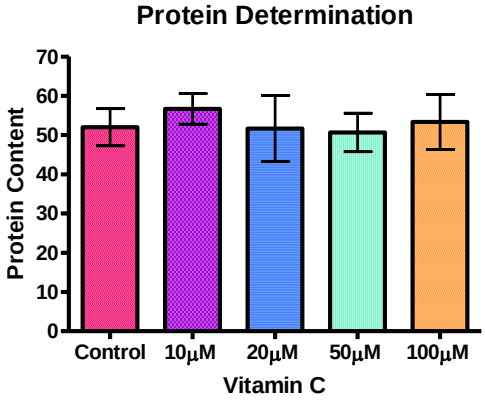


Fig: 5.6 c

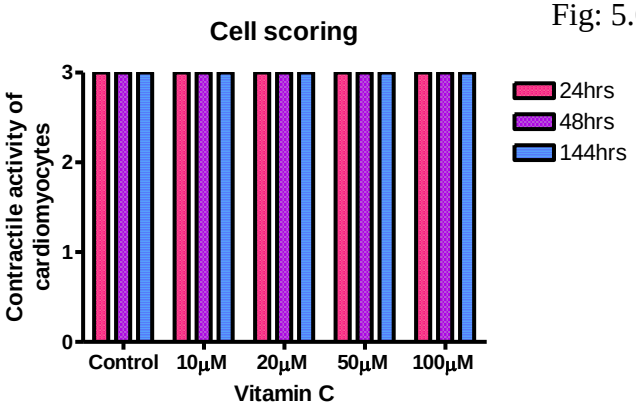


Fig 5.6. Shows resorufin production (a), protein content (b), and contractile activity of cardiomyocytes (c) with different concentrations of vitamin C.

5.3.4. All trans retinoic acid

All-trans retinoic acid is a fat soluble vitamin, and needs a solvent (ethanol) to be soluble in aqueous solutions. The concentration of solvent was kept as low as possible (0.01%). At that concentration ethanol did not show any significant effects on cell viability, protein content and contractile activity of cardiomyocytes. The resorufin produced by micromass cultures exposed to 1µM or above was significantly less as compared to control ($p<0.05$) (Fig 5.7, a, b, c). The maximum concentration at which there was no significant difference between cell viability in the test chemical and the control was 0.8µM. Protein content of cardiomyocytes is also decreased as the concentration of retinoic acid increased as shown in (fig 5.8, a b, c). Statistical analyses showed a significant difference to controls at and above 1µM ($p < 0.05$). The chick cardiomyocyte cultures were observed for contractile activity and rate of beating foci immediately prior to retinoic acid exposure and 24 hours and 120 hrs post exposure. There was no significant difference observed for contractile activity prior to the addition of retinoic acid. However observations at 24 hrs post exposure showed that the cultures exposed to 1µM or above had a significantly decreased contractile activity and beating rate ($p<0.05$) as compared to controls. At 120 hours, cultures with 50µM or more had no contractile activity as compare to controls (fig 5.9, a, b, c).

5.3.4.1. Conclusions

Retinoic acid affected cell viability from as low as 1µM concentrations while total protein was also affected at and above 1µM. Cellular

differentiation was also affected at and above 1µM concentration with no signs of recovery with time.

Fig: 5.7 a

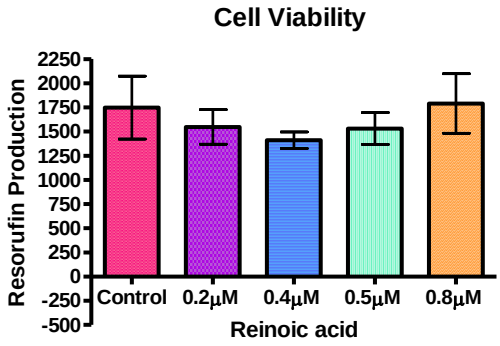


Fig: 5.7 b

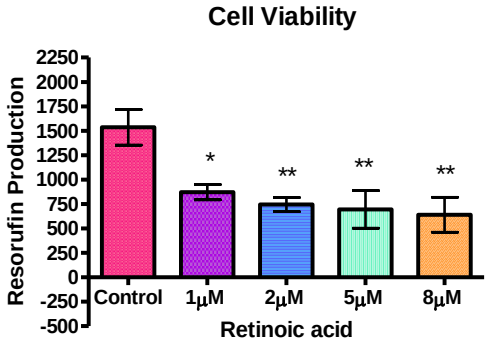


Fig: 5.7 c

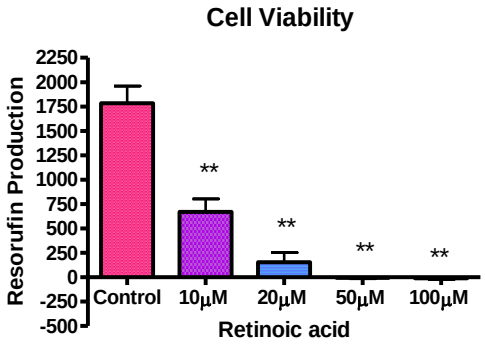


Fig 5.7. Shows resorufin production with different concentrations of retinoic acid (a, b, c). {* ($p<0.05$); ** ($p<0.01$) for Dunnett's post hoc test}.

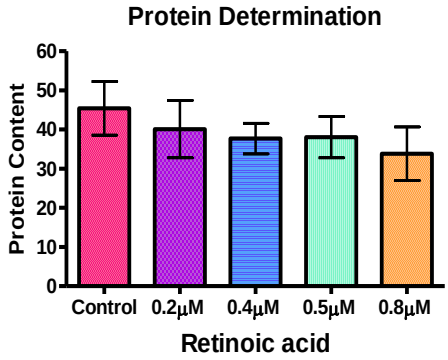


Fig: 5.8 a

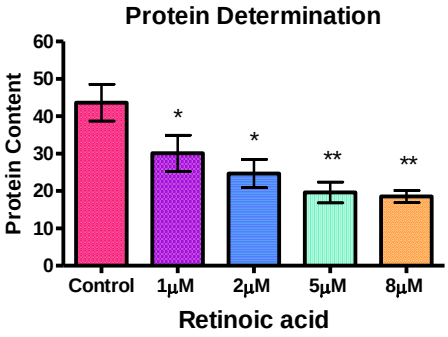


Fig: 5.8 b

Fig: 5.8 c

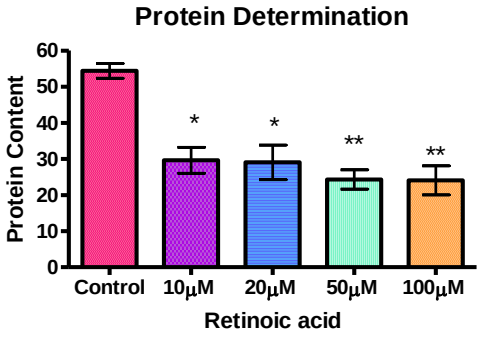


Fig 5.8. Shows protein content with different concentrations of retinoic acid (a, b, c). {* ($p<0.05$); ** ($p<0.01$) for Dunnett's post hoc test}.

Fig: 5.9 a

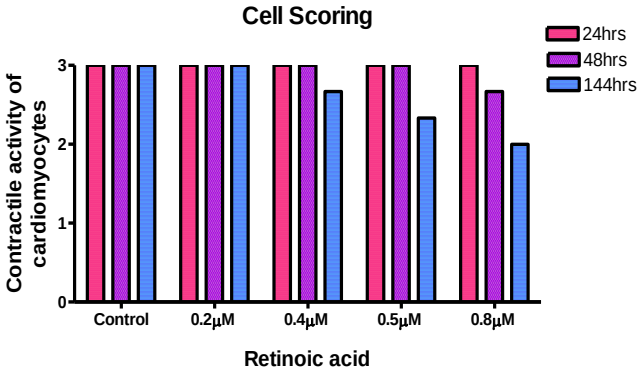


Fig: 5.9 b

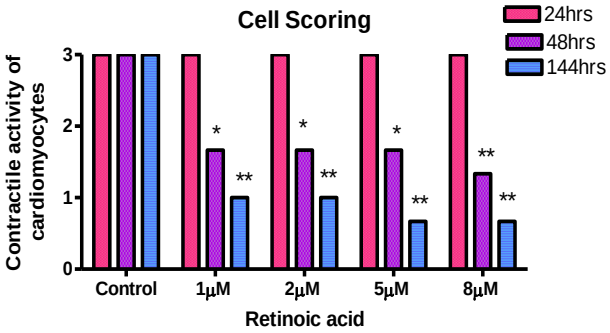


Fig: 5.9 c

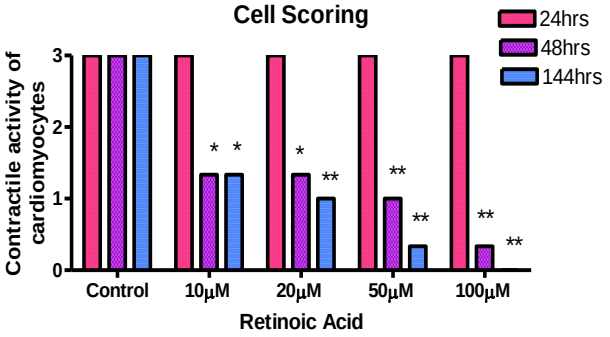


Fig 5.9. Shows contractile activity of cardiomyocytes with different concentrations of retinoic acid (a, b, c). {* ($p<0.05$); ** ($p<0.01$) for Dunn's post hoc test}.

5.3.5. Cadmium Chloride

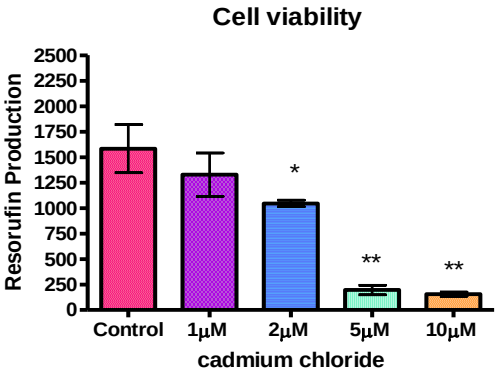
The cardiomyocytes were treated with various concentrations of cadmium chloride ranging from 1µM to 100 µM. The resorufin produced by micromass cultures exposed to 2µM or above was significantly less as compared to the control ($p<0.05$) (fig 5.10, a, b). Protein content of cardiomyocytes is also decreased in direct proportion to increasing concentrations of cadmium chloride as shown in (fig 5.10, c, d). Statistical analyses showed a significant difference to controls at and above 5µM ($p < 0.05$). There was no significant difference observed for contractile activity and rate of beating prior to the addition of cadmium chloride. However observations at 24 hrs post exposure showed that the cultures exposed to 2µM or above had a significantly decreased contractile activity and beating rate as compare to controls. At 120 hours cultures with 50µM or more showed no contractile activity as compare to controls ($p<0.05$) (fig 5.10 e, f).

5.3.5.1. Conclusions

The concentrations of cadmium chloride tested in this study affected cell viability from as low as 2µM concentrations while total protein was affected at and above 5µM. The cellular differentiation was also affected at and above 2µM concentration.

Fig 5.10 a

Fig 5.10 b



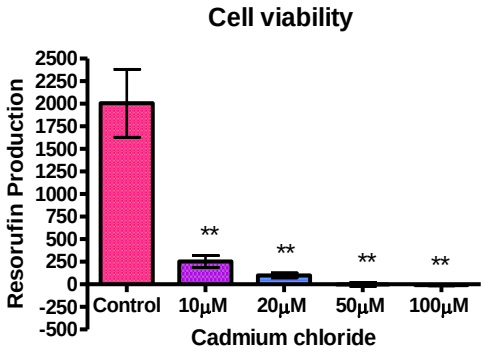
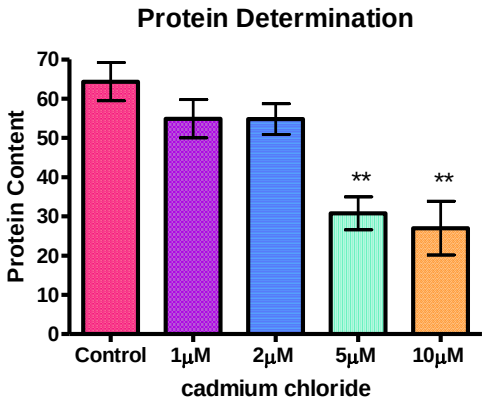


Fig 5.10 c

Fig 5.10 d



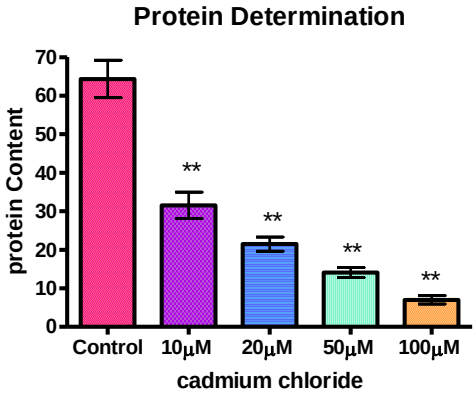
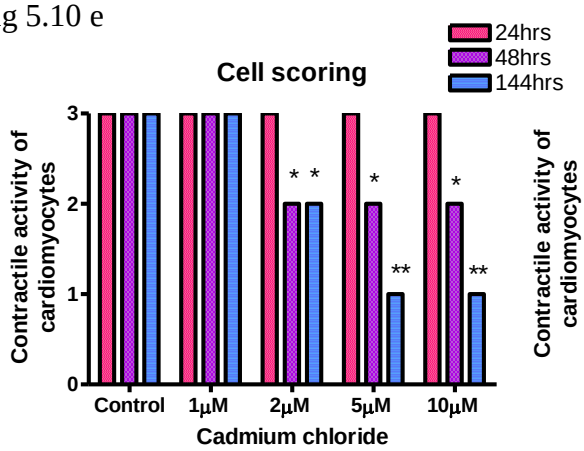


Fig 5.10 f

Fig 5.10 e



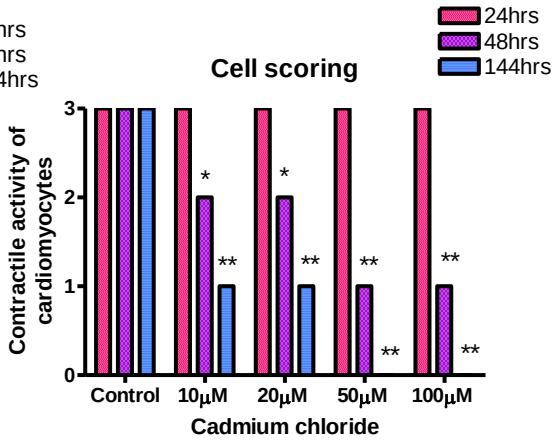


Fig 5.10. Shows resorufin production (a & b), protein content (c & d), and contractile activity of cardiomyocytes (d & e) with different concentrations of cadmium chloride. {* ($p<0.05$); ** ($p<0.01$) for Dunnett's and Dunn's post hoc tests applied to parametric and non parametric data respectively}.

5.3.6. Sodium fluoride

Exposure of chick cardiomyocytes to 10µM to 100µM of sodium fluoride over a period of 144 hours did not cause a reduction in cellular differentiation or resorufin production. The cell proteins were also unchanged when the cells were exposed to the above concentrations of sodium fluoride.

The graphs for resorufin production show that the resorufin produced by cardiomyocytes treated with sodium fluoride is not significantly different to controls at any concentrations used (fig 5.11 a). The protein content of cardiomyocytes is also not significantly different from controls at all concentrations used as shown in fig. 5.11 b. Statistical analyses of 24 hours post culture scores were not significant because of the reason mentioned earlier. However statistical analyses of day 24 and 120 hours post exposure also showed no significant difference to control for sodium fluoride treated cells (fig 5.11 c).

5.3.6.1. Conclusions

The concentrations of sodium fluoride tested did not show any effect on cell viability or protein content. The cellular differentiation and rate of beating were also not effected at all concentration of sodium fluoride.

Fig 5.11 a

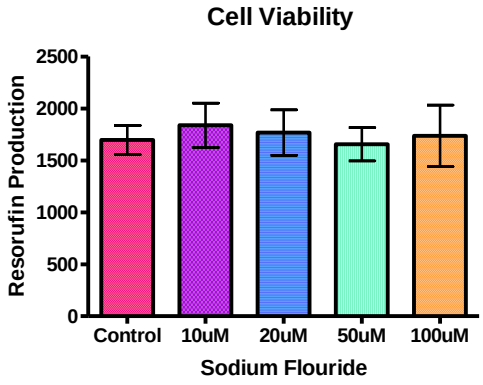


Fig 5.11 b

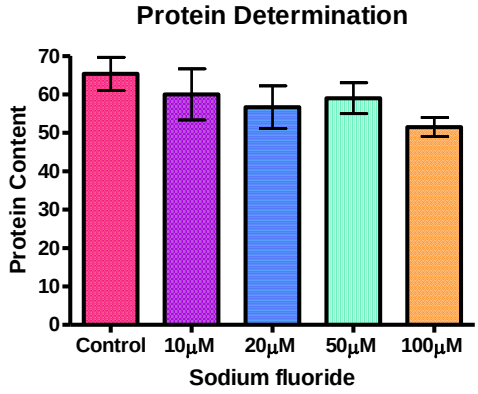


Fig 5.11 c

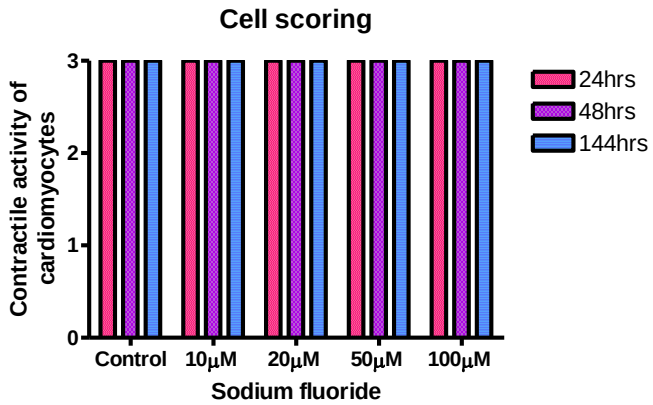


Fig 5.11. Shows resorufin production (a), protein content (b), and contractile activity of cardiomyocytes (c) with different concentrations of sodium fluoride.

5.3.7. Ginseng

Ginsenosides (Rb1) exposure to chick cardiomyocytes in the range between 10µM to 100µM showed no reduction in cellular differentiation or resorufin production during the entire duration of culture. The cellular proteins were also unchanged when exposed to the above concentrations of Rb1. The resazurin assay shows, that the resorufin produced by cardiomyocytes treated with Rb1 is not significantly different to controls at any concentrations used (fig 12 a). Furthermore the protein content of cardiomyocytes is also not significantly different from controls as shown in fig. 5.12 b. Statistical analyses of 24 hours post culture scores were not significant because of the reason mentioned earlier. However statistical analyses of day 24 and 120 hours post exposure also showed no significant difference to control for Rb1 treated cells (fig 5.12 c).

5.3.7.1. Conclusions

The concentrations of ginsenosides used in this study did not show any effect on all endpoints checked.

Fig 5.12 a

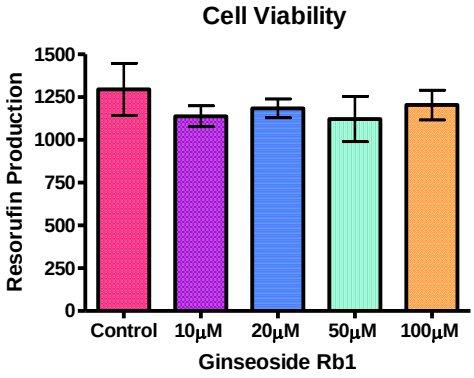


Fig 5.12 b

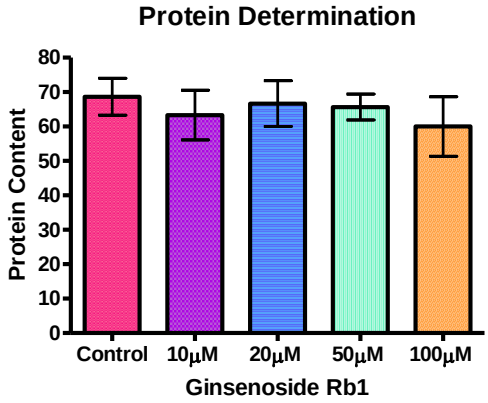


Fig 5.12 c

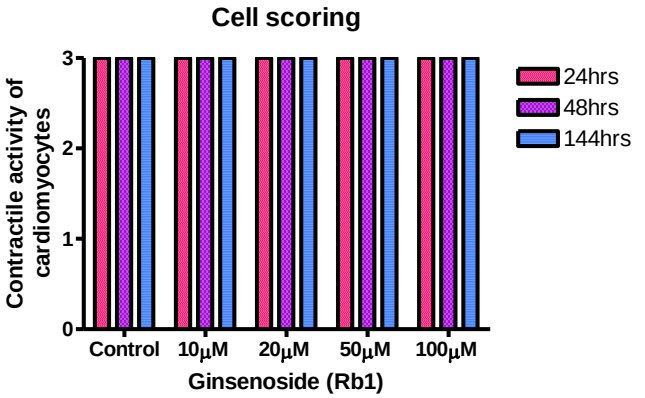


Fig 5.12. Shows resorufin production (a), protein content (b), and contractile activity of cardiomyocytes (c) with different concentrations of ginsenoside (Rb1).

5.3.8. Retinoic acid with folic acid and vitamin C

After investigating different concentrations of retinoic acid with potential cytotoxicity, further experiments were performed where folic acid and vitamin C were added to cultures already flooded with retinoic acid (10µM and 20µM) in order to detect any protective effect offered by administration of folic acid and vitamin C.

Fig 5.13 a and 5.13 b, show the resazurin reduction assay. Here the graph shows that addition of folic acid and vitamin C to retinoic acid can protect the cultures from the teratogenic effects of retinoic acid. Cellular protein levels, affected by retinoic acid were also rescued by addition of folic acid and vitamin C as shown in fig 5.13 c and 5.13 d. Cells were scored at 24, 48 and 144 hrs. Results show that addition of folic acid and vitamin C to retinoic acid treated cells can protect the cells from embryotoxic effects of retinoic acid (Fig 5.13 e and 5.13 f).

5.3.8.1. Conclusion

Addition of folic acid and vitamin C to retinoic acid treated cultures has protective effects against retinoic acid induced developmental toxicity.

Fig 5.13 a

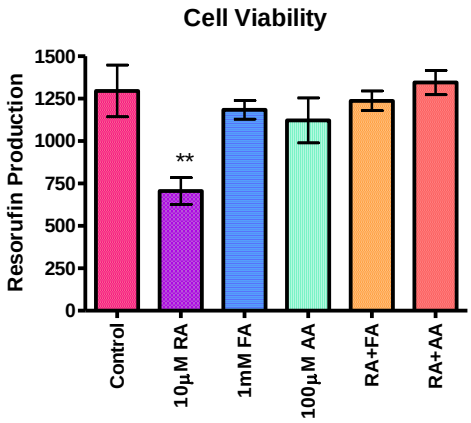


Fig 5.13 b

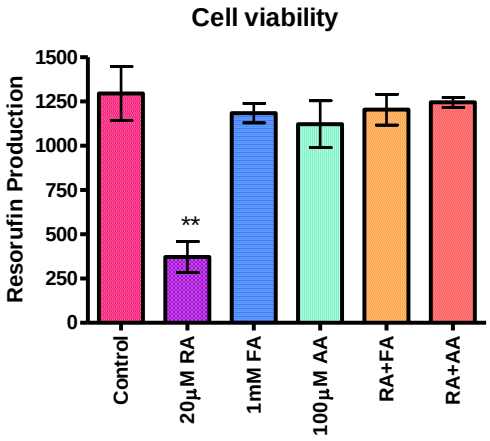


Fig 5.13 c

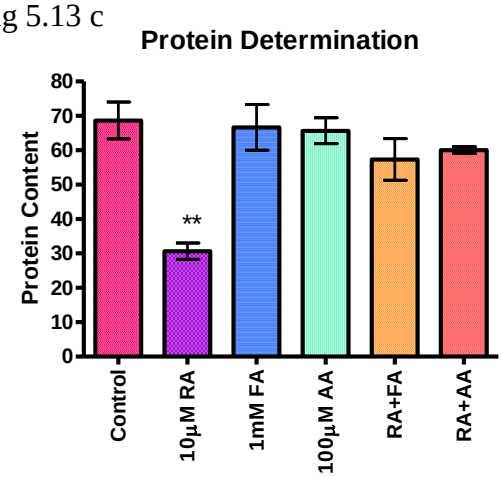


Fig 5.13 d

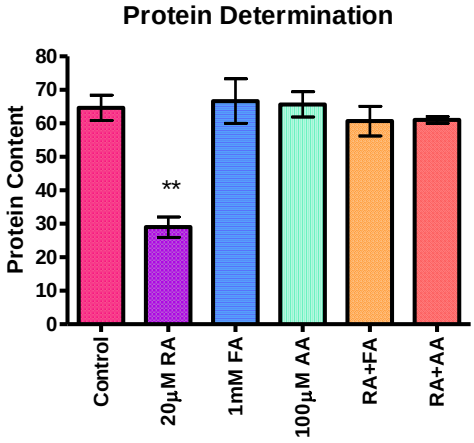


Fig 5.13 e

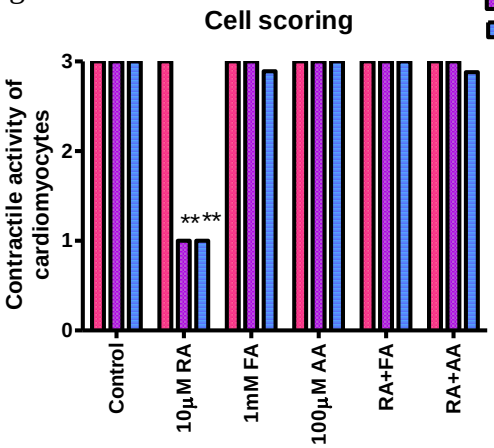


Fig 5.13 f

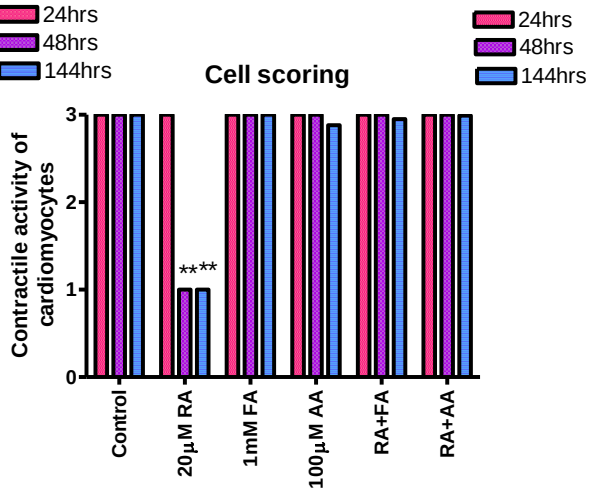


Fig 5.13. Shows resorufin production (a & b), protein content (c & d), and contractile activity of cardiomyocytes (d & e) with different concentrations of retinoic acid plus 1mM folic acid. {** ($p<0.01$) for Dunnett's and Dunn's post hoc tests applied to parametric and non parametric data respectively}.

5.3.9. Cadmium chloride with folic acid and vitamin C

Results show that addition of folic acid and vitamin C to cadmium chloride treated cells could not protect the cells from its embryotoxic effects. Fig 5.14 a, 5.14 b, 5.14 c show resorufin produced by cells. Results shows that addition of folic acid and vitamin C to cadmium chloride treated cells could not reverse the cytotoxic effects of cadmium chloride. Cellular protein levels, affected by cadmium chloride were also not rescued by addition of folic acid and vitamin C as shown in (fig 5.15 a, b, c). Similarly addition of folic acid and vitamin C has no protective effects on contractile activity which is decreased with cadmium chloride as shown in fig 5.16 a, b, c.

5.3.9.1. Conclusions

Addition of folic acid and vitamin C has no protective effects against cadmium chloride induced teratogenicity.

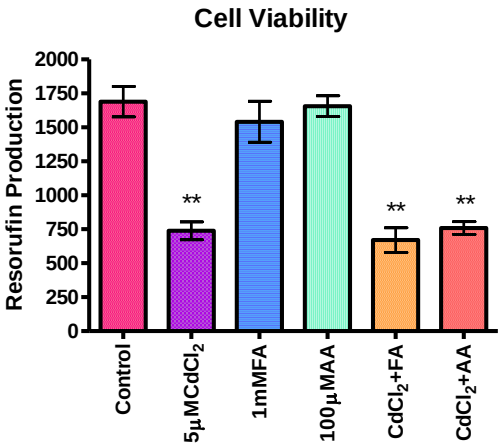


Fig 5.14 a

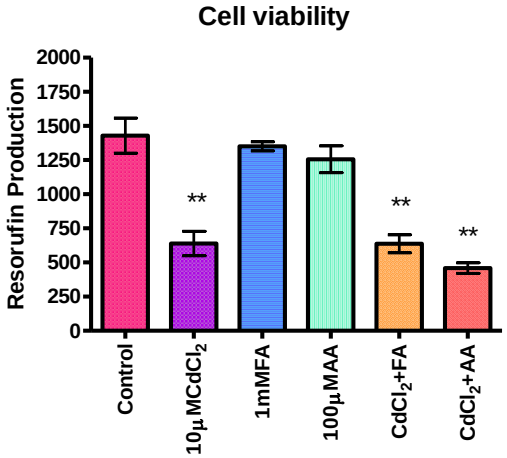


Fig 5.14 b

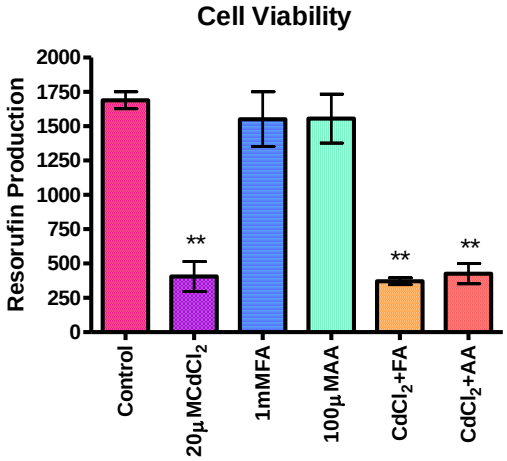


Fig 5.14 c

Fig 5.14. Shows resorufin production by cardiomyocytes with different concentrations of cadmium chloride plus vitamin C and folic acid (a, b, c). { ** ($p<0.01$) for Dunnett's post hoc test}.

Fig 5.15 a

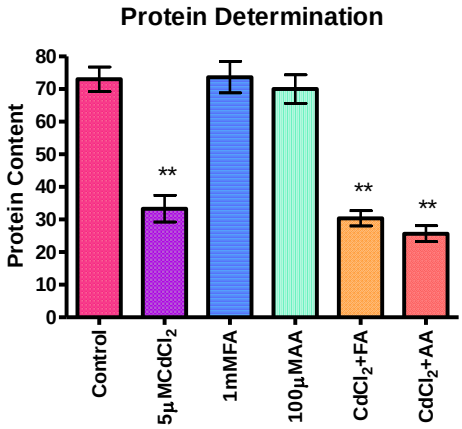


Fig 5.15 b

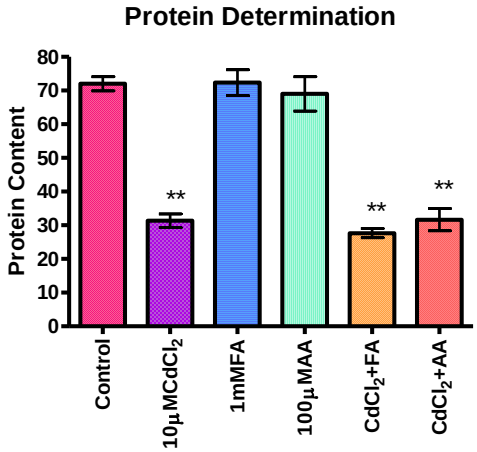


Fig 5.15 c

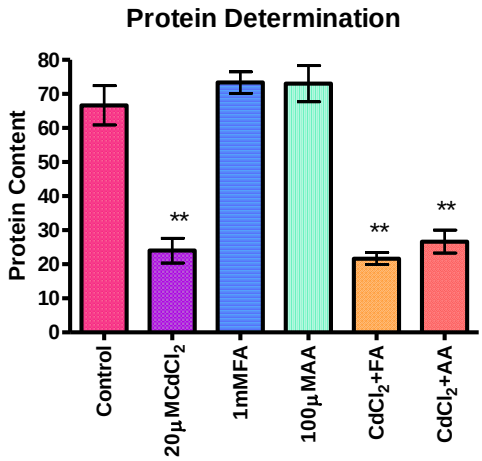


Fig 5.15. Shows protein content of cardiomyocytes with different concentrations of cadmium chloride plus vitamin C and folic acid (a, b, c). {** ($p<0.01$) for Dunnett's post hoc test}.

Fig 5.16 a

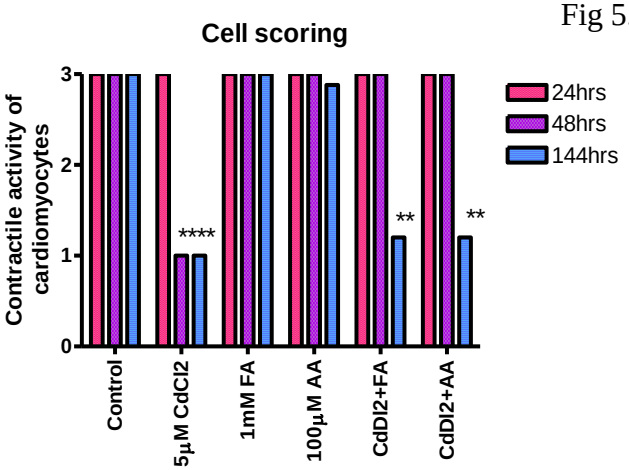


Fig 5.16

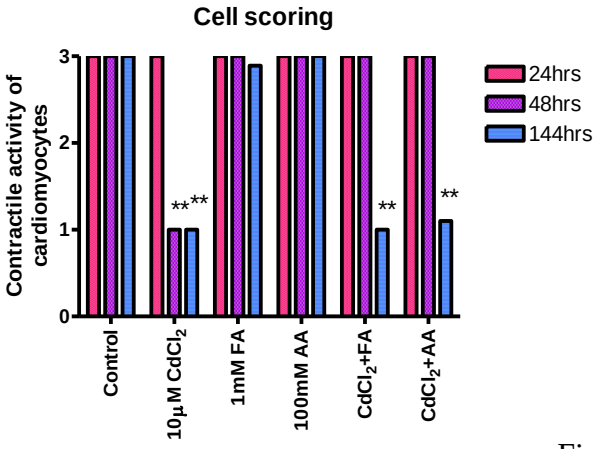


Fig 5.16 c

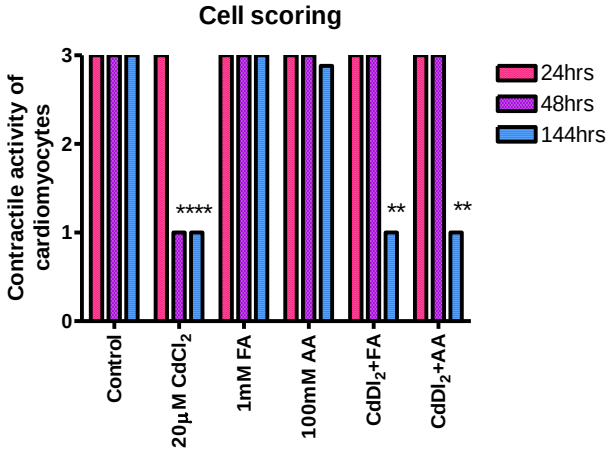


Fig 5.16. Shows contractile activity of cardiomyocytes with different concentrations of cadmium chloride plus vitamin C and folic acid (a, b, c). {** ($p<0.01$) for Dunn's post hoc test}.

5.4. Discussion

5.4.1. Folic acid

In this study chick heart cells were treated with different doses of folic acid ranging from 1µM to 1mM, in order to establish a maximum dose which itself has no any teratogenic effects on cardiomyocytes. We found that even at 1mM folic acid did not show any toxic effects on chick cardiomyocytes, which is consistent with the amount of folinic acid used to treat the malformations of branchial arch derived structures in rats in whole embryo culture by (Zhang *et al.*, 2006).

The relation of folic acid intake during the periconceptional period and reduction of the risk of neural tube defects is very well recognized as evidenced by many studies (Pitkin, 2007). Recently studies conducted on animals as well as on humans suggest that folic acid might also be useful in decreasing other birth defects; in particular congenital heart defects, the most common of all birth defects (Botto & Correa, 2003; Botto *et al.*, 2003; Huhta & Hernandez-Robles, 2005). In an animal study conducted by Li and colleagues in 2005 it was found that in mouse embryos deficiency of maternal folic acid lead to congenital heart defects (Li *et al.*, 2005). Folic acid exerted its effects by affecting proliferation of the embryonic hearts. Several possible mechanisms could be involved in decreasing cellular proliferation, as folic acid is essential for many vital cellular processes including nucleotide synthesis, amino acid synthesis, and methylation reactions (Botto *et al.*, 2004). These processes are essential for rapid mitosis and differentiation of embryonic cardiomyocytes. The

other possible mechanism could be the elevation of homocysteine and decrease in methionine, which in turn affect DNA methylation and thus cell proliferation (Jongbloet *et al.*, 2008).

5.4.2. Vitamin E

In this study experiments were conducted on chick micromass culture to establish a dose of vitamin E a known antioxidant, which has no cytotoxic effect on its own on cardiomyocytes. Vitamin E is a biological antioxidant known to prevent lipid peroxidation of cellular membranes by quenching free radicals thus it functionally protects the cells and tissues from free radical derived damage. It is also required by many animal species such as rats, sheep and chickens to maintain normal growth rate (Brigelius-Flohe *et al.*, 2002).

5.4.3. Vitamin C

Vitamin C, an antioxidant molecule in aqueous environments was also tested in this study, and a desirable dose was established with no toxic effects on chick cardiomyocytes in culture, in order to conduct further experiments, where its protective effects as an antioxidant were checked by adding it with various other chemical teratogens.

5.4.4. Retinoic acid

Retinoids are known human teratogens and therefore have been extensively investigated. The teratogenic potential of all-trans retinoic acid on chick micromass culture was confirmed by our present work. The effects were consistent for any of the three end points measured. The reliability of

chick MM culture was tested by (Wiger *et al.*, 1988), with retinoic acid as one of the test chemicals used to treat the limb bud cells. Results reflected those found in our study, with inhibition of cellular differentiation, cell viability and protein synthesis in chick cardiomyocytes MM cultures exposed to all-trans retinoic acid. Many animal studies have focused the teratogenic effects of retinoids on developing heart cells. In an investigation on the effects of retinoic acid on early heart development by Osmond and colleagues (Osmond *et al.*, 1991), he found that retinoic acid produced a wide range of heart malformation when applied either to the whole embryo culture or implanted in the form of beads to the precardiac mesoderm. In both of these experiments the embryos were treated with concentrations of retinoic acid ranged from 1µM to 100µM. Although supporting the current study in some ways, Osmond et al did not find any killing of cells even at higher concentrations of retinoic acid (Osmond *et al.*, 1991). In contrast our study showed cell death at concentrations ranging from 20µM to 100µM. In another study conducted on mouse whole embryo culture Zhang et al (Zhang *et al.*, 2006), found large number of malformation including cranial NTDs, and branchial arch abnormalities in 100% of embryos exposed to 0.4µM concentration of retinoic acid. The reason of cell death in our study on micromass system could be due to high concentrations of retinoic acid used or increased sensitivity of the micromass to retinoic acid, because in micromass culture the cells are in direct contact with the retinoic acid whereas in whole embryo culture the embryo is protected by the surrounding membranes and yolk sac.

Two families of nuclear receptors, named as retinoic acid receptors (RARs) and retinoic X receptors (RXRs) are involved in retinoic acid induced malformations. Both of these receptors bind to DNA motifs (Retinoic acid response elements RARE) in the form of RAR/RXR heterodimers to function as ligand dependent trans-regulators. RAR/RXRα are the main functional units for retinoid signalling during development and transcriptional activation of both of these partners is often required for most of the physiological effects of retinoic acid during organogenesis (Matt *et al.*, 2003; Niederreither *et al.*, 2003).

5.4.5. Cadmium chloride

Cadmium chloride is one of the environmental toxins; generated as a result of waste disposal, coal combustion, iron and steel production and phosphate fertilizers manufactures. Cadmium is known to produce its teratogenic effects in many species. It is used in this study in a range of 1µM to 100µM, which is in line with other cell culture studies, which used cadmium in micro molar concentrations (Chan & Cheng, 2003b; Yano & Marcondes, 2005). The chick cardiomyocytes exposed to these concentrations stopped beating and cell viability assays showed a reduction in viability and total proteins. Chow and Cheng (Chow & Cheng, 2003) studied cadmium's effects on zebra fish embryos at early stages of development and their results showed that cadmium had toxic effects on early development and these effects are due to apoptosis induced by it. In another study Chan and his colleagues found that zebra fish embryos when exposed to more than 100µM concentrations of cadmium chloride during

the period of gastrulation showed developmental defects in the head and neck region, heart malformations, and had altered axial curvatures (Chan & Cheng, 2003a). Similar types of malformations were also observed in xenopus embryos as well (Sunderman *et al.*, 1992). In our study cadmium shows toxic effects at very low concentrations, and showed an inhibition of beating that might be due to the fact that cadmium possibly alters the intercellular junctions, which result in abnormal rhythm and cell proliferation , Previous work conducted on chick embryo shows that cadmium exposure of chick embryos resulted in epidermal and lateral plate mesoderm defects, possibly due to its interference with intercellular junctions and cadherin molecules (Thompson *et al.*, 2005).

5.4.6. Sodium fluoride

Fluoride is a naturally occurring component of water and high levels of it are usually found in mountainous areas (Izquierdo-Vega *et al.*, 2008). Sodium fluoride was used in a range of 10µM-100µM to detect any potential teratogenic effect on chick embryonic hearts using micromass culture. It was observed that, the concentrations used in this study had no toxic effect on the developing chick heart. Verma and Sherlin, showed in their study that when pregnant rats were administered with 40mg/kg/day of sodium fluoride, they had significant reduction in their weight and had small uterine volume. Furthermore their embryos showed mostly skeletal abnormalities with some subcutaneous haemorrhages (Verma & Sherlin, 2001). In another study no embryonic defects were seen when female rats and rabbits were given 27mg/kg/day sodium fluoride in drinking water.

Effects were observed at higher concentrations due to the reason that concentrations higher than that dose were unpalatable and pregnant rats and rabbits were reluctant to take any food even beyond that dose (Heindel *et al.*, 1996). Our results are consistent with these results as low doses of sodium fluoride used in this study also showed no developmental defects. An in vitro study on frog embryos showed developmental abnormalities in frog embryos at higher doses but not at lower doses. The effect of sodium fluoride on frog embryos when applied directly could be due to its actions as an enzyme inhibitor and it may interact with chromosomes to produce developmental defects. It activates the G- protein which in turn initiates a series of reactions, resulting in developmental defects in different embryonic tissues particularly skeletal and neuromuscular (Goh & Neff, 2003). Also excess sodium fluoride in body tissues causes an abnormality in cellular calcium, and causes hypocalcaemia, that could be a reason that it particularly affects those organs which are rich in calcium and utilize that in order to grow and perform their biological activities.

5.4.7. Ginseng

Ginseng is one of the most well-known natural herbal medicines, used widely in the treatment of various diseases. It is also used as a cooking supplement; daily nourishment and health building remedy (Kitts & Hu, 2000; Fu & Ji, 2003). In this study ginsenoside Rb1 was tested on chick cardiomyocytes for its toxicological effects on developing chicks. No significant teratogenic effects were observed at the doses (10µM-100µM) used in this study. However previous work with mouse and rat embryos

cultured in vitro, showed that both Rb1 and Rg1 (another ginsenoside) when used in the range of 10-50µg/ml, had toxic effects on developing embryos (Liu *et al.*, 2005a; Liu *et al.*, 2005b). However the two species appeared to have toxic effects at different concentrations of ginsenosides. The rat embryos appeared to be more sensitive than mouse embryos. Furthermore heart defects were obvious in rats while mouse embryos did not show any defects in the cardiovascular system (Liu *et al.*, 2006). The inconsistency between our results and previous results on rat and mouse embryos could be due to the dose of ginsenoside used in this study or due to the species difference. The mechanism of ginseng toxicity is still unclear but it might be due to alterations in calcium channels (Poindexter *et al.*, 2006).

5.4.8. Retinoic acid with folic acid and vitamin C

The present study also demonstrates a protective effect of folic acid and vitamin C when added to retinoic acid treated cardiomyocytes. The effective concentration shown to have protective effect is 1mM and 100µM respectively. Epidemiologic evidence also supports the protective effects of folic acid on development of the cardiovascular system in humans. A case control study, conducted in Atlanta suggested a direct relationship for prevention of cardiac defects, especially outflow tract and ventricular septal defects, with periconceptional use of multivitamins containing folic acid (Botto *et al.*, 2003). Similar results were also found in a randomized control trial in Hungary which showed a reduction of 50% in cardiac defects with supplemental folic acid during pregnancy (Gardiner, 2006).

Although folic acid and vitamin C when added to retinoic acid treated cardiomyocytes offered protection against retinoic acid induced cytotoxicity, results also demonstrate that supplemental folate and ascorbic acid do not completely overcome the effects of RA particularly at higher concentrations. This may be due to the fact that more than one mechanism of action of RA is involved, as at present mechanism underlying the effects of folic acid on RA teratogenicity remain unknown (Zhang *et al.*, 2006).

5.4.9. Cadmium chloride with folic acid and vitamin C

In this study we tried to overcome the toxic effects of cadmium chloride by addition of vitamins such as folic acid and vitamin C. The results in this study suggest no preventive effects of folic acid and ascorbic acid against cadmium chloride induced developmental toxicity even at lower concentrations (5µM). There are many mechanisms involved in cadmium induced teratogenicity; one possible mechanism could be the generation of reactive oxygen species as studies conducted on mouse and human promonocytic cells showed that prior treatment with α-tocopherols and *N*-acetyle-L-cysteine could prevent these cells from cadmium induced toxicity (Galán *et al.*, 2001). Another possible mechanism could be due to activation of calcium dependant endonucleases, as cadmium cation may mimic calcium channels and treatment with a calcium channel blocker could protect the cells from cadmium induced cell death (Azzouzi *et al.*, 1994). In our study antioxidants did not show any protection against cadmium toxicity in micromass culture, due to the possibility of other mechanisms involved in cadmium induced cardiovascular abnormalities.

Chapter 6

Effect of social drugs on chick cardiomyocyte micromass culture and chick embryos cultured in ovo, and potential protective effects of vitamin supplementation

6.1. Introduction

This chapter evaluates the effects of two social drugs (Ethanol and Nicotine), which have known potential to produce teratogenic effects when consumed in excess during pregnancy. Also this chapter aims to evaluate the potential protective effects of vitamins particularly folic acid and vitamin C in prevention of developmental defects of the heart brought about by these drugs. Two different techniques (chick heart micromass and in ovo culture), using chick embryos were employed to investigate the effects.

6.1.1. Ethanol

Alcohol or ethanol, one of the most common environmental teratogens, when consumed in excess during pregnancy is associated with a wide spectrum of embryonic and fetal malformations in humans as well as in various animal species (Wentzel & Eriksson, 2006). The effects, often called fetal alcohol syndrome, usually result in early embryo resorption, second trimester miscarriage, growth retardation of the embryo, craniofacial abnormalities, and malformations of the central nervous system. Apart from affecting the brain it also involves other vital organs like the liver, kidney and heart. The most common heart abnormalities include atrial and ventricular septal defects, Fallot's tetralogy, and abnormalities of the great vessels (Cavieres & Smith, 2000). The majority of ethanol embryotoxicity effects are dose dependant and more obvious when it is consumed during the period of organogenesis, which corresponds to the third to the eighth week post implantation in humans

(Parnell *et al.*, 2006). According to the UK department of Health guidelines, the recommended dose of alcohol for pregnant women, or for one who is trying to become pregnant, should not exceed 1 or 2 UK units once or twice a week (1 UK unit contain 8g of alcohol). Despite all the guidelines regarding alcohol consumption, data shows that alcohol consumption has increased during the last two decades (Henderson *et al.*, 2007).

The precise mechanism for ethanol induced embryonic dysmorphogenesis has not yet been completely clarified. However, various experimental studies suggest more then one factor is involved. One possible mechanism could be the action of metabolic end products of ethanol such as acetaldehyde, which in turn produce excessive amounts of reactive oxygen radicals and generate oxidative stress and could result in abnormal development of the embryo (Aberle *et al.*, 2003). However some studies also argue that it is the ethanol itself which is responsible for the production of excessive amounts of free radicals, rather than its end products (Reimers *et al.*, 2004). Reactive oxygen radicals are produced as a by-product of many metabolic reactions and are regulated at physiological levels by antioxidant defence mechanisms of the body, as well as by antioxidants present in the diet, when consumed in the recommended range. At physiological levels these reactive oxygen radicals participate in various cell processes especially in tissue remodelling, hormone signalling, steroidogenesis and germ cell function in adults as well as in embryos (Hossein *et al.*, 2007). If antioxidants are not sufficient to maintain their physiological limits, these oxidative radicals

cause damage to lipids, DNA and proteins and result in malformations of the embryo (Jauniaux *et al.*, 2004). Studies have also suggested that fetal tissues have a more compromised or less active antioxidant system than adult tissue; thus embryos would be more sensitive to oxidative injury (Reimers *et al.*, 2006).

6.1.2. Nicotine

Maternal smoking is considered to be one of the leading causes of congenital malformations. Active and passive smoking is an important risk factor for the developing fetus in every trimester of pregnancy (Jauniaux & Burton, 2007). Smoking during the periconceptional period affects the development of major organs of the fetus including, the central nervous system, cardiovascular system and skeletal system. Also it results in perinatal complications such as preterm delivery, stillbirth, low birth weight, and high perinatal mortality (Kallen, 1999). Moreover, children of mothers who smoke during pregnancy have an increased risk of developing behavioural problems, attention deficits, hyperactivity and learning disabilities and nicotine dependence later in life (Morales-Suarez Varela *et al.*, 2009). Cigarette smoke introduces at least two thousand toxins into the blood stream, including cyanides, sulphides, cadmium, carcinogenic hydrocarbons and nicotine (Zhang & Ratcliffe, 1993). Among all these toxins, nicotine is considered to be the major teratogenic constituent. Nicotine is a low molecular weight substance and is quickly absorbed into the blood stream from the lungs and oral cavity. It can also easily cross the placenta, with resultant relatively higher concentration of nicotine and its metabolites in the fetus (Yildiz, 2004). Nicotine exerts its effects by acting

on nicotinic acetylcholine receptors. These receptors in turn increase ion influx including Ca^{2+}. Increased concentrations of calcium ions, disturbs intracellular signalling and cell function. As a result of disruption of cellular function, there is increased production of reactive oxygen species (ROS), leading to oxidative stress (Ermak & Davies, 2002). Furthermore it has also been shown that increased concentrations of nicotine in the body compartments result in decreased activities of free radical scavenging enzymes. As a consequence of these events, excessive free radicals exert their toxic effects on many cellular organelles in different tissues and cause deleterious injury. In one study conducted on rat brain, Gumustekin, K. and his colleagues found a protective effect of vitamin E on nicotine induced damage (Gumustekin *et al.*, 2003). Similarly, protective effects of vitamin C, another antioxidant, were also observed on nicotine induced oxidative stress (Kelly, 2003).

This study is aimed at detecting any toxic effects of ethanol and nicotine on chick cardiomyocytes cultured in vitro and chick embryos in ovo. Also to investigate any possible protective effects of antioxidants vitamin C and folic acid on ethanol and nicotine induced toxicity.

6.2. Materials and Methods

6.2.1. Micromass Culture

Chick heart cells were seeded in 24 well plates at a concentration of 3×10^6 in each well in the form of 20µl droplets. They were incubated for 2 hours to allow cellular attachment. Then 500µl of DMEM growth medium was added and the cultures were left for 24 hours (Chapter 2, section;

2.1.4.5.2). The DMEM medium was replaced by different concentrations of test chemicals after 24 hours.

6.2.1.1. Test chemical preparation

The test chemicals were serially diluted from their stock solutions (Chapter 2 table 2.1) in DMEM supplemented culture medium to give a desired concentration (Ethanol 10µl/ml, 20µl/ml, 50µl/ml, 100µl/ml; Nicotine 2µM, 5µM, 10µM, 20µM; vitamin C 100µM; folic acid 1mM; superoxide dismutase (SOD) 2µM. All chemicals were prepared and applied to micromass cultures within 1 hour of preparation.

6.2.1.2. End points

The differentiation assay, resazurin reduction assay and kenacid blue assay were performed as described in (Chapter 2, sections2.1.5.1 and 2.1.5.2). The cultures were inspected morphologically for cardiomyocyte contractile activity at 24, 48 and 144 hours, while resazurin and kenacid blue assays was performed at 144 hours.

6.2.2. In Ovo culture

White leghorn fertilized chicken eggs were incubated as described in (Chapter 2, section 2.2.3.1. At day 3 of incubation, eggs with viable embryos were either kept untouched or injected with 100µl of ethanol or nicotine (100µl/ml or 10µM respectively), vitamin C (100µM), folic acid (1mM), ethanol plus vitamin C or folic acid, nicotine plus vitamin C or folic acid or PBS, by making a hole on their blunt end. Eggs were then sealed, labelled and re-incubated with the blunt end up till day 9 of incubation. On day 9, embryos were taken out under sterile conditions and

examined under a dissecting microscope for any gross malformations and scored according to the criteria shown in table 4.1 (Chapter 4).

6.2.2.1. Test chemical preparation

The test chemicals were serially diluted from their stock solutions (Chapter 2 table 2.1) in PBS to give a desired concentration (ethanol 100µl/ml, nicotine 10µM, folic acid 1mM, and vitamin C 100µM. All chemicals were prepared and applied to chick embryos in ovo within 1 hour of preparation.

6.2.3. Statistical analysis

All statistical analysis for this chapter was performed using Prism 5 (Graphpad Software Inc. San Diego, USA). All results were compared using one way ANOVA with Dunnett's and Dunn's multiple comparison post hoc tests, where $p < 0.05$ was considered statistically significant.

6.3. Results

6.3.1. Results of micromass culture

6.3.1.1. Results of ethanol treatment

The aim of this part of the study was to detect the developmental toxicity of ethanol in chick cardiomyocytes cultured in the micromass system and also to see any protective effects of antioxidant vitamin C and folic acid. Furthermore to confirm antioxidant activity of folic acid and vitamin C, SOD (an antioxidant enzyme) was added to ethanol treated cultures.

Cells were scored at 24, 48 and 144 hours and results are shown in fig 6.1 (a). The graph shows a clear trend of decreased cardiomyocytes beating at

all concentrations of ethanol. Statistical analyses were performed on results for all the days on which the cells were scored. At 24 hours there was no significant difference between cell scores but this was to be expected as scoring was performed just before addition of chemicals. However this does confirm the reliability of results as all the wells were starting at the same level of differentiation prior to treatment with chemical.

Statistical analyses of cell scores on day 2 showed a significant difference ($p < 0.05$) between cell scores of ethanol treatment and controls. Results also show that addition of folic acid and vitamin C to ethanol treated cells can protect the cells from the embryotoxic effects of ethanol (fig 6.2 a and 6.2 b). Results also show that addition of SOD to ethanol treated cultures had protective effects against ethanol induced toxicity (fig 6.3 a).

Fig 6.1 (b) shows the results of the resazurin reduction assay. Here again the graph shows a highly significant difference ($p<0.05$) in the amount of resorufin being produced, and hence the cell viability, at 50 and 100µl/ml ethanol. This would suggest that ethanol is cytotoxic to cells. The results also show that addition of 100µM vitamin C or 1mM folic acid to 100µl/ml ethanol (the highest concentration of ethanol used in this study) can protect the cultures from the toxic effects of ethanol, as the results with addition of folic acid and vitamin C were comparable to the control respectively (fig 6.2 c and 6.2 d). Moreover addition of SOD to ethanol treated cardiomyocytes has the same effects as folic acid and vitamin C (fig 6.3 b).

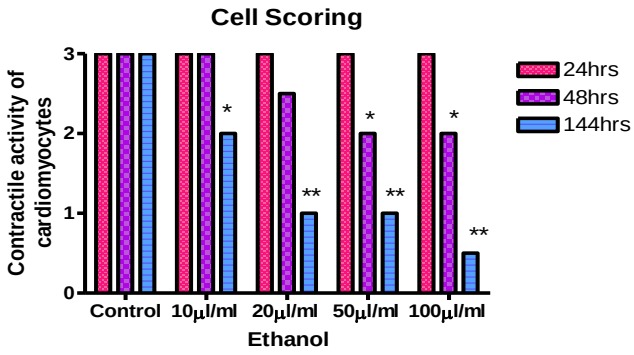
The protein content of cardiomyocytes is also decreased at 50 and 100µl/ml ethanol as shown in fig 6.1 c. Statistical analyses showed a

significant difference to controls ($p < 0.05$). The results also show that addition of 100μM folic acid and vitamin C to ethanol can protect the cultures from the cytotoxic effects of ethanol as the values were comparable to controls (fig 6.2 e and 6.2 f). Here again addition of SOD to ethanol has protective effects and proteins were comparable to the control (fig 6.3 c).

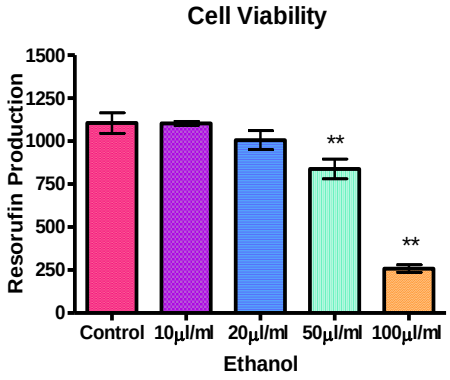
6.3.1.1.1. Conclusion

The micromass results indicate that addition of vitamin C and folic acid could protect the heart cells from toxic effects of ethanol.

6.1 a



6.1 b



6.1 c

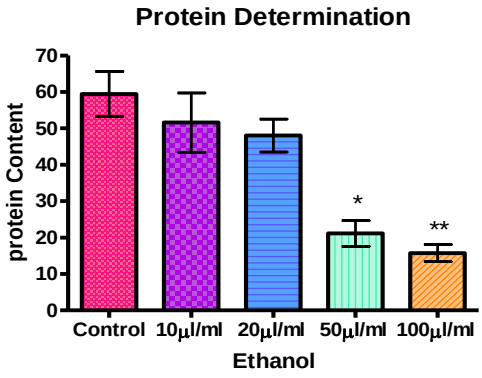


Figure 6.1: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with different concentrations of ethanol. {* ($p<0.05$); ** ($p<0.01$) for Dunnett's and Dunn's post hoc tests applied for parametric and non parametric data respectively}.

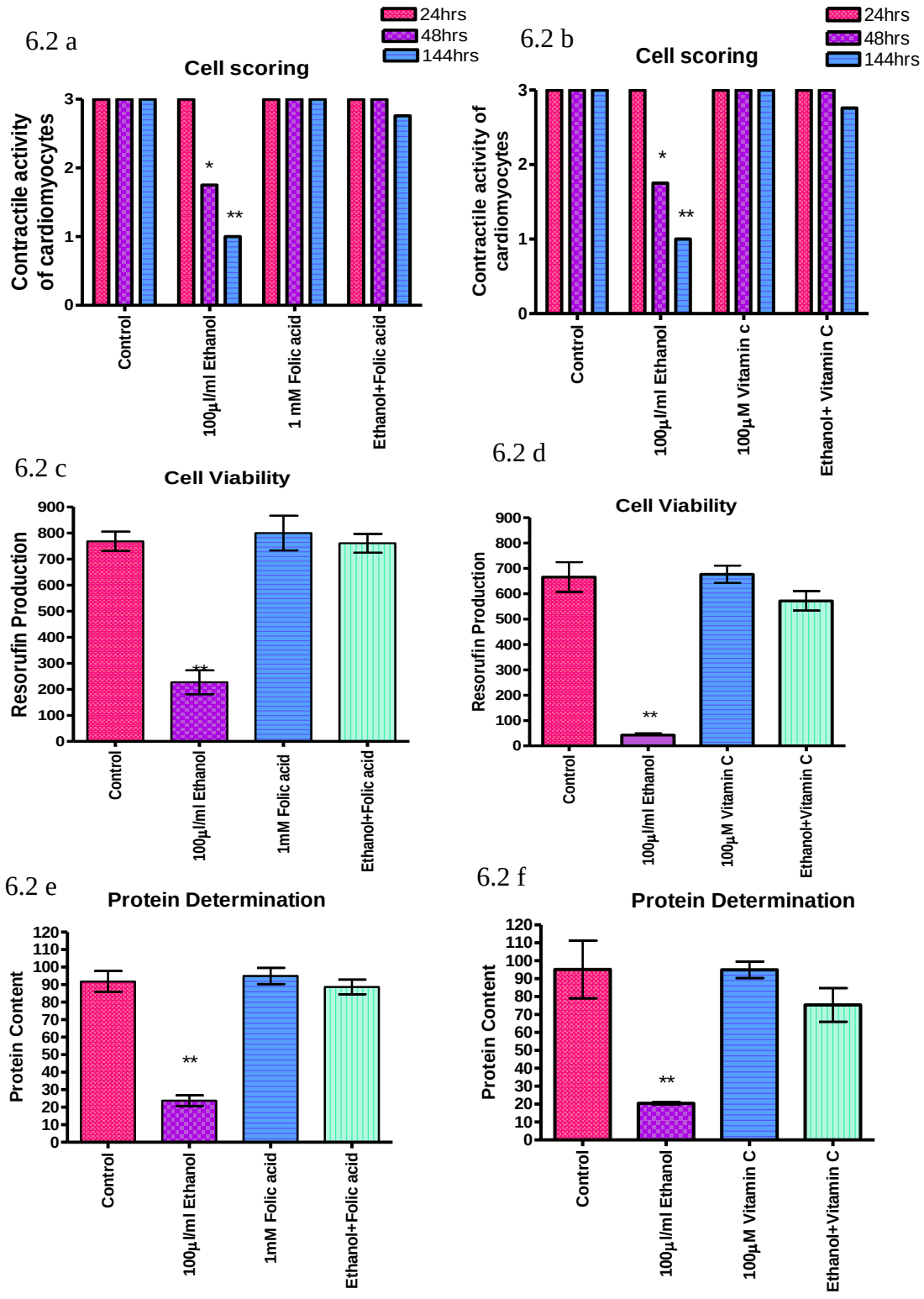


Figure 6.2: Shows cell scoring (a,b), resazurin assay (c,d), and protein assay (e,f) with ethanol plus folic acid and vitamin C. {* ($p<0.05$); ** ($p<0.01$) for Dunnett's and Dunn's post hoc tests applied for parametric and non parametric data respectively}.

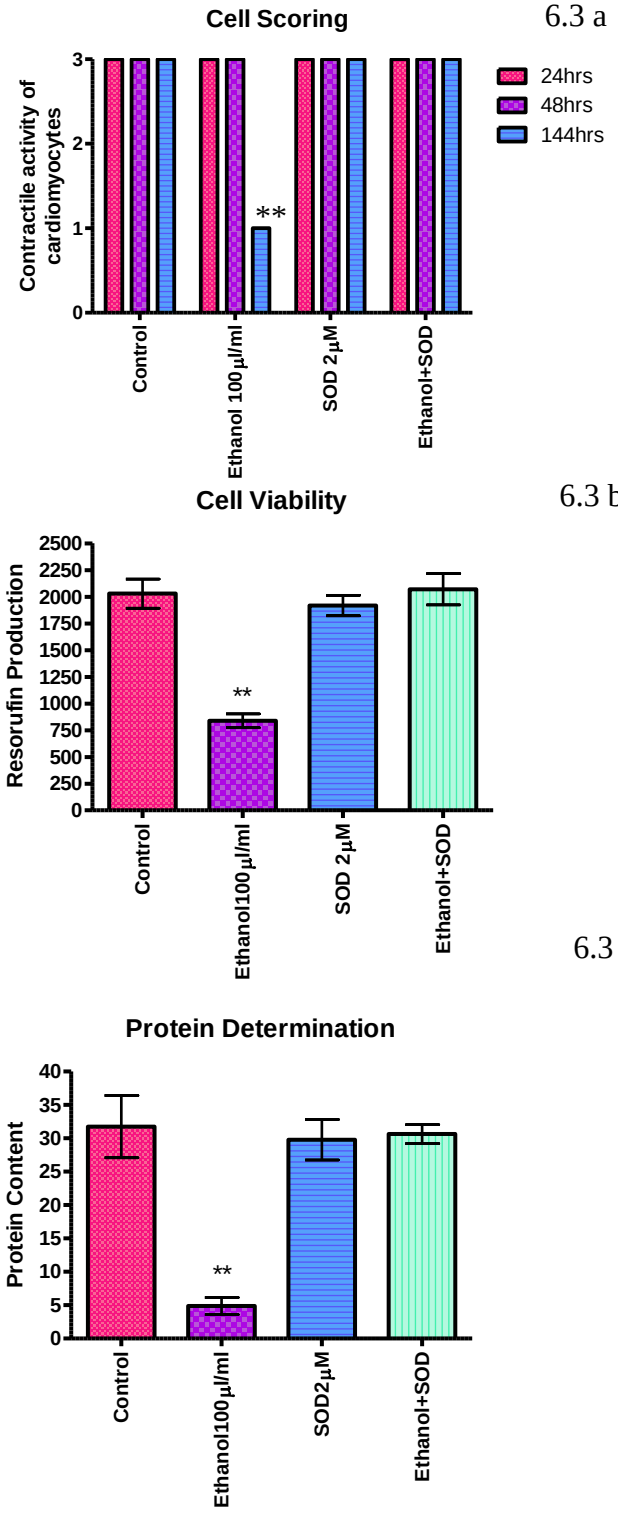


Figure 6.3: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with ethanol plus SOD. {** ($p<0.01$) for Dunnett's and Dunn's post hoc tests applied for parametric and non parametric data respectively}.

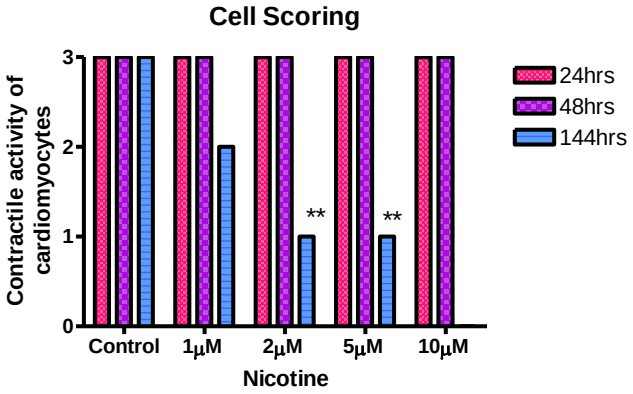
6.3.1.2. Results of Nicotine treatment

The aim of this part of the study was to detect the developmental toxicity of nicotine in chick cardiomyocytes cultured in the micromass system and also to see any protective effects of antioxidant vitamin C and folic acid.

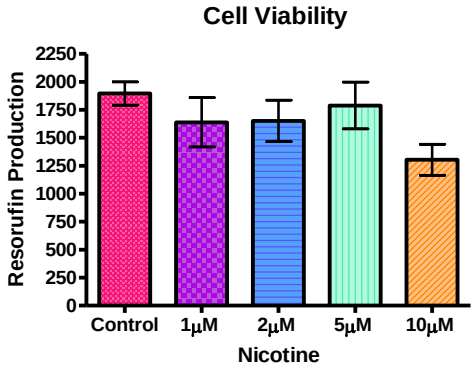
Cells were scored at 24, 48 and 144 hours and results are shown in fig 6.4 a. The graph shows that at 10µM nicotine, cardiomyocytes showed less contractile activity on day 2 and had no any beating on day 6. Statistical analyses showed that at 24 hours there was no significant difference between cell scores but this was to be expected as scoring was tested just before addition of chemicals. Statistical analyses of cell scores on day 2 and 6 showed a significant difference ($p < 0.05$) between cell scores at nicotine (10µM) and controls. Results also show that addition of folic acid and vitamin C to nicotine treated cells can protect the cells from the embryotoxic effects of nicotine (fig 6.5 a and 6.6 a).

The graph for the resazurin reduction assay shows a decrease in the amount of resorufin produced by nicotine treated cells (10µM), but that is not significantly different to controls (fig 6.4 b). The results also show that the cells treated with addition of 1mM folic acid or 100µM vitamin C to nicotine are comparable to controls (fig 6.5 b and 6.6 b). The protein content of cardiomyocytes is also decreased at 10µM nicotine as shown in fig 6.4 (c). However statistical analyses showed no significant difference to controls. The results also show that the cells treated with addition folic acid and vitamin C in addition to nicotine were comparable to controls (fig. 6.5 c and 6.6 c).

6.4 a



6.4 b



6.4 c

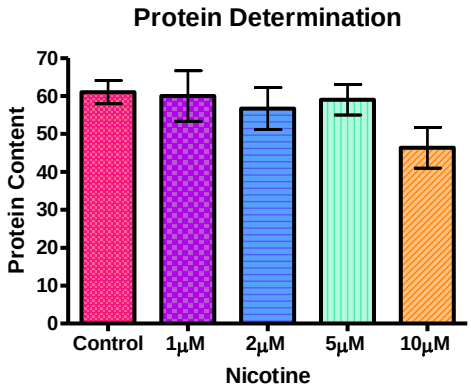


Figure 6.4: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with different concentrations of nicotine. {** ($p<0.01$) for Dunn's post hoc test }.

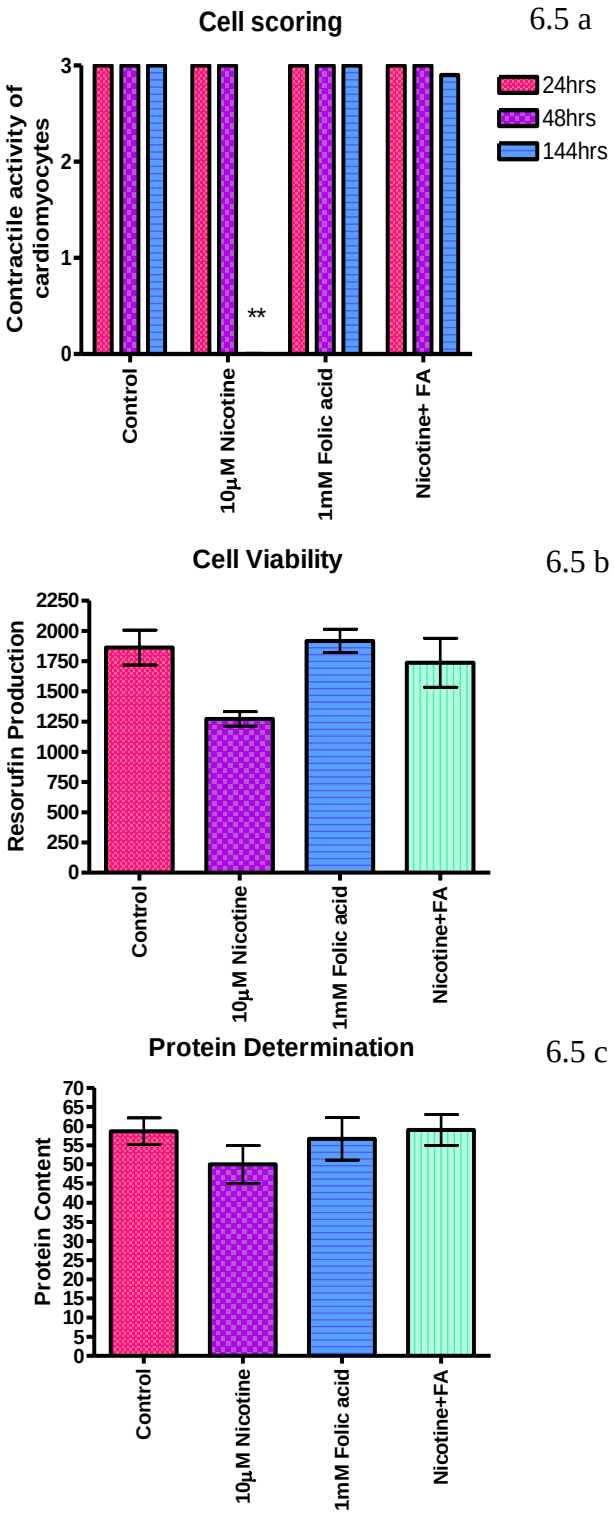
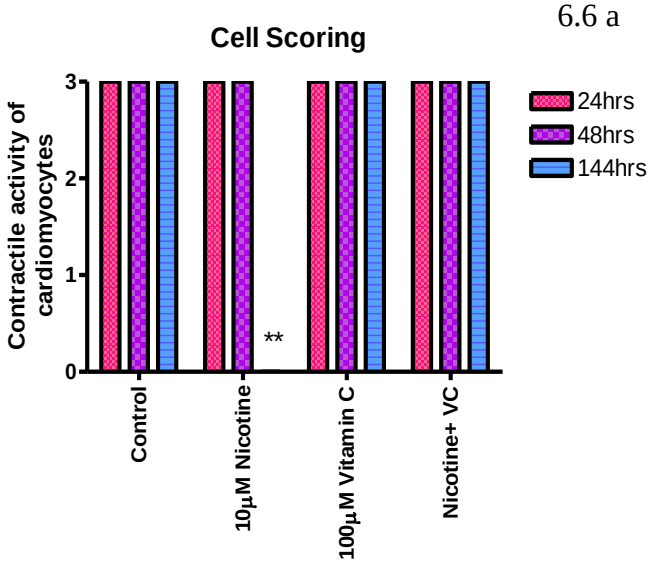
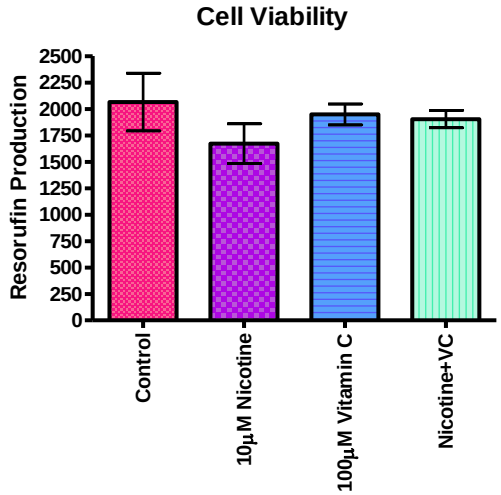


Figure 6.5: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with nicotine plus folic acid. {** ($p<0.01$) for Dunn's post hoc test }.

6.6 a



6.6 b



6.6 c

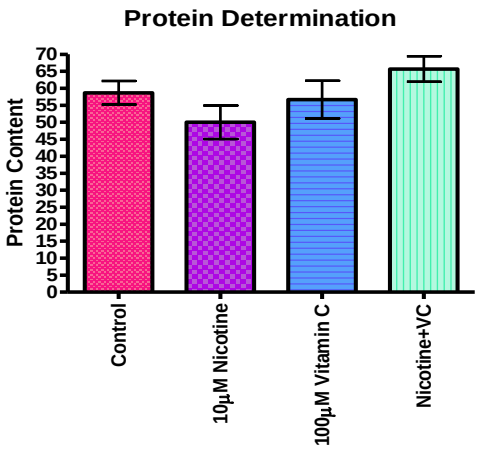


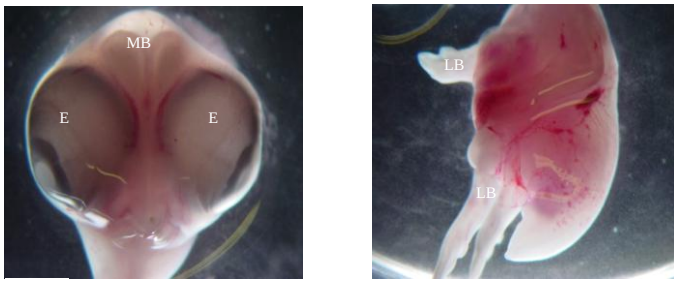
Figure 6.6: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with nicotine plus vitamin C. {** ($p<0.01$) for Dunn's post hoc test}.

6.3.2. Results of In Ovo culture

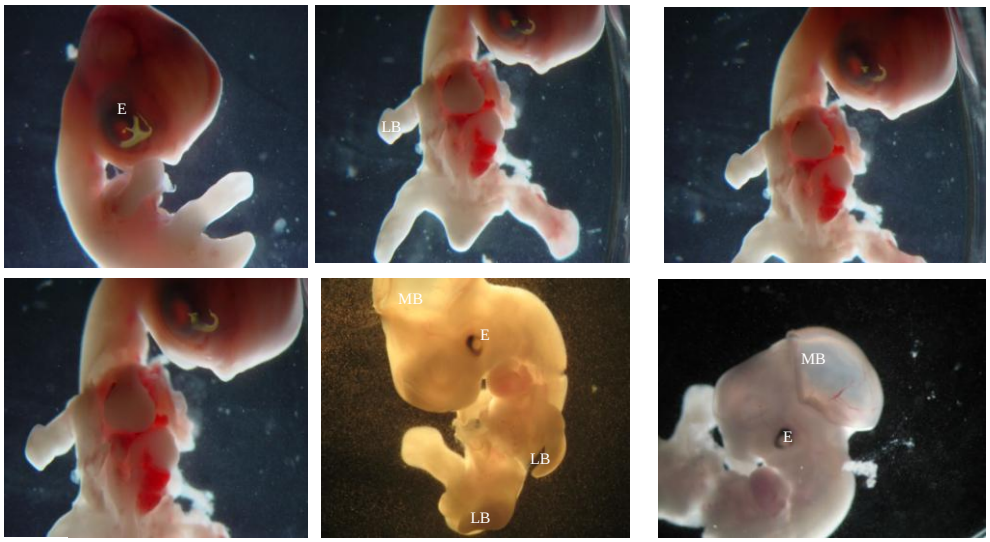
6.3.2.1. Results of Ethanol

Ethanol significantly reduced overall survival of embryos to less than 60%, while the survival rate of the remaining groups was more than 90%. Also embryos which were treated with PBS, showed no gross abnormalities (fig 6.7 a), while embryos cultured with ethanol were growth retarded and showed obvious gross malformations of the face, anterior thoracic and abdominal wall and limbs as compared to controls (fig 6.7 b). However embryos treated with vitamin C and folic acid in addition to ethanol showed no gross malformations and none of them was growth retarded as shown in fig 6.7 c and d respectively. Histological examination of the hearts clearly shows that whereas in the control there are clearly three chambers visible (fig 6.8 a), in the ethanol treated embryo the heart has failed to develop correctly and only one chamber is visible (fig 6.8 b). Treatment with additional vitamin C (fig 6.8 c) or folate (fig 6.8 d) restores normal development. Statistical analyses of crown rump length of embryos showed a decrease in length of ethanol treated embryos ($p<0.05$), while embryos with vitamin C and folic acid in addition grew normally and were comparable to control, as shown in fig 6.9. Moreover Statistical analyses of other parameters including vitelline circulation (fig 6.10 a), flexion of the embryo (fig 6.10 b), heart development (fig 6.10 c), craniofacial structures (fig 6.11 a), brain development (6.11 b) and limb development (6.11 c) show a significant difference between ethanol treated embryos and control ($p<0.05$). Here again addition of vitamin C and folic acid restored normal

development of all organ systems such that the embryos were comparable to control.



a



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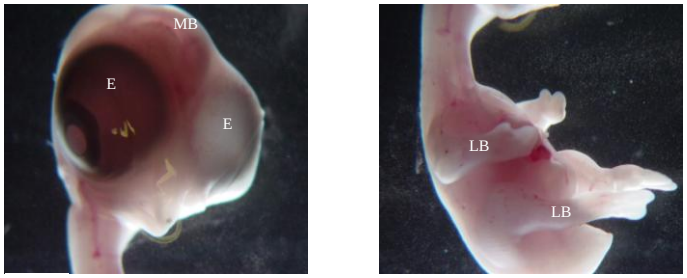
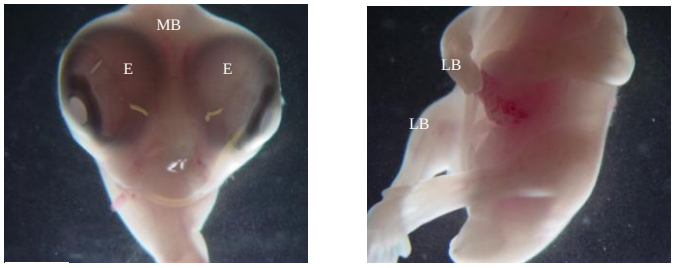
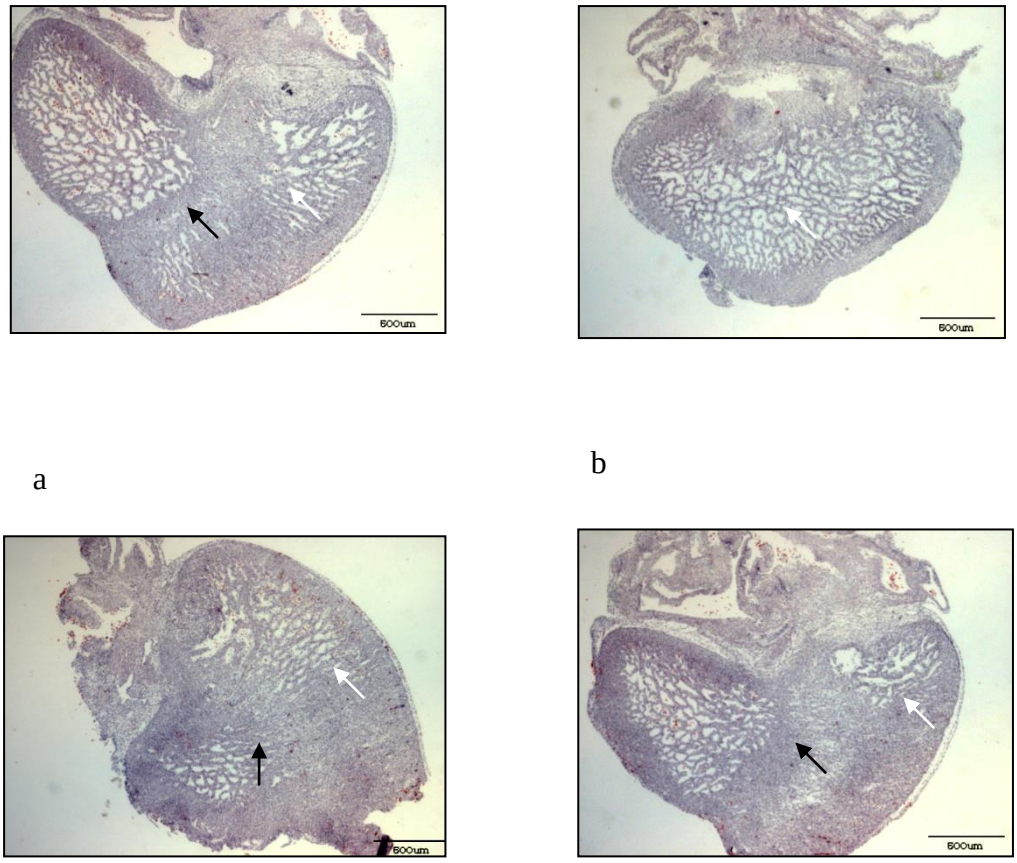


c



d

Figure 6.7: Shows control embryos (a), embryos treated with ethanol (b), ethanol plus vitamin C (c) and folic acid (d). The figure clearly demonstrates the abnormal development of eyes (E), midbrain (MB), and limb buds (LB) with ethanol treatment, however addition of folic acid and vitamin C rescued the embryos and they are comparable to control. Original magnification 200x.



a b

c d

Figure 6.8: Shows H&E staining of control hearts (a), treated with ethanol (b), ethanol plus vitamin C (c) and folic acid (d). Black arrows indicate interventricular septum which is not developed in ethanol treated hearts and white arrows indicate ventricular muscular wall which is also very thin in ethanol treated hearts as compared to controls and the hearts treated with vitamin C and folic acid in addition to ethanol. Original magnification 200x.

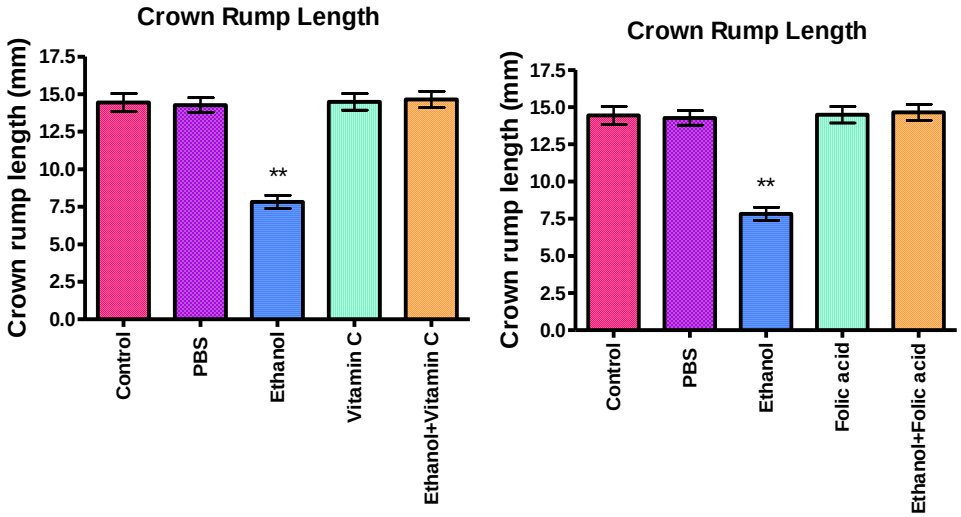
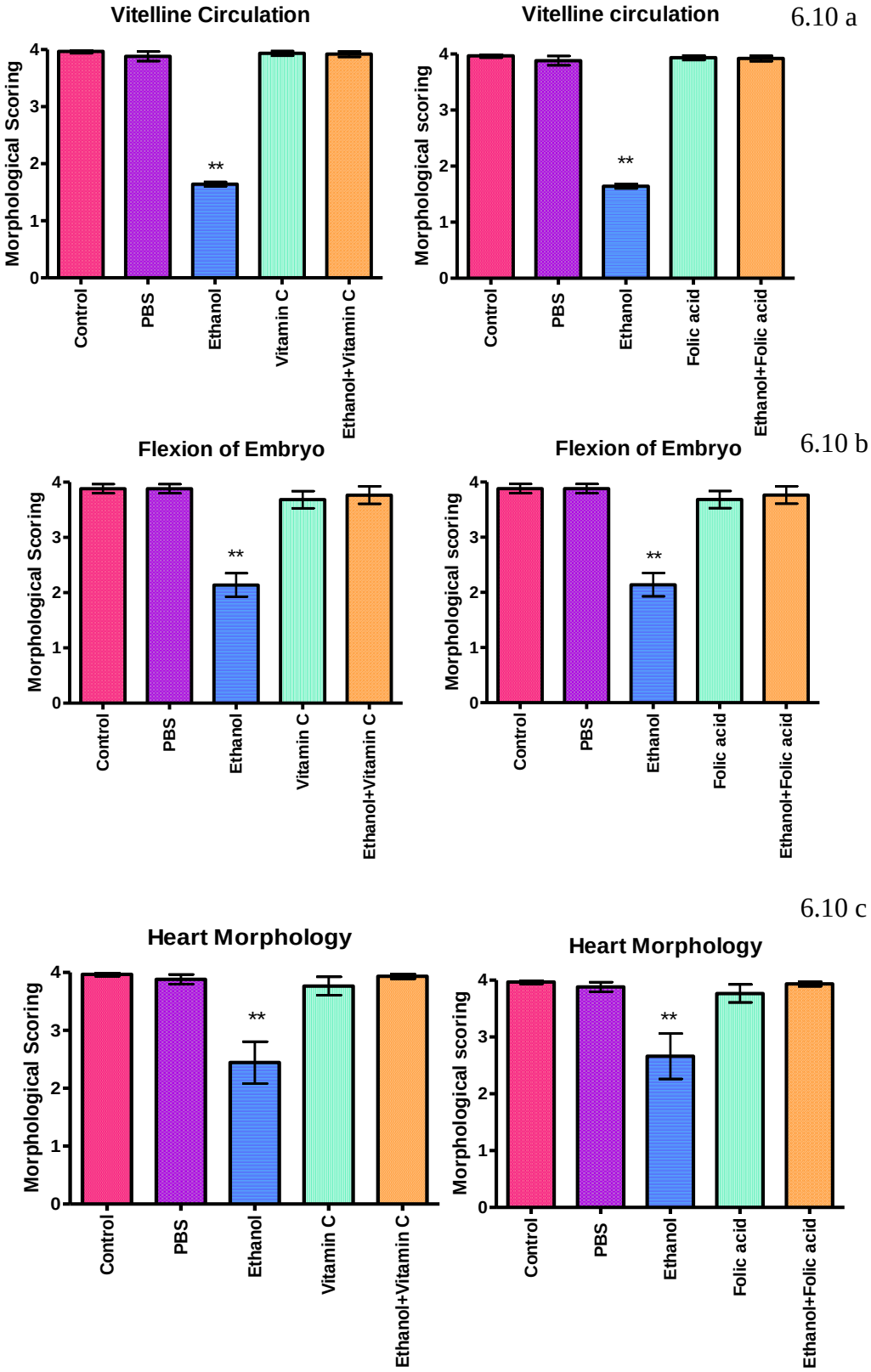


Figure 6.9: Shows crown rump length of embryos treated with ethanol, plus vitamin C and folic acid. {** ($p < 0.01$) for Dunnett's post hoc test}.



6.10 a

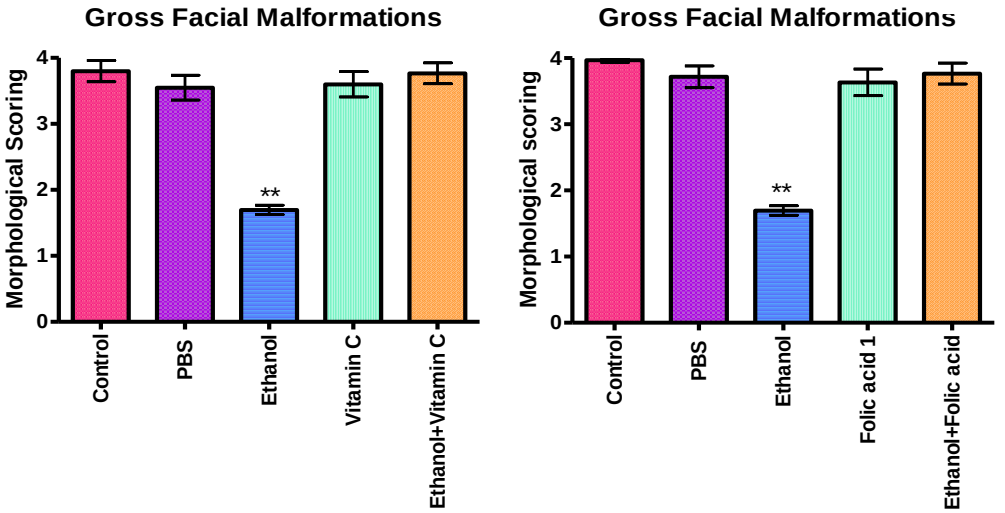
6.10 b

6.10 c

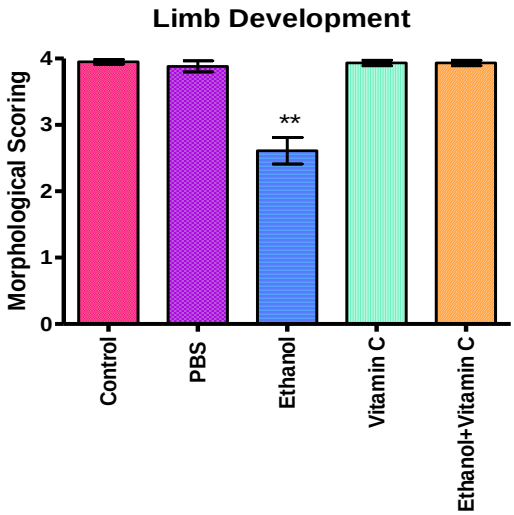
Figure 6.10: Shows vitelline circulation (a) flexion of embryo (b) and heart malformations (c) of embryos treated with ethanol, plus vitamin C and folic acid. {** ($p < 0.01$) for Dunnett's post hoc test}.

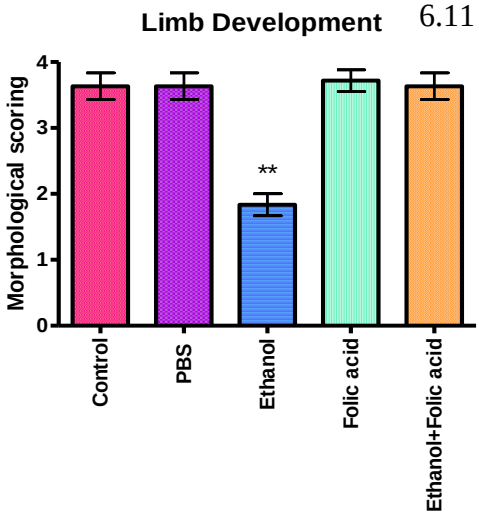
Figure 6.11: Shows facial malformations (a) and brain development (b) and limb development (c) of embryos treated with ethanol, plus vitamin C and folic acid. {** ($p < 0.01$) for Dunnett's post hoc test}.

6.11 a

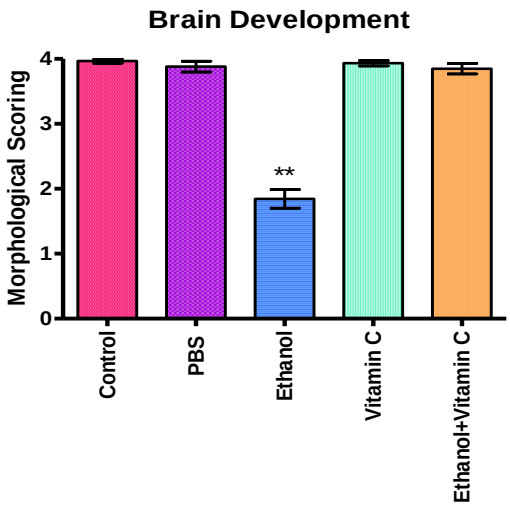


6.11 b





6.11 c



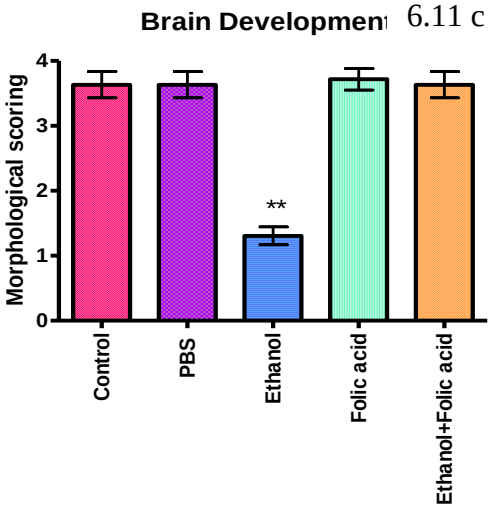
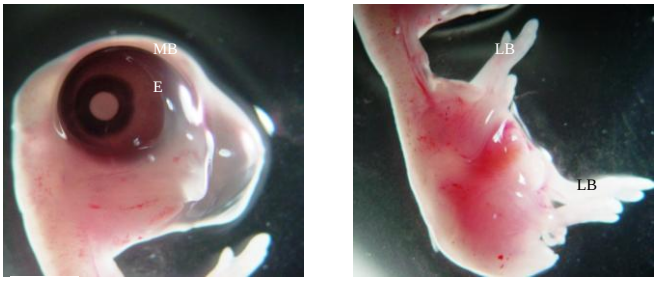


Figure 6.11: Shows facial malformations (a) and brain development (b) and limb development (c) of embryos treated with ethanol, plus vitamin C and folic acid. {** ($p < 0.01$) for Dunnett's post hoc test}.

6.3.2.2. Results of Nicotine treatment

The embryos treated with nicotine were growth retarded (fig 6.12 b) as compared to embryos treated with PBS, which showed no gross abnormalities (Fig 6.12 a), However treatment of embryos with vitamin C and folic acid in addition to nicotine showed no gross malformations and none of them was growth retarded as shown in fig 6.12 c & d respectively. Histological examination of the hearts shows that hearts of nicotine treated embryos (fig 6.13 b) show no abnormalities and were comparable to controls (fig 6.13 a). Also histological examination of hearts treated with additional vitamin C or folate were comparable to controls (fig 6.13 c & d respectively). Statistical analyses of the crown rump length of embryos showed a decrease in length of nicotine treated embryos ($p<0.05$), while embryos with vitamin C and folic acid in addition grew normally and were comparable to control, as shown in fig 6.14. Moreover Statistical analyses of other parameters including vitelline circulation (fig 6.15 a), flexion of the embryo (fig 6.15 b), heart development (fig 6.15 c), cranio facial structures (fig 6.16 a), brain development (fig 6.16 b) and limb development (fig 6.16 c) show no significant difference between nicotine treated embryos, control and vitamin C and folic acid treated embryos.



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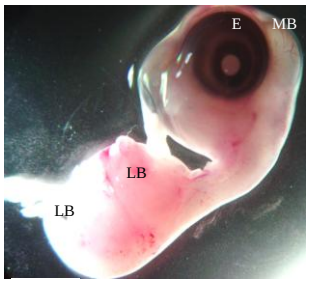

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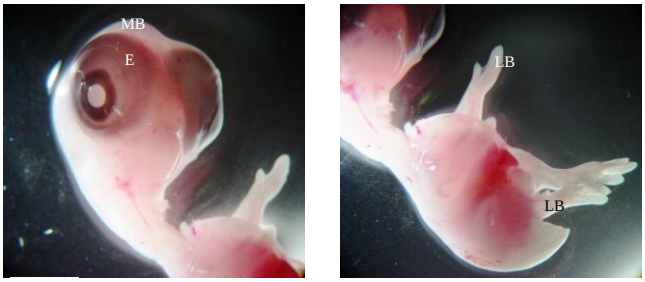

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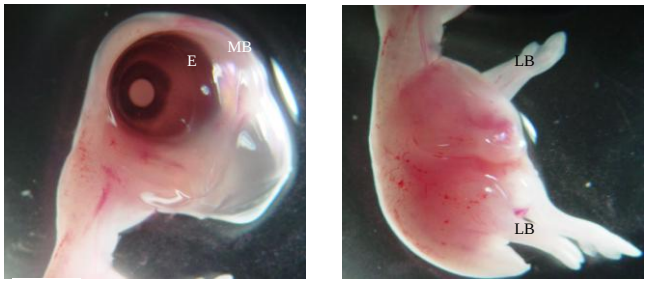
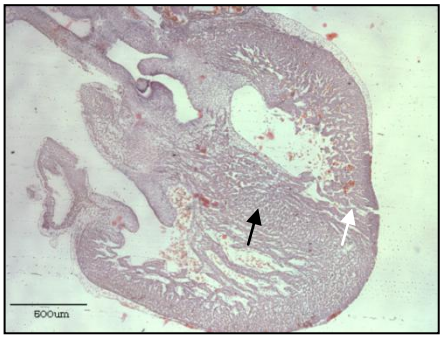
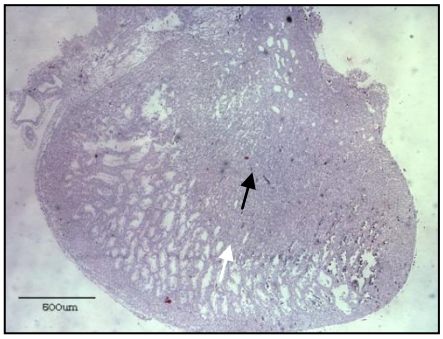


d

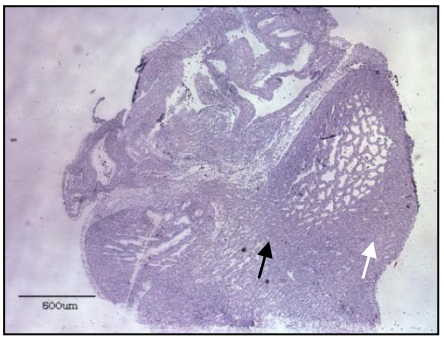
Figure 6.12: Shows control embryos (a), embryos treated with nicotine (b), nicotine plus vitamin C (c) and folic acid (d). This figure demonstrates that the development of the eyes (E), midbrain (MB) and limb buds (LB) is not affected by nicotine treatment as these features are comparable in nicotine treated embryos and rest of the groups, however nicotine treated embryos are smaller in size as compare to other groups. Original magnification 200x.



b



a



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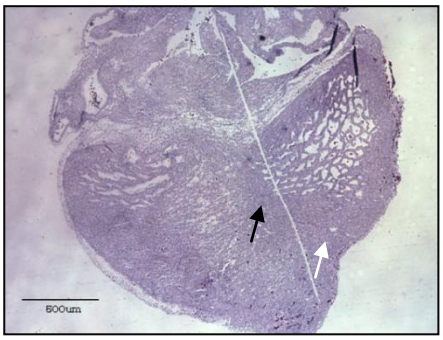


d

Figure 6.13: Shows H&E staining of control hearts (a), treated with nicotine (b), nicotine plus vitamin C (c) and folic acid (d). Black arrows indicate interventricular septum and white arrows indicate ventricular muscular wall. The figure shows that there are no histological abnormalities observed in nicotine treated embryonic hearts as compare to other groups. Original magnification 200x.

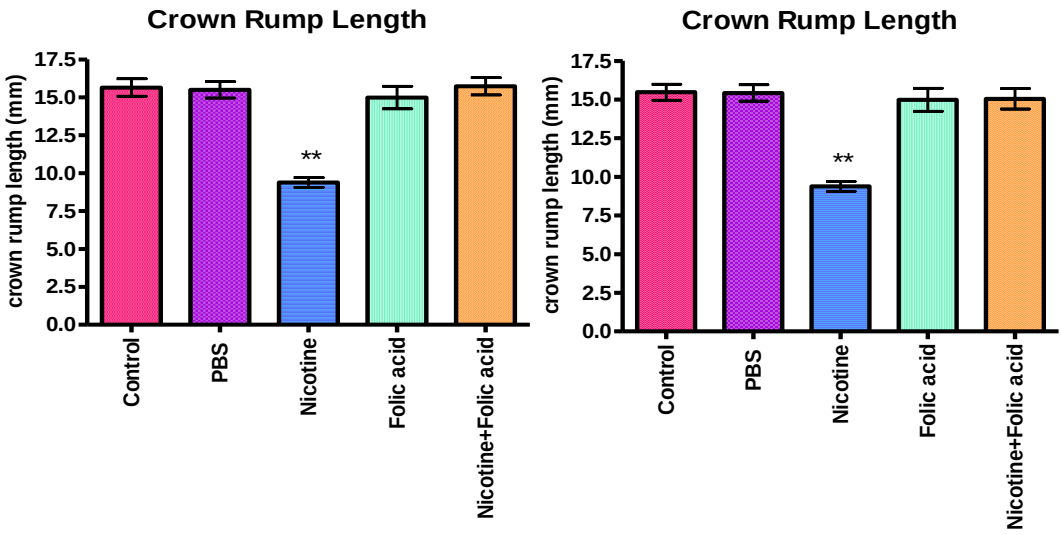
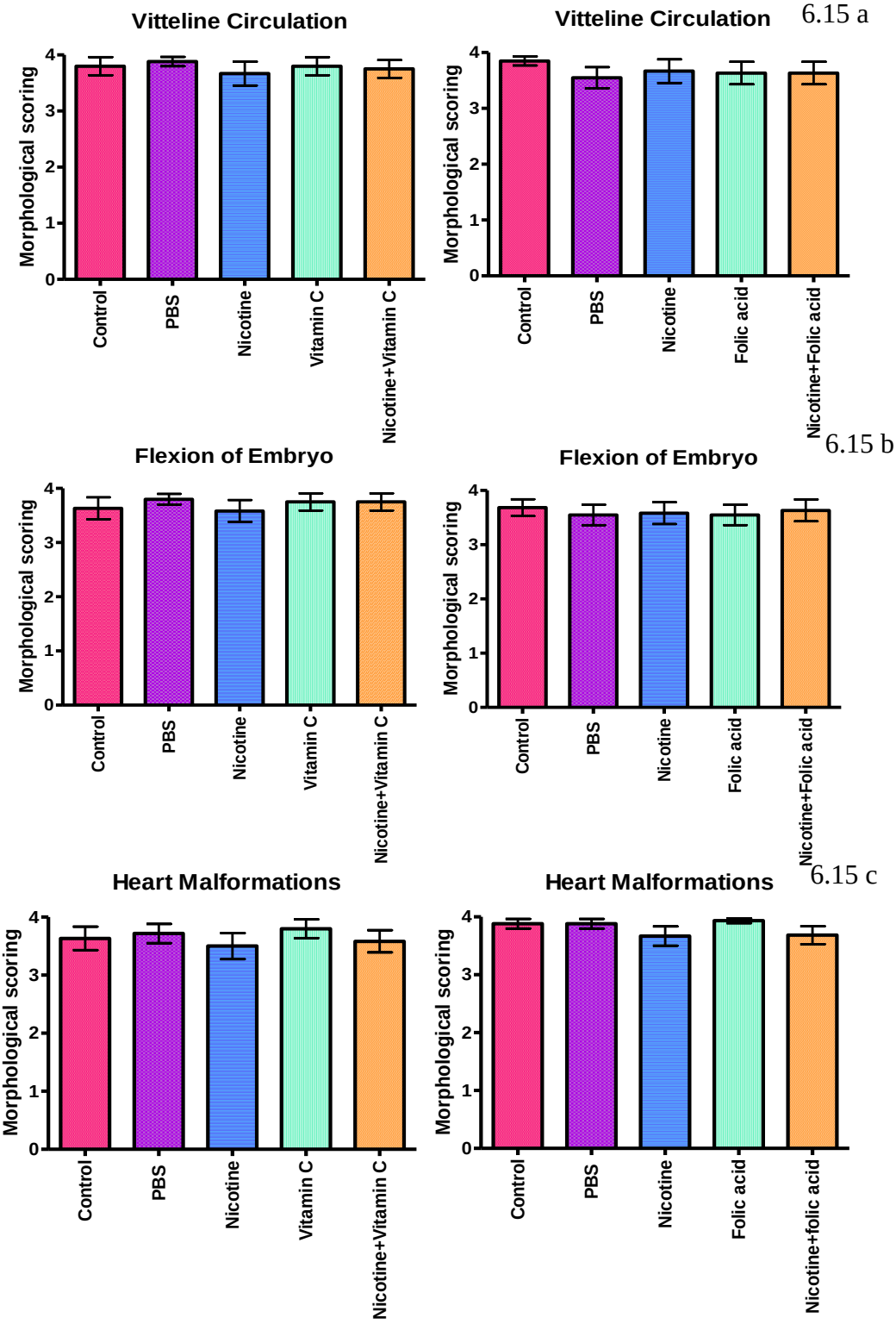


Figure 6.14: Shows crown rump of embryos treated with ethanol, plus vitamin C and folic acid. {** ($p < 0.01$) for Dunnett's post hoc test}.



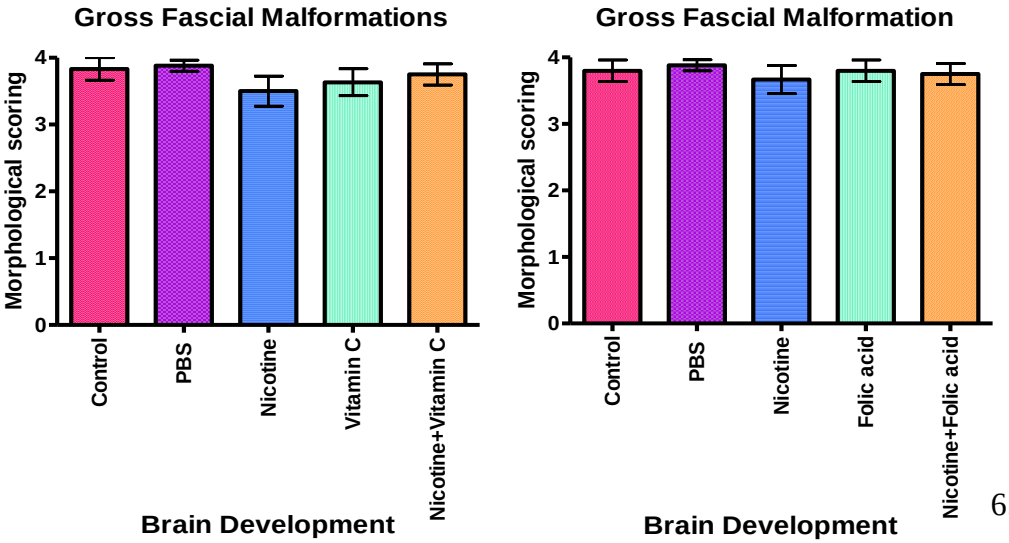
6.15 a

6.15 b

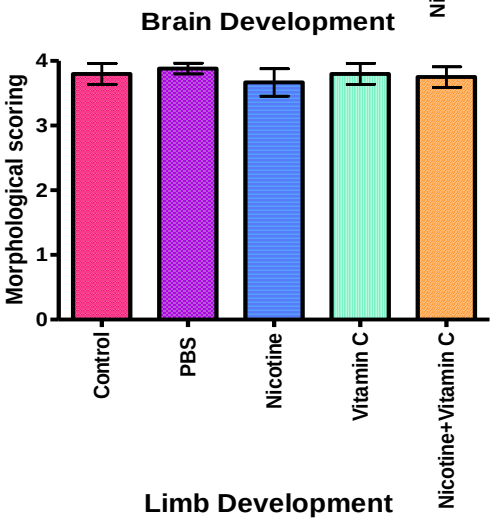
6.15 c

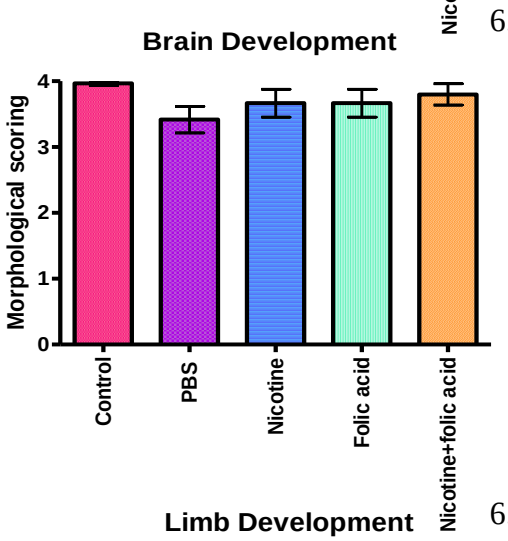
Figure 6.15: Shows vitalline circulation (a) flexion of embryo (b) and heart malformations (c) of embryos treated with nicotine, plus vitamin C and folic acid.

6.16 a

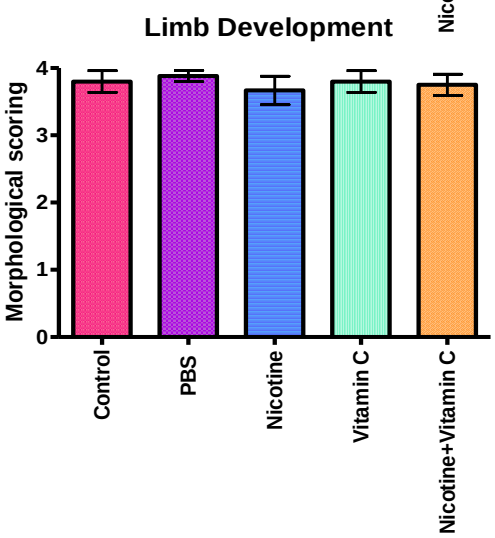


6.16 b





6.16 c



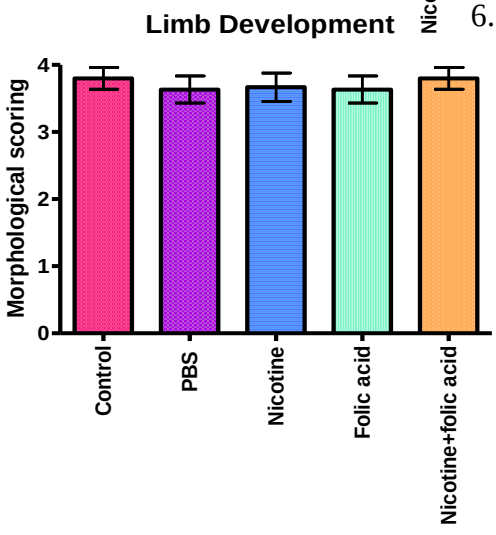


Figure 6.16: Shows facial malformations (a) brain development (b) and limb development (C) of embryos treated with nicotine, plus vitamin C and folic acid.

6.4. Discussion

Ethanol and nicotine are known human teratogens and therefore have been extensively investigated. This study was conducted to study ethanol and nicotine induced developmental toxicity and also to investigate any protective effects of vitamin C and folic acid in preventing teratogenic effects brought about by ethanol and nicotine, both in vitro and in ovo using chick embryos. Avian embryos were chosen as a model system for these studies as these are cost effective, easy to handle, less time consuming, involve no maternal sacrifice and provide reliable results. The teratogenic potential of ethanol on chick micromass culture, as well as in ovo culture, was confirmed by our present work. The effects were consistent for both culture systems used. While teratogenic effects of nicotine on chick micromass was confirmed in this study, the dose of nicotine which showed toxic effects on cardiomyocytes in culture had not shown toxic effects on chick embryos cultured in ovo, except growth retardation.

Using the chick micromass system the effects observed were consistent for any of the three end points measured. The reliability of chick micromass culture was tested by Wiger et al (Wiger *et al.*, 1988) with many toxic chemicals used to treat the limb bud cells. The main finding of our in ovo study for ethanol was that ethanol when injected caused a destruction of many organ systems of chick embryos. With ethanol about half of the embryos were dead and almost all remaining embryos were underdeveloped and showed features of fetal alcohol syndrome including

gross craniofacial malformations. These findings are consistent with previous studies performed on different animal species including rats, mouse and zebra fish, which showed similar types of abnormalities when treated with ethanol (Reimers *et al.*, 2006; Wentzel & Eriksson, 2006; Wentzel *et al.*, 2006). Our findings also suggest that most embryos had optic defects, which are similar to those found by Yelin et al (Yelin *et al.*, 2007), when he applied ethanol to Xenopus embryos. It is not possible to relate our findings of developmentally toxic levels of ethanol precisely with humans; however, studies conducted on humans showed a direct relationship of ethanol consumption in excess of the recommended dose and potential malformations in the offspring (Jones & Smith, 1973). Ethanol consumption is also one of the major risk factors for non-inherited causes of congenital heart disease especially ventricular and septal heart defects. However two case control studies conducted in Atlanta and Finland showed no significant difference in fetal cardiac malformation between alcoholic and non alcoholic mothers (Jenkins *et al.*, 2007). This could be due to the smaller study size or the dose of alcohol consumed.

Nicotine administration has teratogenic effects on many animal species. In one study conducted by Zhao and Reece on mouse embryos, nicotine was shown to have teratogenic effects in a dose dependant manner. The mouse embryos treated with nicotine were growth retarded (Zhao & Reece, 2005). These findings are consistent with this study as the majority of embryos treated with nicotine were growth retarded. However in another study conducted on rat embryos show that carbon monoxide was the major contributing factor for low fetal birth weight (Carmines & Rajendran,

2008). The epidemiological studies conducted on humans also showed a relationship between tobacco smoking and congenital malformations. The results of one study conducted in the Baltimore–Washington area, show an association between maternal cigarette smoking of more than one pack per day and cardiovascular malformations, including: transposition of great vessels with ventricular septal defect and pulmonary stenosis. Similarly a relationship between maternal smoking and increased risk of neonates born with congenital heart diseases was observed in a case-control study conducted in Lithuania (Kučienė & Dulskienė, 2008). However some epidemiological studies also show no association between smoking and congenital malformations (Kallen, 1999; Scherbak Y. *et al.*, 2007).

The present study also demonstrates a protective effect of Vitamin C and folic acid on ethanol and nicotine treated cardiomyocytes in vitro as well as in ovo. The effective concentration of vitamin C shown to have a protective effect is 100µM which is consistent with the amount of vitamin C used by Peng et al (Peng *et al.*, 2005) to treat ethanol induced growth retardation and microcephaly in Xenopus laevis embryos. The concentration of folic acid with protective effect is 1mM which is similar to the amount of folinic acid used to treat the malformations of branchial arch derived structures in rats in whole embryo culture by Zhang et al by (Zhang *et al.*, 2006). In a case control study, conducted in Atlanta a direct relationship of prevention of cardiac defects, especially outflow tract and ventricular septal defects, with periconceptional use of multivitamins containing folic acid and vitamin C was suggested (Botto *et al.*, 2003; Botto *et al.*, 2004; Willcox *et al.*, 2008). Similar results were also found in

a randomized control trial in Hungary which showed a reduction of 50% in cardiac defects with supplemental folic acid during pregnancy (Gardinar, 2006).

The present study also confirmed this as results with addition of the potent antioxidant enzyme SOD were similar to those with addition of these two vitamins. The observations that supplemental vitamin C and folic acid offered a remarkable protection against the effects of ethanol and nicotine might be due to their antioxidant properties, as has been shown by animal studies conducted on chick and rat embryos (Henderson *et al.*, 1995; Nasha *et al.*, 2007). These studies proposed an involvement of oxidative stress produced by the generation of free oxygen radicals when embryos were exposed to ethanol, and suggest a critical role for reactive oxygen species in the teratogenic actions of ethanol. Under these conditions, reactive oxygen species may be continuously formed as a by product of metabolic reactions. Furthermore such reactive oxygen species have also been linked with embryonic heart defects (Hobbs *et al.*, 2005) caused by a variety of environmental toxins and maternal diseases. However the extent of reactive oxygen species production and the subsequent likelihood of a developmentally toxic effect depend on the time and dose of exposure. Therefore, in order for normal development of embryos to be achieved under conditions where free radicals or reactive oxygen species are generated at abnormally high levels, there must be a balance between free radical generation and Vitamin C, which may be useful in promoting degradation of free radicals (Barrow *et al.*, 2000). In fact embryos have less protective mechanisms against free radicals compared to adults

(Reimers *et al.*, 2006), therefore antioxidant supplements, like folic acid and Vitamin C, may be useful in protecting embryos against damage.

Chapter 7

Effect of diabetic condition on chick cardiomyocyte micromass culture and chick embryos cultured in ovo, and potential protective effects of vitamin supplementation

7.1. Introduction

Diabetes mellitus (DM) is characterized by abnormalities in carbohydrate, protein and lipid metabolism, caused by the complete or relative inadequacy of insulin secretion and/or insulin action. Diabetes in pregnancy is associated with an increased risk of maternal and fetal morbidity and mortality, and remains a significant medical challenge. Diabetes in pregnant women may be divided into clinical diabetes or pregestational diabetes (women previously diagnosed with type 1 or type 2 diabetes) and gestational diabetes (GDM), which is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (Forsbach-Sanchez *et al.*, 2005; AmericanDiabetesAssociation, 2009). GDM represents nearly 90% of all pregnancies complicated by diabetes (AmericanDiabetesAssociation, 2006), and it affects 2-5% of all pregnancies (Crowther *et al.*, 2005). Both type 1 and type 2 pregestational diabetic conditions are associated with early and late complications of pregnancy, while gestational diabetes is associated with complications in the second-half of pregnancy. However all types of diabetes are related to metabolic complications in the newborn (Hanson & Persson, 1993; Kjos & Buchanan, 1999; Platt *et al.*, 2002). Maternal diabetes significantly increases the risk for embryonic malformations (Vercheval *et al.*, 1990). These malformations arise at the early stages of organogenesis, approximately the first 8 weeks of pregnancy (Evers *et al.*, 2004). The most frequently observed malformations are congenital heart and central nervous system defects (Macintosh *et al.*, 2006). The cardiac

malformations include endocardial cushion (EC) defects, persistent truncus arteriosus (PTA) and ventricular septal defects (VSD) (Loffredo *et al.*, 2001). Despite all clinical interventions to achieve a good glycemic control during and before pregnancy, the rate of congenital malformations is still higher in the offspring of diabetic mothers than non-diabetic mothers. The underlying mechanisms of these defects are not known, several teratological pathways are involved in diabetic induced malformations (Zhiyong, 2010). These teratogenic pathways include alterations of metabolic and signalling pathways such as metabolism of arachidonic acid, inositol, sorbitol, folic acid, reactive oxygen species and alterations in the activation of protein kinase C (PKC). Many experimental studies suggest a partial role for reactive oxygen species (ROS) in diabetes induced congenital defects (Eriksson & Borg, 1991). Accumulation of reactive oxygen species is a result of either excessive free oxygen radical formation or decreased ability of scavenging enzymes or both. Excessive glucose metabolism in embryonic and fetal cells, exposed to increased levels of glucose could be a triggering factor behind diabetic induced oxidative stress. Another possibility of diabetic embryopathy is accumulation of excessive ketone bodies. One primary source of production of ROS is mitochondria which receive excessive amounts of pyruvate and oxygen and produce a large quantity of free radicals in electron transport chain reactions (Yang *et al.*, 1997). These ROS, particularly superoxide, leak from the mitochondria and initiate a cascade of events, which result in the formation of other radicals including hydrogen peroxide hydroxyl radicals. This oxidative stress may induce

DNA damage by either altering expression of key genes or enhancing apoptosis (Lee *et al.,* 1999). These events result in dysmorphogenesis of different organ systems in diabetic embryopathy.

This chapter aims to investigate the effects of high glucose and ketone body (β hydroxybutyric acid) concentrations on chick cardiomyocytes cultured in vitro, and chick embryos in ovo. This chapter also investigates the protective effects of antioxidants (folic acid and Vitamin C) on diabetic induced heart defects in chick embryos.

7.2. Materials and Methods

7.2.1. Micromass Culture

Chick heart cells were seeded in 24 well plates at a concentration of 3×10^6 in each well in the form of 20µl droplets. They were incubated for 2 hours to allow cellular attachment. Then 500µl of DMEM growth medium was added and the cultures were left for 24 hours (chapter 2 section 2.1.4.5.2). The DMEM medium was replaced by different concentrations of test chemicals after 24 hours.

7.2.1.1. Test chemical preparation

The test chemicals were serially diluted from their stock solutions (Chapter 2 table 2.1) in DMEM supplemented culture medium to give a desired concentration glucose 20 mM, β hydroxybutyric acid 10mM, folic acid 1 mM and vitamin C 100µM. All chemicals were prepared and applied to micromass cultures within 1 hour of preparation.

7.2.1.2. End points

The differentiation assay, resazurin reduction assay and kenacid blue assay were performed as described in chapter 2 sections 2.1.5.1 and 2.1.5.2. The cultures were inspected morphologically for cardiomyocyte contractile activity at 24, 48 and 144 hours, while resazurin and kenacid blue assays was performed at 144 hours.

7.2.2. In Ovo culture

White leghorn fertilized chicken eggs were incubated as described in chapter 2 section 2.2.3.1. At day 3 of incubation, eggs with viable embryos were either kept untouched or injected with 100µl of glucose +β hydroxybutyric acid (20mM+10mM respectively), vitamin C (100µM), folic acid (1mM), glucose +β hydroxybutyric acid plus vitamin C or folic acid, or PBS, by making a hole on their blunt end. Eggs were then sealed, labelled and re-incubated with the blunt end up till day 9 of incubation. On day 9, embryos were taken out under sterile conditions and examined under a dissecting microscope for any gross malformations and scored according to the criteria shown in table 4.1 Chapter 4.

7.2.2.1. Test chemical preparation

The test chemicals were serially diluted from their stock solutions (Chapter 2 table 2.1) in PBS to give a desired concentration (glucose 20 mM, β hydroxybutyric acid 10mM, folic acid 1 mM and vitamin C 100µM). All chemicals were prepared and applied to chick embryos in ovo within 1 hour of preparation.

7.2.3. Statistical analysis

All statistical analysis for this chapter was performed using Prism 5 (Graphpad Software Inc. San Diego, USA). All results were compared using one way ANOVA with Dunnett's and Dunn's multiple comparison post hoc tests, with $p < 0.05$ was considered statistically significant.

7.3. Results

7.3.1. Micromass culture

7.3.1.1. Effects of high glucose and β-hydroxybutyric acid concentrations

The aim of this in vitro study was to detect the teratogenicity of high concentrations of glucose and β-hydroxybutyric acid (ketone body) on chick heart cells cultured in the micromass system.

A range of different concentrations of glucose and β-hydroxybutyric acid was investigated, with the potential to produce teratogenic effects on cardiomyocytes in micromass culture. Concentrations of glucose and β-hydroxybutyric acid tested ranged between 5mM to 20mM for both chemicals.

Cells were scored at 24, 48 and 144 hours. Statistical analyses were performed on results for all the days on which the cells were scored. At 24 hours there was no significant difference between cell scores but this was to be expected as scoring was tested just before addition of chemicals. This does confirm the reliability of results as all the wells were starting at

the same level of differentiation prior to treatment with chemical. Statistical analysis of 48 hrs and 122 hrs post exposure shows that glucose has no effect on contractile activity of cardiomyocytes at all concentrations used 7.1 (a), while cells exposed to higher concentration (20mM) of β hydroxybutyric acid showed minimal or no beating at 122 hours post exposure (fig 7.2 a). However when cells were exposed to both of them together in culture at concentrations (20mM glucose and 15mM β-hydroxybutyric acid) which have no effect independently, a clear trend of decreased cardiomyocytes beating is observed at 122 hours post exposure (fig 7.3 a).

Fig 7.1 (b) 7.2 (b) and 7.3 (b) show the results of the resazurin reduction assay. Here again the graph shows no significant difference in the amount of resorufin being produced, and hence the cell viability, with glucose treatment (7.1 b). With 20mM β- hydroxybutyric acid treatment, a reduction in resorufin production and hence cell viability was observed (fig 7.2 b). The results also show that synergistic effects of combined glucose and β-hydroxybutyric acid result in decrease cell viability (fig 7.3b). The cells exposed to combined glucose and β-hydroxybutyric acid treatment were significantly different to controls ($p < 0.05$).

The protein content of cardiomyocytes was also decreased with synergistic effects of glucose and β-hydroxybutyric acid (fig 7.3 c), which was not observed with individual treatment at any concentration as shown in fig 7.1 c and 7.2 c respectively. Statistical analyses of cells treated with glucose and β-hydroxybutyric acid showed a significant difference to controls ($p < 0.05$).

7.3.1.2. Effects of folic acid and vitamin C on high glucose and β-hydroxybutyric acid treated chick cardiomyocytes.

The aim of this part of study was to investigate any protective effects of antioxidant vitamin C and folic acid on chick cardiomyocytes, cultured in high glucose and β-hydroxybutyric acid concentrations, in micromass system.

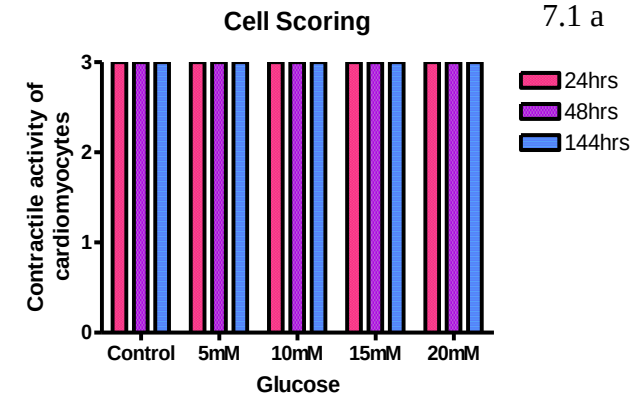
Cells were scored as described in the previous section at 24, 48 and 144 hrs. Results show that addition of vitamin C and folic acid to cells, treated with combined glucose and β hydroxybutyric acid, can protect the cells from the embryotoxic effects of combined glucose and β hydroxybutyric acid treatment (fig 7.4 a and 7.5 a). Statistical analysis showed that contractile activity of cells exposed to combined glucose and β hydroxybutyric acid treatment was significantly different to controls ($p < 0.05$). However cells treated with folic acid and vitamin C in addition to combined glucose and β hydroxybutyric acid treatment were comparable to controls (fig 7.4 a and 7.5 a).

Results of resazurin assay also show that addition of folic acid and vitamin C can protect the cultures from the toxic effects of diabetic conditions, as the results with addition of folic acid or vitamin C and were comparable to the control (fig 7.4 b and 7.5 b).

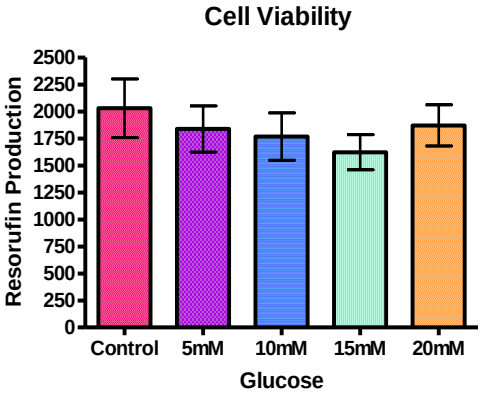
The kenacid blue protein assay results also show that addition of folic acid and vitamin C to cultures treated with combined glucose and β hydroxybutyric acid can protect the cultures from their cytotoxic effects as the values were comparable to controls (fig 7.4 c and 7.5 c).

7.3.1.2.1. Conclusion

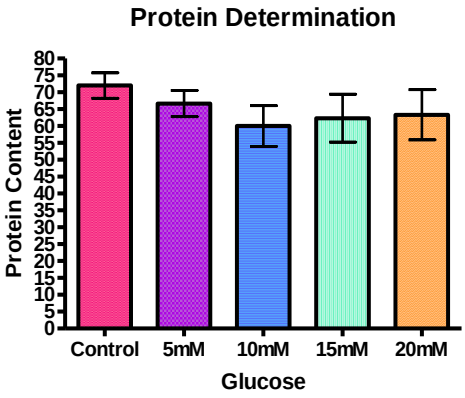
It is concluded from these results that diabetic conditions (high glucose and β-hydroxybutyric acid) result in cardiovascular malformations. Moreover results also showed that addition of antioxidants such as folic acid and vitamin C reversed toxic effects of diabetic environment.



7.1 a

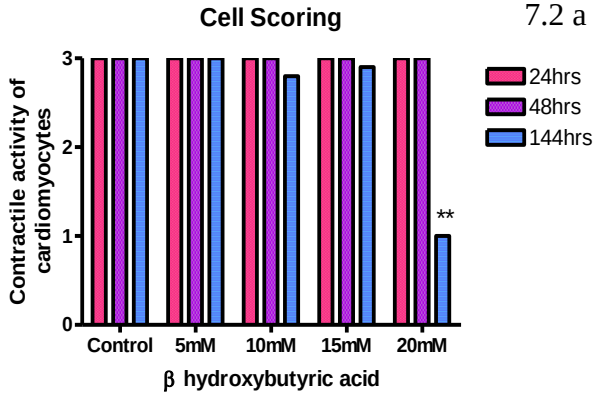


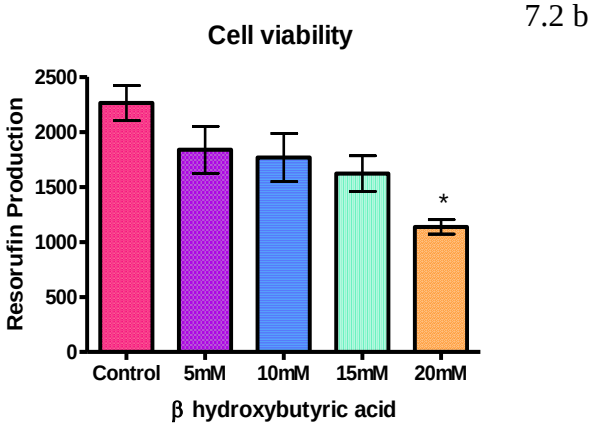
7.1 b



7.1 c

Figure 7.1: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with different concentrations of glucose





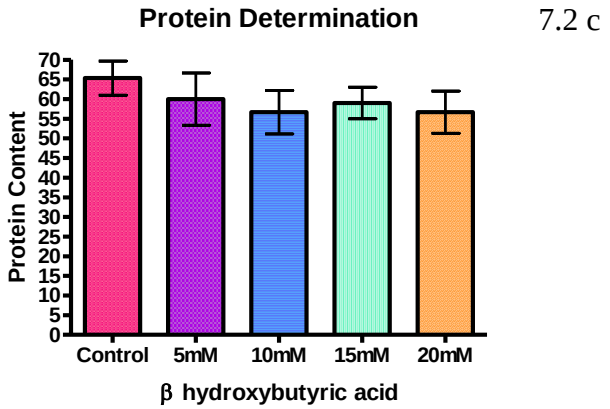
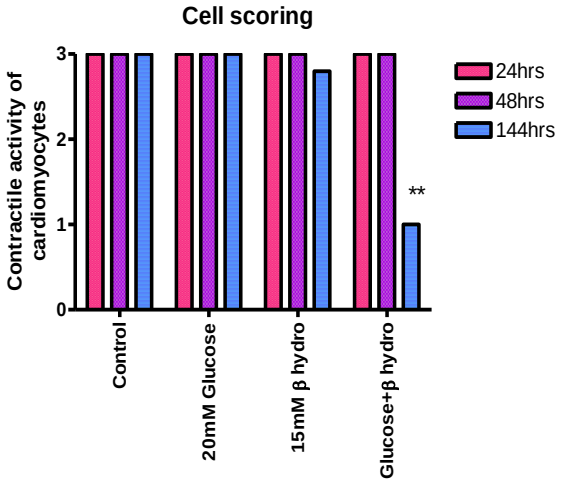


Figure 7.2: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with different concentrations of β-hydroxybutyric acid. {* ($p<0.01$); ** ($p<0.01$) for Dunnett's and Dunn's post hoc test applied to parametric and non parametric data respectively}.

7.3 a



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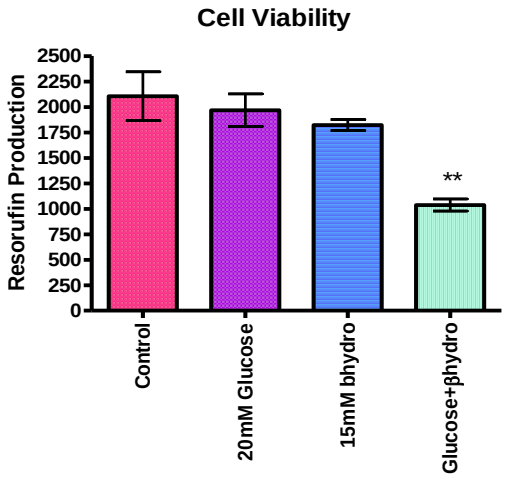

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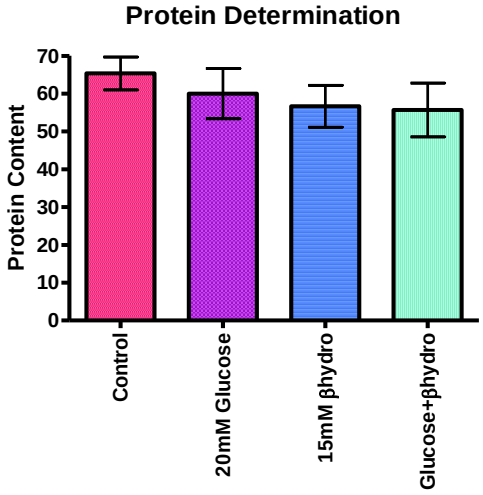
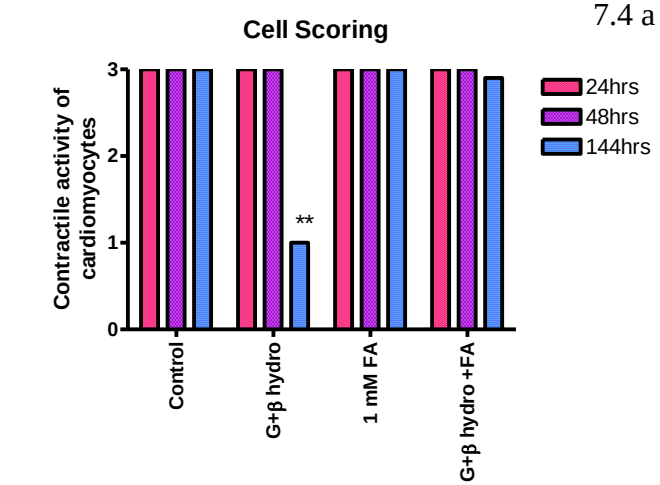


Figure 7.3: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with different concentrations of β-hydroxybutyric acid and glucose. {** ($p < 0.01$) for Dunnett's and Dunn's post hoc test applied to parametric and non parametric data respectively}.

7.4 a



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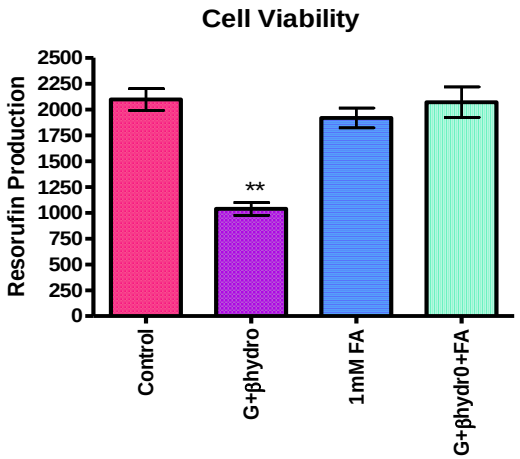


7.4 c

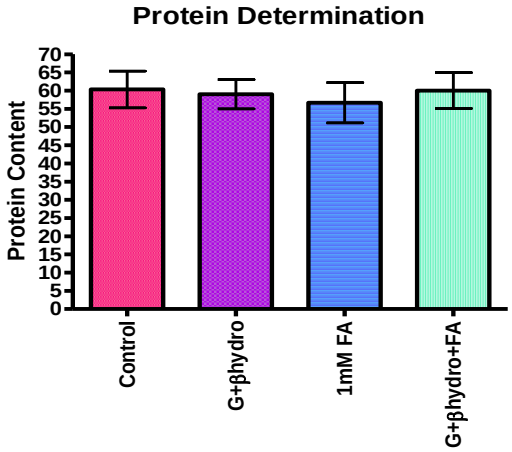
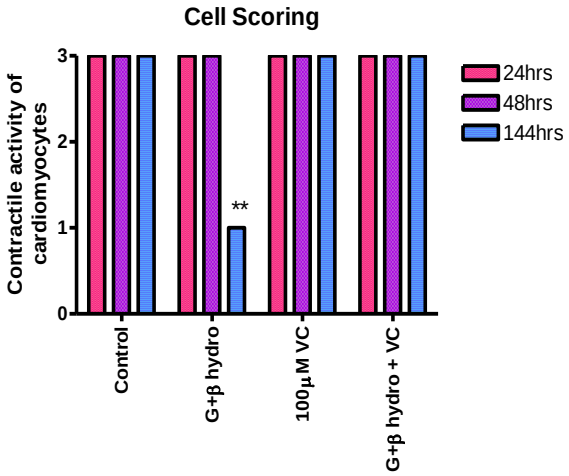


Figure 7.4: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with combined β-hydroxybutyric acid and glucose, plus folic acid. {** ($p < 0.01$) for Dunnett's and Dunn's post hoc test applied to parametric and non parametric data respectively}.

7.5 a



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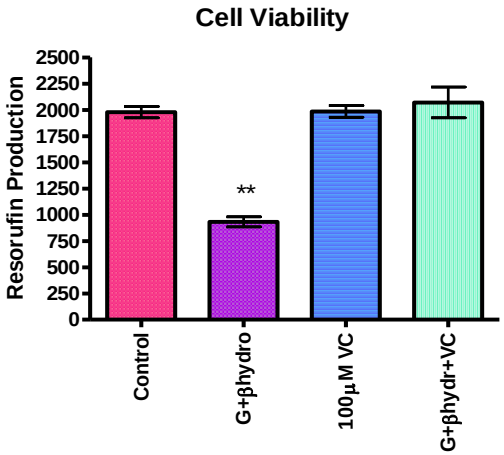


7.5 c

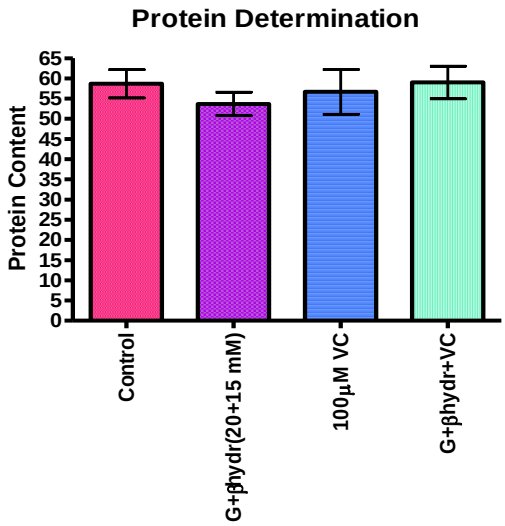


Figure 7.5: Shows cell scoring (a), resazurin assay (b), and protein assay (c) with combined β-hydroxybutyric acid and glucose, plus vitamin C. {** ($p < 0.01$) for Dunnett's and Dunn's post hoc test applied to parametric and non parametric data respectively}.

7.3.2. Results of In Ovo Culture

Diabetic condition (glucose and β hydroxybutyric acid) significantly reduced overall survival of embryos. Control embryos showed no gross abnormalities, while embryos with diabetic condition were growth retarded and showed obvious external malformations as compared to controls (fig 7. 6 a, 7.6 b). However treatment of embryos with vitamin C and folic acid in addition to diabetic conditions showed no gross malformations and none of them was growth retarded as shown in fig 7.6 c and 7.6 d respectively.

H& E staining of the hearts (Fig 7.7 a) shows that in control embryos the heart chambers are clearly visible, while in embryos treated with glucose and β hydroxybutyric acid fig 7.7 b, the heart has failed to develop correctly. Treatment with additional folate or vitamin C restores normal development (fig 7.7 c and 7.7 d). Statistical analyses of crown rump length of embryos showed a decrease in length of glucose and β hydroxybutyric acid treated embryos ($p<0.05$), while embryos with folic acid and vitamin C in addition grew normally as shown in fig 7.8. Moreover Statistical analyses of other parameters including vitelline circulation (fig 7.9 a), flexion of the embryo (fig 7.9 b), heart development (7.9 c), gross facial abnormalities (fig 7.10 a), brain development (fig 7.10 b), and limb development (fig 7.10 c), show a significant difference between glucose and β hydroxybutyric acid treated embryos and control ($p<0.05$). Here again addition of folic acid and vitamin C restored normal development of all organ systems such that the embryos were comparable to the control.

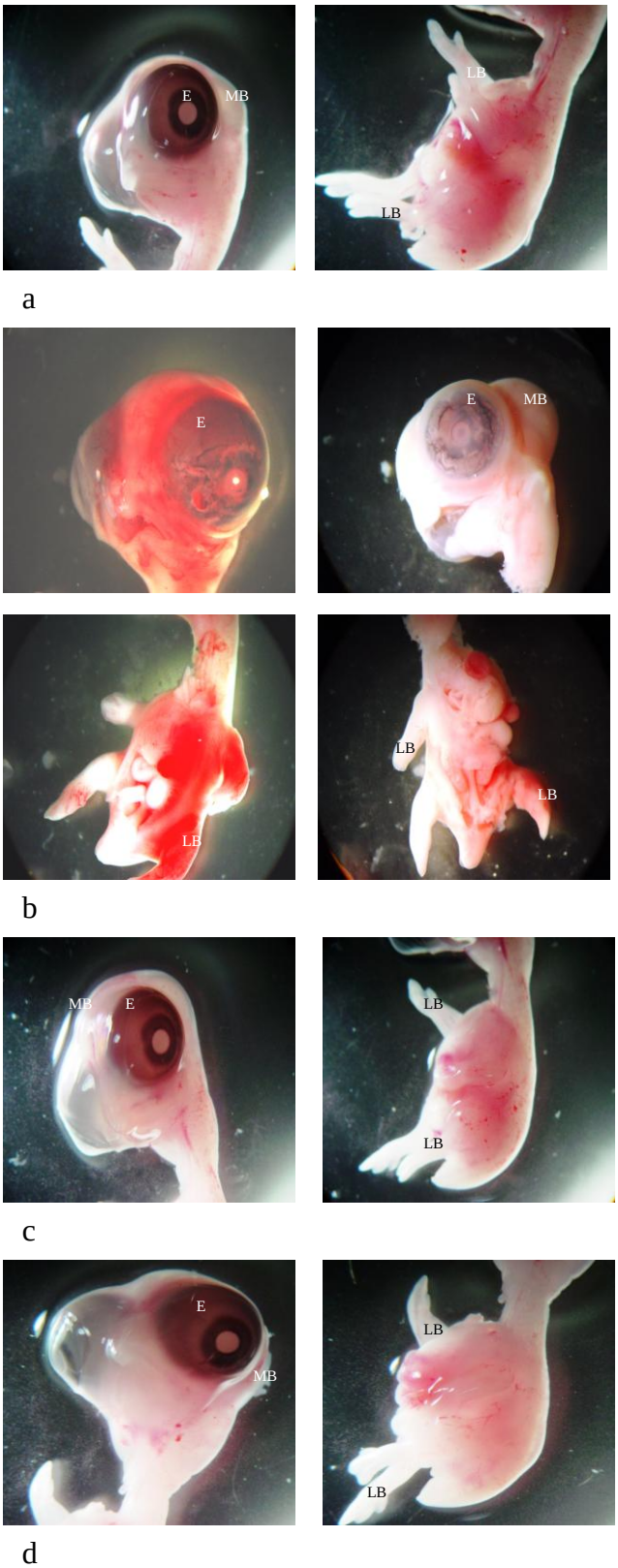


Figure 7.6: Shows control embryos (a), embryos treated with glucose and β-hydroxybutyric acid (b), glucose and β-hydroxybutyric acid plus vitamin C (c), glucose and β-hydroxybutyric acid plus folic acid (d). The figure clearly demonstrates the abnormal development of eyes (E), midbrain (MB), and limb buds (LB) with glucose plus β-hydroxybutyric acid treatment, however addition of folic acid and vitamin C rescued the embryos and they are comparable to control. Original magnification 200x.

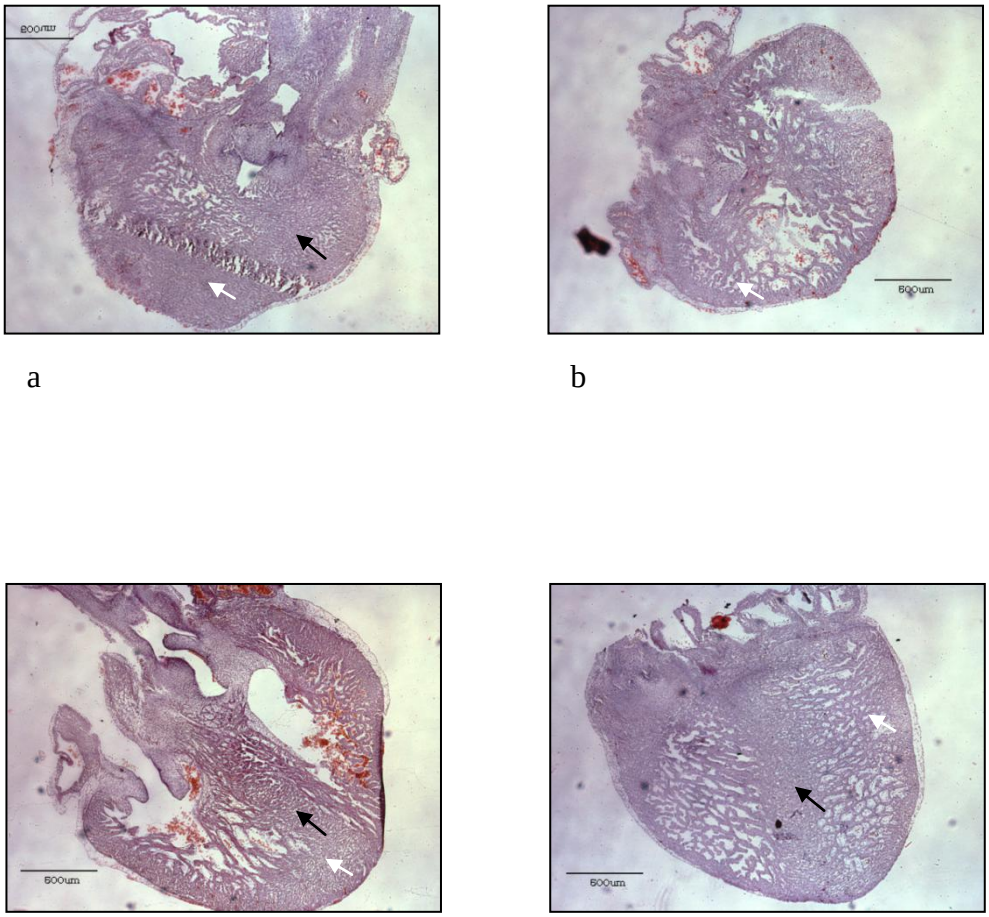


Figure 7.7: Shows H&E staining of control hearts (a), treated with glucose and β-hydroxybutyric acid (b), glucose and β-hydroxybutyric acid plus vitamin C (c) and folic acid (d). Black arrows show interventricular septum which is not developed normally in glucose plus β hydroxybutyric acid treated embryonic hearts as compare to other groups while white arrows indicate ventricular muscle which is also very thin in glucose plus β hydroxybutyric acid treated embryonic hearts as compare to other groups. Original magnification 200x.

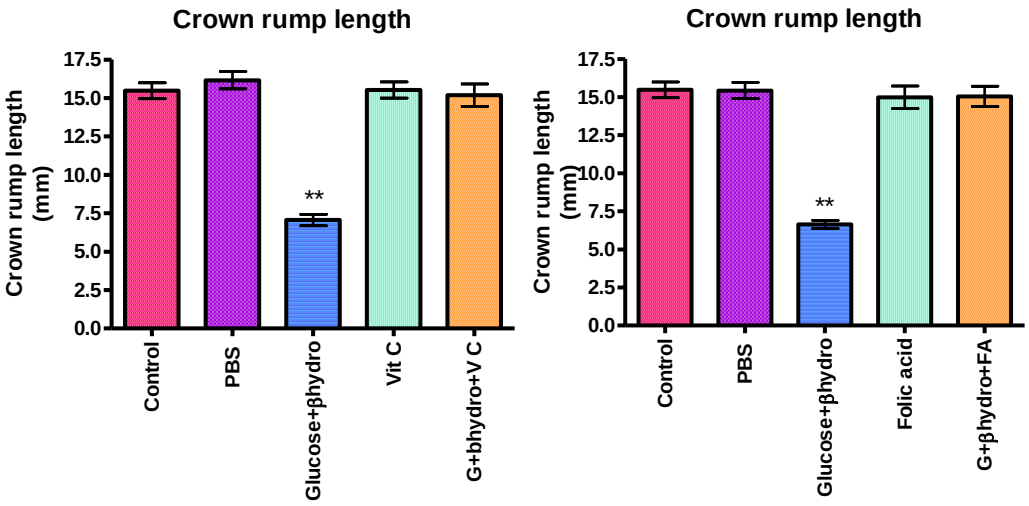


Figure 7.8: Shows crown rump length of embryos treated with glucose and β-hydroxybutyric acid, plus vitamin C and folic acid. {** ($p < 0.01$) for Dunnett's post hoc test}.

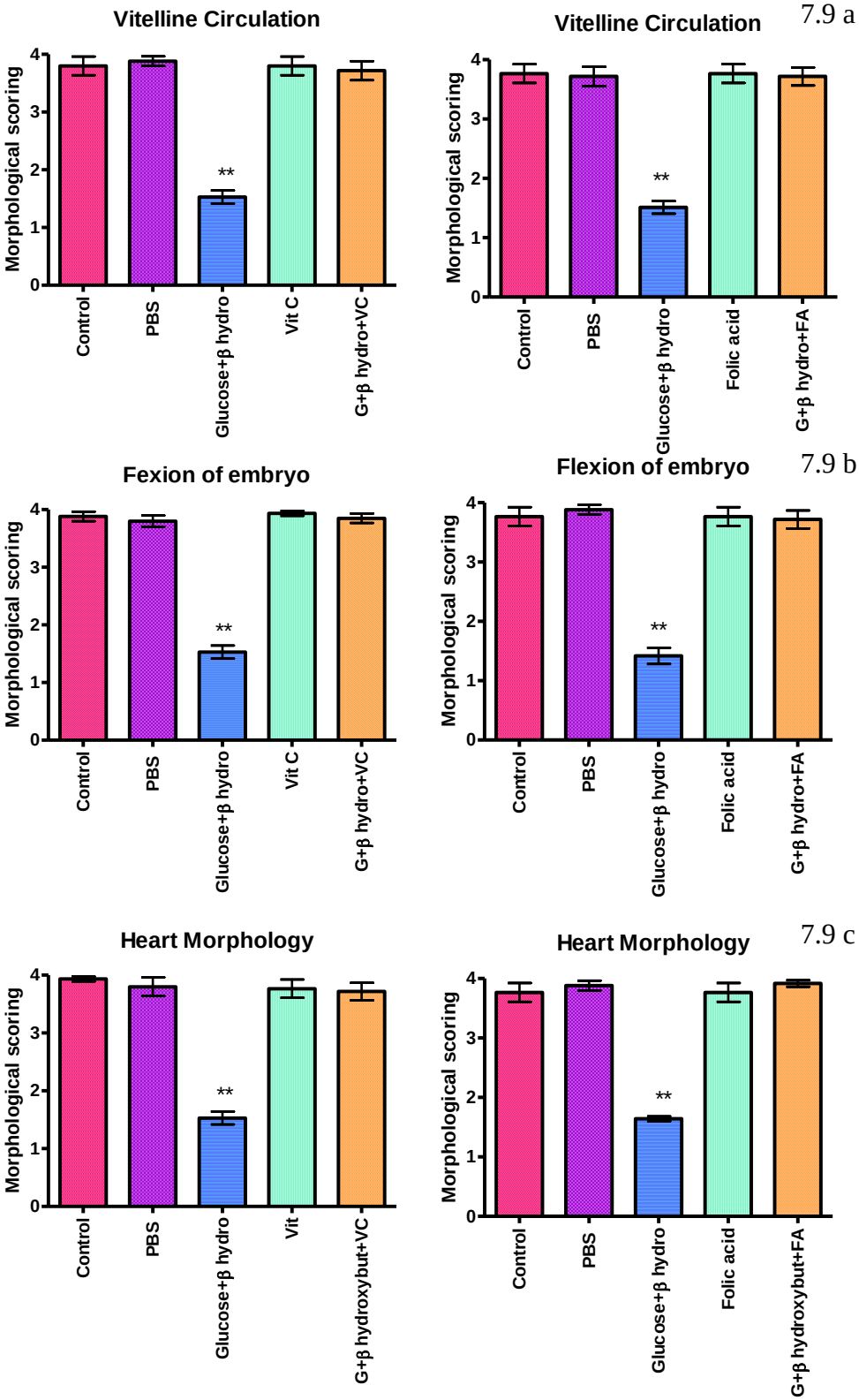
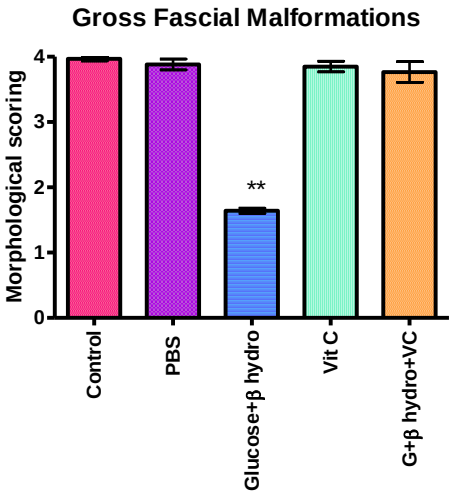
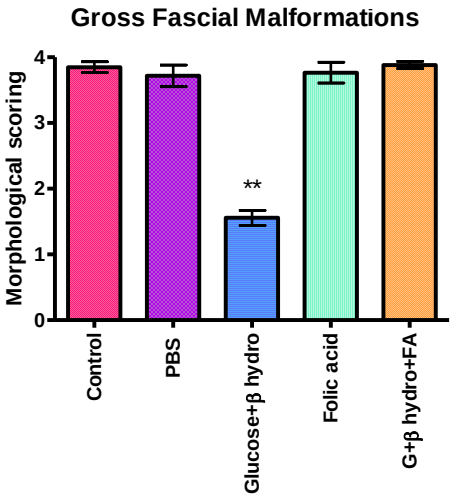


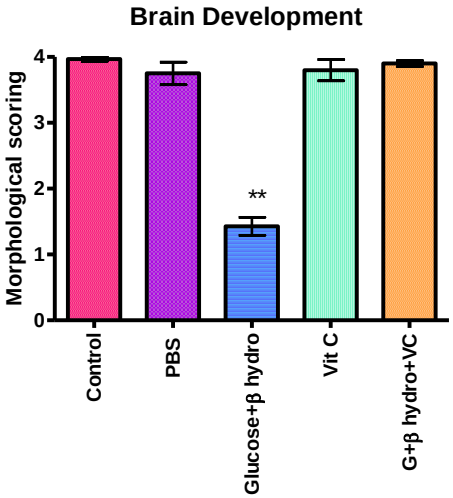
Figure 7.9: Shows vitelline circulation (a) flexion of embryo (b) heart morphology (c) treated with glucose and β-hydroxybutyric acid, plus vitamin C and folic acid. {** ($p < 0.01$) for Dunnett's post hoc test}.

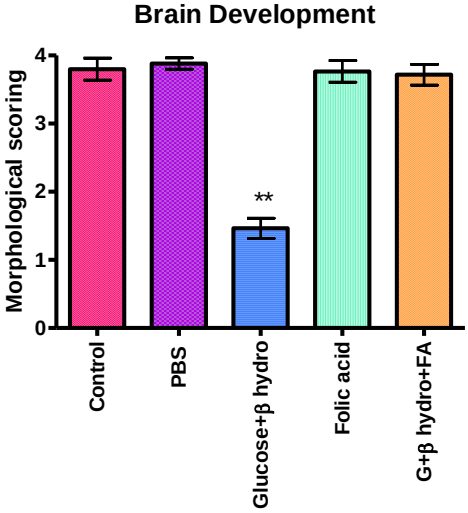
7.10 a



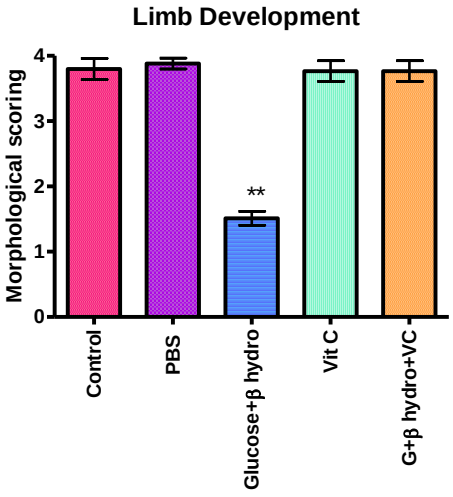


7.10 b





7.10 c



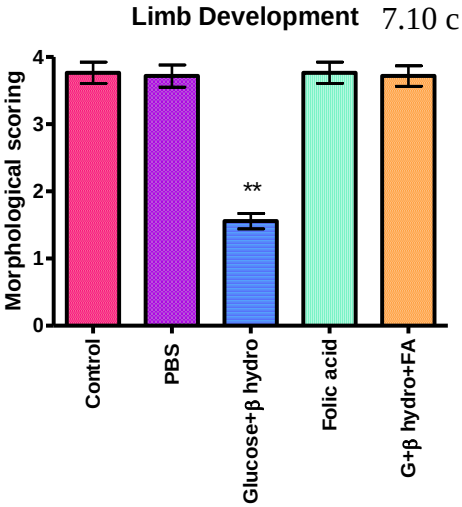


Figure 7.10: Shows gross facial malformations (a) brain development (b) limb development (c) treated with glucose and β-hydroxybutyric acid, plus vitamin C and folic acid. {** ($p < 0.01$) for Dunnett's post hoc test}.

7.4. Discussion

Diabetes mellitus is one of the frequent metabolic pregnancy complications associated with an increased risk of maternal and neonatal morbidities. In fact, uncontrolled diabetes during pregnancy is associated with serious medical conditions in the fetus. If it remains uncontrolled during the period of organogenesis, it leads to severe congenital malformations of major organ systems including the central nervous system and cardiovascular system. Late pregnancy complications of uncontrolled diabetes result in neonatal morbidity and mortality. Several epidemiological studies show that despite good glycemic control, the number of congenital malformations in diabetic mothers is still higher than in non-diabetics (Evers *et al.*, 2004; Macintosh *et al.*, 2006).

This study was carried out to investigate possible teratogenic effects of diabetic environment on development of the cardiovascular system of chick embryos. Also to evaluate potential preventive effects of two antioxidant vitamins; vitamin C and folic acid. Two different techniques (micromass culture and in ovo culture) were employed to detect the possible toxicity of diabetes. Results of micromass culture show that experimental diabetic conditions (high glucose and ketone body) result in a decreased contractile activity and viability of heart cells. Moreover results of in ovo culture also show that with experimental diabetic conditions survival of embryos was affected. Also embryos show gross abnormalities of the central nervous system and facial structures. The findings of this study indicate that glucose treatment even at higher concentrations (20mM) has no toxic effect

on heart cells; this could be due to the fact that cells rely on glucose for their nourishment and use it as an energy source. However, previous studies on animal models show that a high glucose concentration is a sole teratogenic factor for the development of congenital malformations (Datar & Bhonde, 2005; Gäreskog *et al.*, 2007). Some studies suggested that other metabolic factors may also be responsible for the higher malformation rates. The diabetic state is characterized by elevated levels of glucose as well as ketones, amino acids, lipids, glycerol and lactate, all of which might also contribute to the development of anomalies and early fetal loss (Shum & Sadler, 1988). In fact, some experimental studies have shown higher number of teratogenic effects when embryos (mouse, rats) were cultured in high concentrations of ketones (Moley *et al.*, 1994; Fujimoto *et al.*, 1997; Eriksson, 2009).

The mechanism of teratogenicity of high levels of glucose and ketone bodies in diabetes involves many factors. One of the key factors is oxidative stress, which is proved in many experimental studies. The effects of free radicals are normally controlled by administration of wide range of antioxidants (Viana *et al.*, 2000; Zhiyong, 2010). In this study chick heart cells and chick embryos were also treated with antioxidants vitamin C and folic acid. Our results show that addition of both of these vitamins to chick heart cells and chick embryos treated with diabetic conditions reversed the toxic effects of high glucose and β-hydroxybutyric acid. Our results are consistent with previous studies conducted on rat embryos (Cederberg & Eriksson, 2005; Wentzel & Eriksson, 2005). Supplementation by antioxidants in diabetic mothers during pregnancy may be helpful in

preventing diabetes induced malformations, and thus reduce overall mortality and morbidity in the offspring.

Chapter 8

Effect of various teratogens and vitamin supplementation on expression and distribution of Cx43

8.1. Introduction

8.1.1. Gap junctions and connexins

Gap junction channels form conduits between adjacent cells and thus they allow the transfer of small molecules of less than 1000Da (Söhl & Willecke, 2004). They also allow intercellular propagation of current carrying ions between excitable cells, including cardiac muscle, smooth muscle and nerves. Gap junction mediated communication between cells is vital for many cellular processes, such as cellular differentiation, embryonic development and growth (Musil & Goodenough, 1991).

Gap junctions are made up of a family of integral membrane proteins called connexins (Cx). The first gene of this family of proteins was cloned and sequenced from rat hepatocytes, in 1986 by Paul and his colleagues (Paul, 1986). Since then, at least 21 members in humans and 19 members in mouse have been cloned (Willecke *et al.*, 2002; Willecke *et al.*, 2005). It is well known that almost all cells in vertebrates contain gap junctions. Some connexins are expressed in specific tissues while some connexions, like connexion 43, are expressed in a variety of tissues (Solan & Lampe, 2005).

The most widely used nomenclature for connexins is according to their molecular weight in kilo Daltons preceded by the suffix Cx. There are some other ways of denoting connexins and these depend on connexin subfamilies e.g. α, and β etc (Dbouk *et al.*, 2009).

8.1.1.1. Structure of Connexins

The connexin polypeptide contains four transmembrane domains (M1, M2, M3 and M4), separated by a cytoplasmic loop between M2 and M3, and two extra cellular loops (E1 and E2) between M1 and M2, M3 and M4 respectively (Veen *et al.*, 2001). It also has a short cytoplasmic amino terminal (NT) and a carboxyl cytoplasmic terminal (CT) as shown in fig 8.1. The two extracellular loops are joined together by disulfide bonds and are involved in docking and functional assembly of gap junction channels. While most connexins exhibit similarities in their transmembrane domain, extracellular loop and amino terminal, they differ considerably in terms of the size of their cytoplasmic loop and the length of carboxyl terminal (Beyer *et al.*, 1995; Laird, 2005).

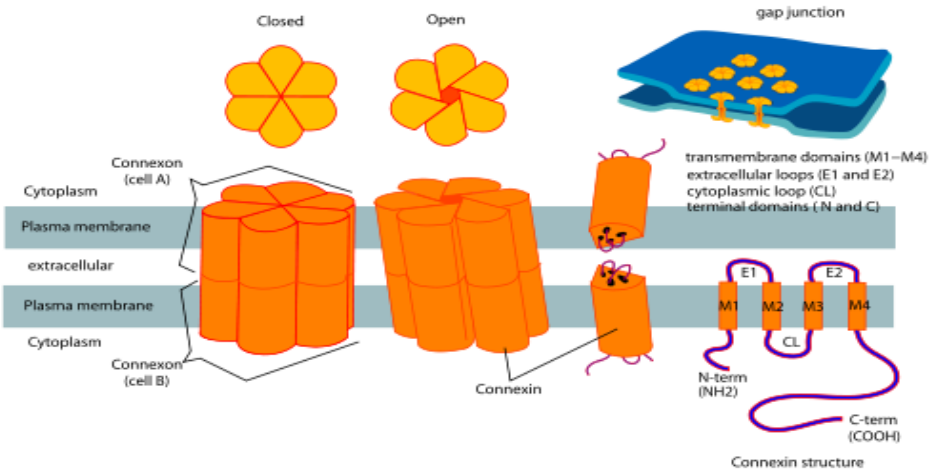


Fig 8.1. Structure and arrangement of connexins at gap junction plaques.

http://en.wikipedia.org/wiki/Connexin

8.1.1.2. Formation and degradation of Connexins and Gap junctions

Once translated by ribosomes, connexins are incorporated in endoplasmic reticulum where they are aligned and folded properly to be used in the formation of hemichannels (Huang *et al.*, 2009). All misfolded connexins are degraded by proteosomes. Once correctly folded, these proteins follow a classic secretory pathway, which involves transport through the Golgi apparatus. Most of the connexins, in particular Cx 43, oligomerize in to connexons, while in the *trans*-Golgi network rather than in the endoplasmic reticulum. Oligomerization of six connexin monomers in to half an intercellular channel (a connexon) is necessary for gap junction formation (Musil & Goodenough, 1991). However, some studies also suggest that oligomerization of some connexins e.g. Cx 32 and 26 takes place within the endoplasmic reticulum or in the Golgi apparatus. Newly formed connexons are transported in a closed state, via vesicles or tubules towards plasma membranes, where they appear as hemichannels. These hemichannels would pair with connexons from the neighbouring cell and form gap junction channels (Sosinksky, 1995). Some of the adhesion molecules particularly Ca^{2+} dependant cadherins play a crucial role in the formation of gap junction channels. Gap junction channels cluster to form gap junction plaques, which are the functioning units of gap junctions. Gap junction channels typically form between the same type of cells and are called homocellular gap junctions. But there is some evidence of gap junction channels between different cell types and these are termed heterocellular gap junctions (Sandow *et al.*, 2003). In vitro and in vivo studies also show evidence of homomeric (composed of same connexin)

and heteromeric (composed of more then one connexin) connexons within the same cells due to the presence of more than one connexin type in the same cell.

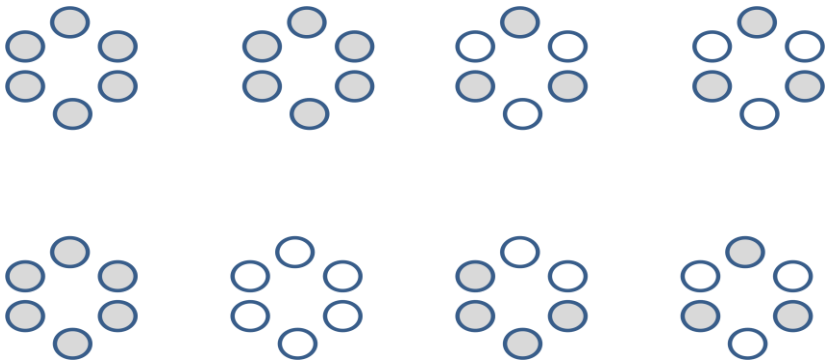


Fig. 8.2. Arrangement of homomeric and heteromeric connexons.

Intercellular communication via gap junctions is regulated by connexin synthesis and degradation. Connexins are very dynamic structures with a very short half life. These proteins follow two major pathways for their degradation; the lysosomal pathway and the proteosomal pathway. Most of the studies on degradation pathways utilized connexin 43 due to the fact that it is the most abundant. Degradation by the proteosomal pathway is used for those proteins which are either misfolded or not properly oligomerized and unable to take part in the formation of gap junctions. Degradation by the lysosomal pathway usually involves connexons from the centre of gap junction plaques, which detach and are internalized to form membrane bounded structures called annular gap junctions. These annular gap junctions are also a substrate for endosomes and are degraded by lysosomes.

8.1.1.3. Phosphorylation of connexin43

All connexins studied until now are phosphoproteins except Cx26. Phosphorylation is involved in regulation of intercellular communication via a number of mechanisms; including connexin synthesis, transport, assembly, channel gating, internalization and degradation (Lamp & Lau, 2004). Cx43 is the most extensively studied protein amongst the connexin family, in terms of phosphorylation. Initial work done by Musil and Goodenough, has shown that Cx 43 is differentially phosphorylated throughout its life cycle in homeostatic cells (Musil & Goodenough, 1991). When analysed by polyacrylamide gel electrophoresis (SDS-PAGE), Cx43 shows different electrophoretic forms, including a non phosphorylated or faster migrating form (NP or P0) with MW ~42 kd, and two slower migrating forms (P1 and P2) with MW ranged from 44 to 46 kd respectively (Pahuja *et al.,* 2007). The majority of phosphorylation events occur on serine residues as evidenced by phosphor amino acid analysis (Solan *et al.*, 2003). The primary site for Cx43 to be phosphorylated is the C-terminal.

8.1.1.4. Role of connexin 43 during embryonic heart development

The connexins also play an important role during development of the heart (Willecke *et al.*, 2002; Houghton, 2005). Out of the 20 connexins identified thus far in vertebrates, four (Cx37, Cx40, Cx43, and Cx45) have been detected in the developing and mature heart (Gros & Jongsma, 1996). Connexin 43 is expressed throughout the development of the heart (Alcolea *et al.*, 1999), and is essential for normal development. Cx43 knock down

mice die at birth due to conotruncal malformations and pulmonary outflow obstruction (Reaume *et al.*, 1995; Ya *et al.*, 1998).

Since connexins play an important role during embryogenesis, this could raise the possibility that teratogens affect embryonic development and growth through disruption of connexins. Therefore effects of different teratogens on connexin expression were examined in order to detect possible mechanism of action of these teratogens.

8.2. Materials and methods

8.2.1. Immunohistochemistry

Chick cardiac cells were cultured in 8 well chamber slides at a concentration of 3×10^6 in each well in the form of 20µl droplets. They were incubated for 2 hours to allow cellular attachment. The supplemented culture medium (DMEM) was added to the cultures and cultures were left for 24 hours (Chapter 2, section 2.1.4.5.2). The different concentrations of test chemicals were added after 24 hours in culture. The cultures were fixed in paraformaldehyde solution (2% w/v in PBS) for 1 hour at room temperature on day 6 of incubation. The cells were washed three times with PBS each for 5 minutes, before being permeabilised with 0.2% Triton X-100 in PBS (v/v) for 10 minutes. The cells were incubated in 5% goat serum in PBS for 1 hour to block nonspecific bindings of secondary antibody. The cells were incubated with 200 µl of the primary antibody (connexin 43 Cx43) (Table2.2) overnight at 4^oC. The secondary antibody (Table 2.3) diluted in 0.1% BSA in PBS was applied and incubated for 1 hour in the dark. Cells were washed in PBS (3 x 5 minutes) to remove

excess unbound secondary antibody before mounted with Vectorshield™ containing DAPI (Chapter 2, section 2.3.5.1). Sections were viewed under a Leica confocal microscope.

8.2.2. Western blotting

The samples were cultured as described in (chapter 2 section 2.1.4.5.2). Cell lysates were prepared as described in chapter 2 section 2.4.3.1. The proteins were denatured by heating the samples to 95^{o}C for 5 minutes in a heating block. Then 20µl of sample (20mg protein) was loaded in to the precast gels, along with 1µl of marker. The electrophoresis was performed by running the gel in 1X electrode buffer for approximately 45 minutes at 200V. The gel was then transferred to nitrocellulose membrane. The transfer was done in cold transfer buffer for 60 minutes at 100V. Once transfer was complete nitrocellulose was blocked in 1.5% fish skin gelatine in TBST (v/v) using Snap i.d. The primary antibody (connexin 43 and GAPDH) was added for 10 minutes, which was followed by three quick washes in TBST. The secondary antibodies were applied for ten minutes in the dark. The blot was analysed using the Odyssey scanner after quick washes with TBST and distilled water (chapter 2 section 2.4.5).

8.2.3. Controls

8.2.3.1. Positive controls

Day 9 in ovo chick hearts were used as positive control, as the expression of Cx43 is increased with advancing embryonic age (Kirchhoff *et al.*, 2000). TPA treatment was used as a negative control. TPA is a protein

kinase activator and through PKC it causes an increase in connexin phosphorylation and thus decreases gap junction intercellular communication. A stock solution of 50µM of TPA was formed by diluting it into DMSO. The stock solution was then further diluted to final test concentration (50nM) in complete culture medium. The final concentration of DMSO as a solvent was less than 0.01%. A positive control, cyclic AMP treatment, was used in this study as well. The cyclic AMP acts through Protein kinase A and increases the number of gap junctions and Connexins.

8.2.3.2. Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)

Glyceraldehyde 3-phosphate dehydrogenase is one of the key enzymes involved in glycolysis. It serves to break down glucose for energy and carbon molecules by catalyzing the conversion of glyceraldehyde 3-phosphate. In addition to its metabolic function, GAPDH has also been implicated in several non-metabolic processes, such as transcription, apoptosis, and ER to Golgi vesicle shuttling. The GAPDH is stably and constitutively expressed at high levels in almost all tissues and cells of the body, and because of this reason this gene is used as a house keeping gene. The house keeping genes are commonly used as a loading control for protein normalization and as a loading control in western blotting and many other molecular assays such as RT-PCR. The GAPDH is detected as a band of approximately 36-40 kDa on western blots.

8.3. Results

8.3.1. Day 9 controls, Day 5 controls, TPA, vitamin C, folic acid and cyclic AMP

8.3.1.1. Immunohistochemistry

The day 9 chick hearts stained with anti-Cx43 show a bright green staining as shown in fig 8.3 and figure 8.4 shows negative control stained with secondary antibody in absence of Cx43 antibody. Figure 8.5 shows day 5 chick cardiomyocyte controls. The day 5 chick heart micromass cultures treated with TPA show a decreased expression of Cx43 (fig 8.6), while cultures treated with cyclic AMP show brighter staining with more bright spots on the surface of the cells (fig 8.7). The results also illustrate that in the cells treated with folic acid (1mM) and vitamin C (100µM), brighter staining is observed on the surface of the cells (fig 8.8 and 8.9 respectively).

8.3.1.2. Western blotting

The protein analyses with western blotting shows that the ratio of Cx43 to GAPDH in day 9 chick embryonic hearts and day 5 chick cardiomyocytes is comparable. Also chick micromass cultures treated with vitamin C and folic acid showed same ratio as day 5 controls. However cyclic AMP caused an increase in Cx43 to GAPDH ratio. TPA treatment results show a decrease in Cx43 to GAPDH ratio as compare to day 9 and day 5 controls (fig 8.10 a & b). The expression of GAPDH remained constant in all culture groups (fig 8.10 c).

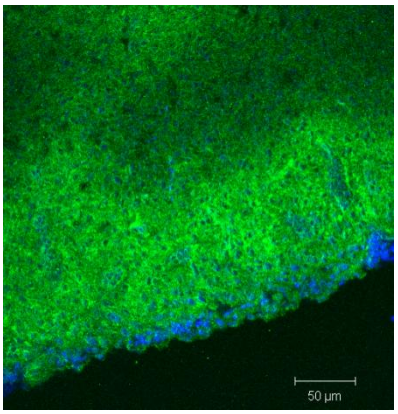


Fig 8.3: Day 9 heart expressing Cx43 (200x magnification)

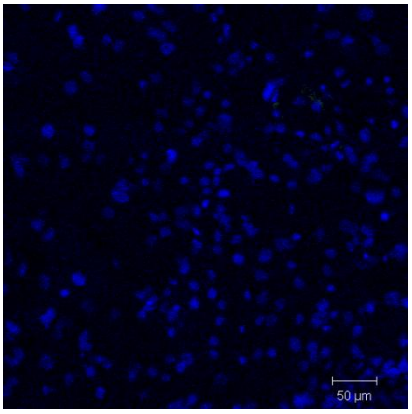


Fig 8.4: Day 9 heart, –ve control Alexa 488 (200x magnification)

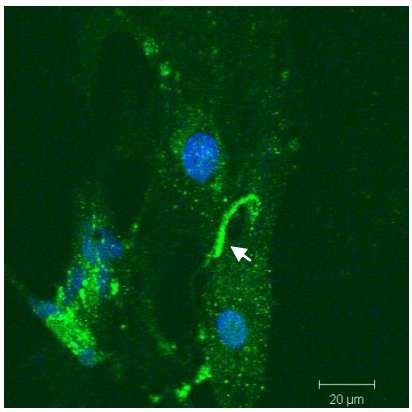


Fig 8.5: Day 5 control cardiomyocytes expressing Cx43 (600x magnification oil immersion)

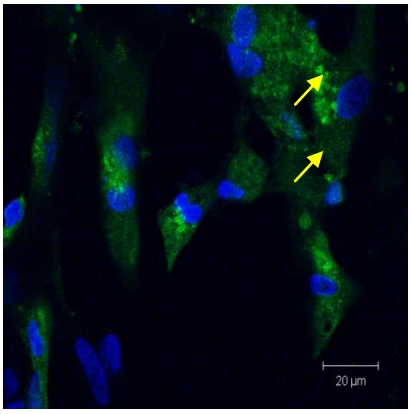


Fig 8.6: Day 5 cardiomyocytes treated with TPA expressing Cx43 (600x magnification oil immersion)

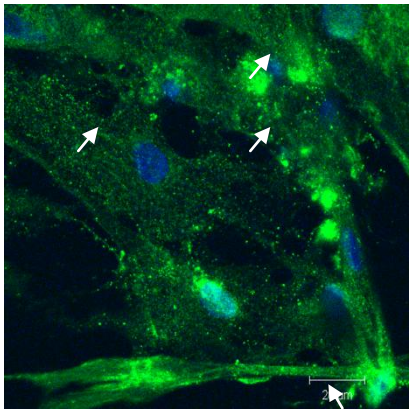


Fig 8.7: Day 5 cardiomyocytes treated with cAMP expressing Cx43 (600x magnification oil immersion)

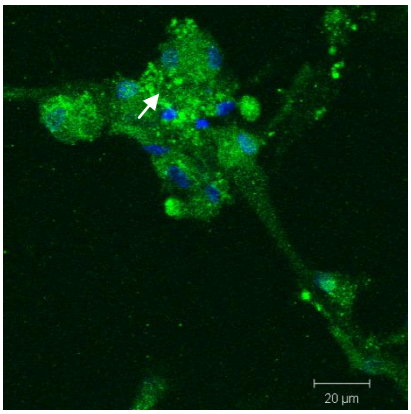


Fig 8.8: Day 5 cardiomyocytes treated with VC expressing Cx43 (600x magnification oil immersion)

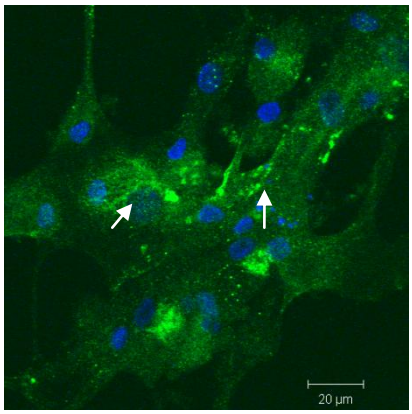


Fig 8.9: Day 5 cardiomyocytes treated with FA expressing Cx43 (600x magnification oil immersion)

{TPA (12-*O*-tetradecanoylphorbol 13-acetate); VC (vitamin C); FA (folic acid); cAMP (cyclic AMP)}. **White arrows** indicate expression of Cx43 on cell surface; Y**ellow arrows** indicate Cx43 expression inside the cells

Fig. 8.10 a

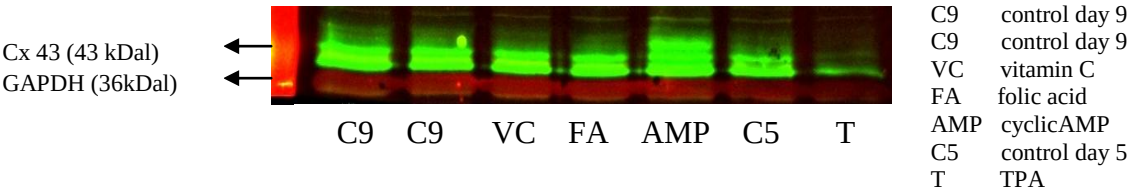


Fig. 8.10 b

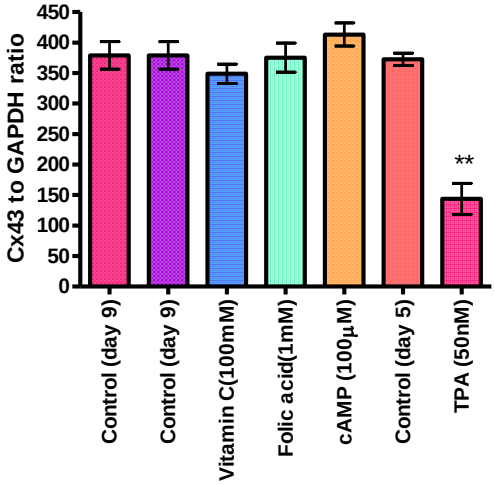


Fig. 8.10 c

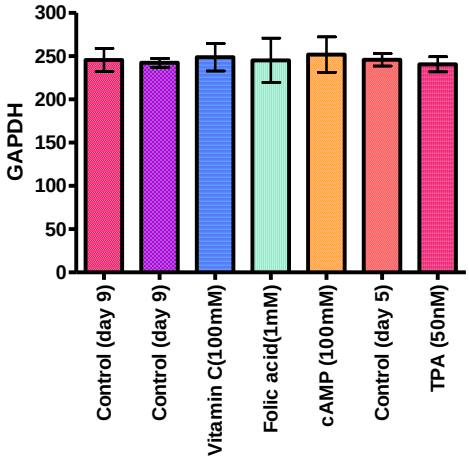


Fig. 8.10: Shows protein analysis by Western blot (a), graphical representation of ratio of Cx43 to GAPDH (b) and GAPDH (housekeeping gene) only (c). {** ($p<0.01$) for Dunnett's post hoc test}.

8.3.2. Ethanol plus folic acid and vitamin C

8.3.2.1. Immunohistochemistry

In cultures treated with ethanol, the overall expression of Cx43 is less as compared to controls (fig 8.11 b). The addition of folic acid and vitamin C in addition to ethanol significantly increased the expression of Cx 43 as shown in fig 8.11 c and 8.11d and are comparable to controls (fig 8.11 a).

8.3.2.2. Western blotting

Ethanol at a concentration of 100µl/ml significantly reduced the ratio of Cx 43 to GAPDH (<0.05) at 96 hrs post exposure in micromass cultures (fig 8.12 a & b) and day 9 chick hearts (fig 8.13 a & b). While the cell cultures as well as day 9 hearts treated with vitamin C (100µM) and folic acid (1mM) the ratio of connexin 43 to GAPDH is not significantly different to that of controls.

Addition of vitamin C and folic acid to ethanol treated cells and day 9 hearts improved the ratio of Cx43 to GAPDH and were also comparable to controls (fig 8.12 a & b) and (8.13 a & b) respectively.

Treatment of cells with TPA (50nM) showed significant difference in the expression of Cx 43 (<0.05) to that of control cultures. While expression of Cx 43 to GAPDH in the cells which were treated with 100µM of cyclic AMP was comparable to controls and didn't show any decrease (fig 8.12 a & b).

The expression of GAPDH (house keeping gene) remained constant in all culture groups (fig 8.12 c and 8.13 c).

8.3.2.3. Conclusion

Immunohistochemistry and western blotting results showed that the expression of Cx 43 was significantly decreased by ethanol administration. However addition of multivitamins folic acid and vitamin C reversed the effects of ethanol, and the expression of Cx 43 in cultures treated with folic acid and vitamin C in addition to ethanol was comparable to control.

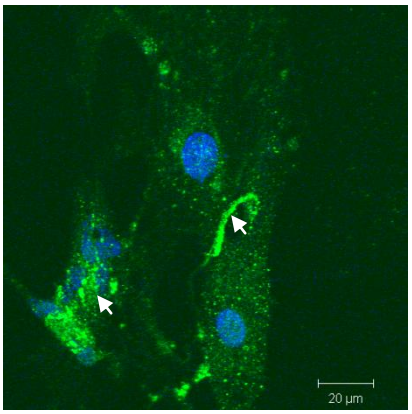


Fig 8.11 a: Day 5 control, showing expression of Cx43 (600x magnification oil immersion)

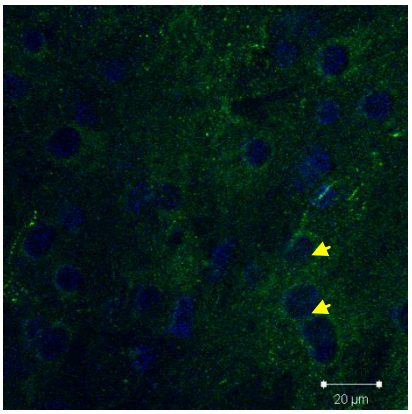


Fig 8.11 b: Day 5 ethanol treated cells, showing expression of Cx43 (600x magnification oil immersion)

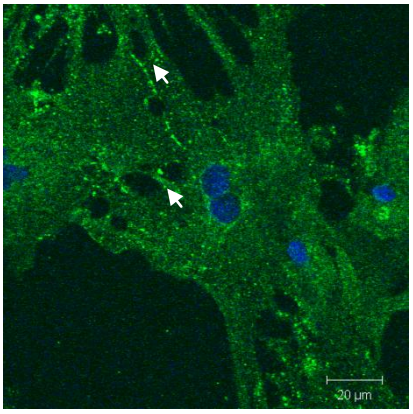


Fig 8.11 c: Day 5 ethanol plus vitamin C treated cells, showing expression of Cx43 (600x magnification oil immersion)

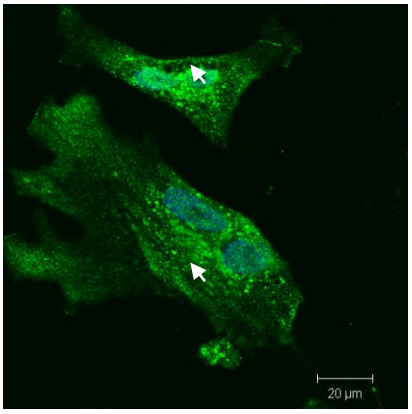
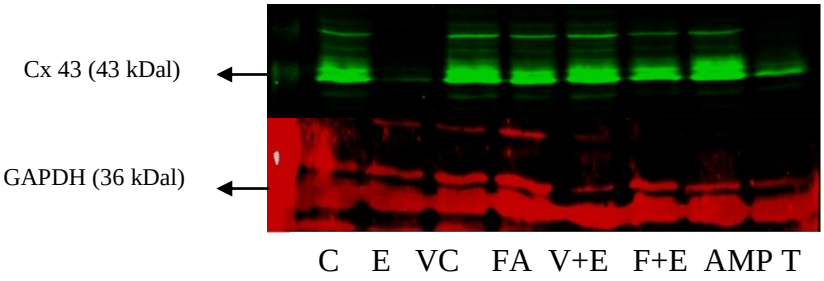


Fig 8.11 d: Day 5 ethanol plus folic acid treated cells, showing expression of Cx43 (600x magnification oil immersion)

Fig 8.11: Shows expression of connexin 43 (Cx43) in day 5 controls (a), ethanol treated (b), ethanol and vitamin C treated, (d) ethanol and folic acid treated.
White arrows indicate expression of Cx43 on cell surface; Y**ellow arrows** indicate Cx43 expression inside the cells

Fig 8.12 a



C control
E ethanol
VC vitamin C
FA folic acid
V+E VC+E
F+E FA+E
T TPA
AMP cyclic AMP

Fig 8.12 b

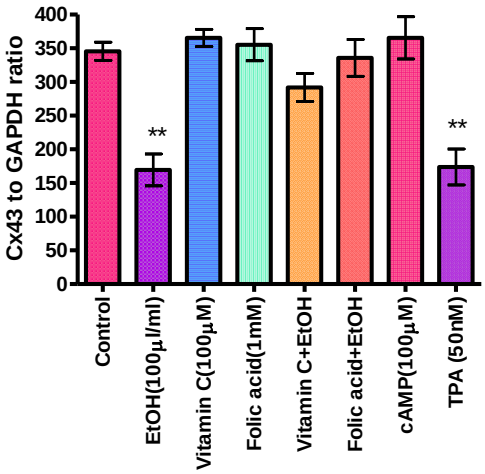


Fig 8.12 c

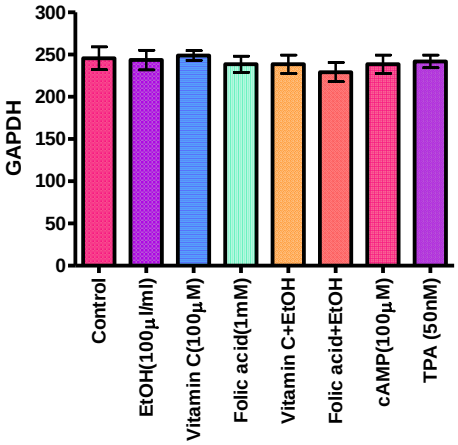


Fig. 8.12: Shows protein analysis of day 5 chick cardiomyocytes cultures by Western blot (a), graphical representation of ratio of Cx43 to GAPDH (b) and GAPDH (housekeeping gene) only (c). {** ($p<0.01$) for Dunnett's post hoc test}.

Fig 8.13 a

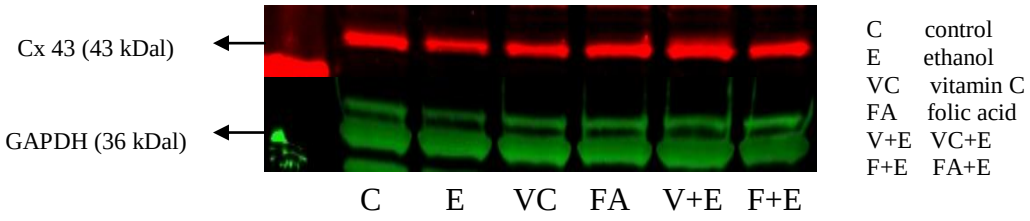


Fig 8.13 b

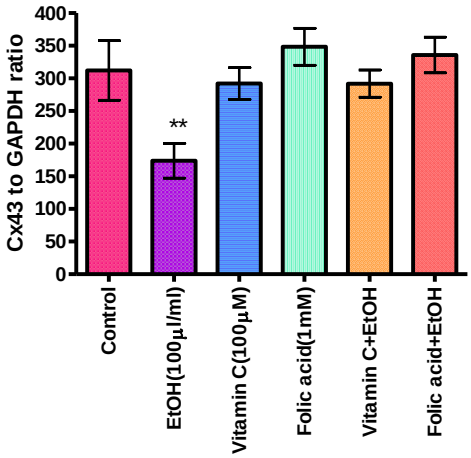


Fig 8.13 c

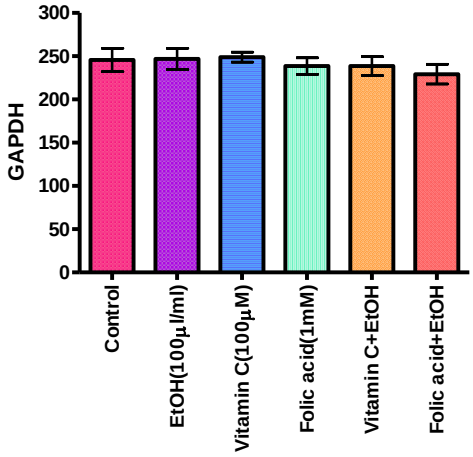


Fig. 8.13: Shows protein analysis in day 9 chick embryonic hearts by Western blot (a), graphical representation of ratio of Cx43 to GAPDH (b) and GAPDH (housekeeping gene) only (c). {** ($p<0.01$) for Dunnett's post hoc test}.

8.3.3. Nicotine plus folic acid and vitamin C

8.3.3.1. Immunohistochemistry

The expression of Cx 43 is less on the surface as well as inside the cytoplasm of cells treated with nicotine (fig 8.14 b), as compare to controls (fig 8.14 a). The addition of folic acid and vitamin C in addition to nicotine increased the expression of Cx 43 as shown in fig 8.14 c & d respectively and are comparable to controls.

8.3.3.2. Western blotting

The Cx 43 to GAPDH ratio is significant reduced with nicotine (10µM) treatment at 96 hrs post exposure in micromass cultures ($p < 0.05$) (fig 8.15 a & b). However, in cultures treated with folic acid (1mM) and vitamin C (100µM), the ratio of Cx 43 to GAPDH is not significantly different to that of controls.

Moreover the cells treated with folic acid and vitamin C, in addition to nicotine expression of Cx43 was comparable to controls (fig 8.15 a & b).

The cells treated with TPA showed significant difference in the ratio of Cx 43 to GAPDH (<0.05) to that of control cultures. While an increased ratio of Cx 43 to GAPDH was observed in the cells which were treated with 100µM of cyclic AMP and are comparable to controls. The GAPDH remained constant in all culture groups (fig 8.15 c).

8.3.3.3. Conclusion

The ratio of Cx 43 to GAPDH which was significantly decreased by nicotine, was reversed with addition of folic acid and vitamin C and cultures are comparable to controls.

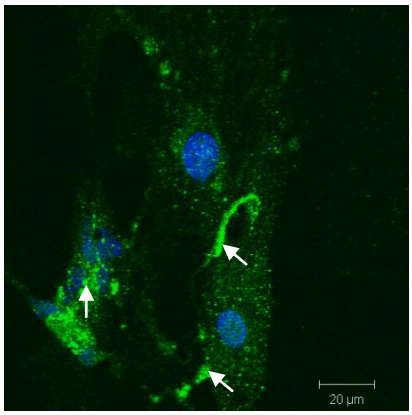


Fig 8.14 a: Day 5 control, showing expression of Cx43 (600x magnification oil immersion)

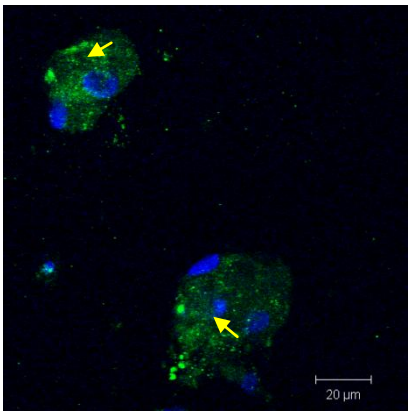


Fig 8.14 b: Day 5 nicotine treated cells, showing expression of Cx43 (600x magnification oil immersion)

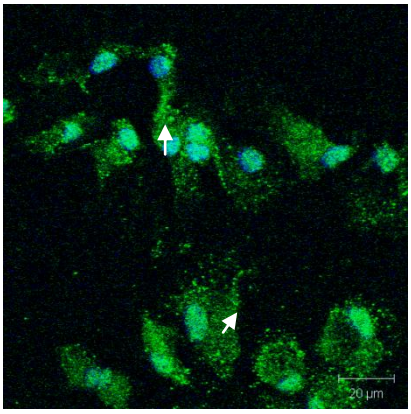


Fig 8.14 c: Day 5 nicotine plus vitamin C treated cells, showing expression of Cx43 (600x magnification oil immersion)

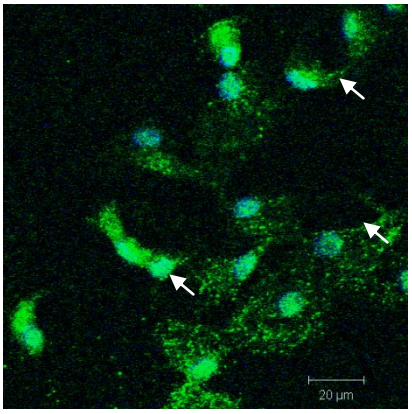
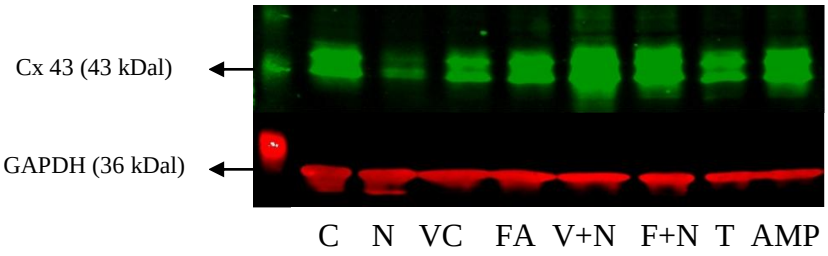


Fig 8.14 d: Day 5 nicotine plus folic acid treated cells, showing expression of Cx43 (600x magnification oil immersion)

Fig 8.14: Shows expression of connexin 43 (Cx43) in day 5 controls (a), nicotine treated (b), nicotine and vitamin C treated, (d) nicotine and folic acid treated.
White arrows indicate expression of Cx43 on cell surface; Y**ellow arrows** indicate Cx43 expression inside the cells

Fig 8.15 a



C control
N nicotine
VC vitamin C
FA folic acid
V+N VC+N
F+N FA+N
T TPA
AMP cyclic AMP

Fig 8.15 b

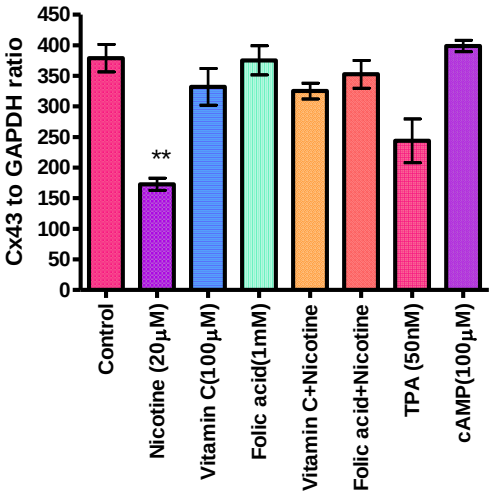


Fig 8.15 c

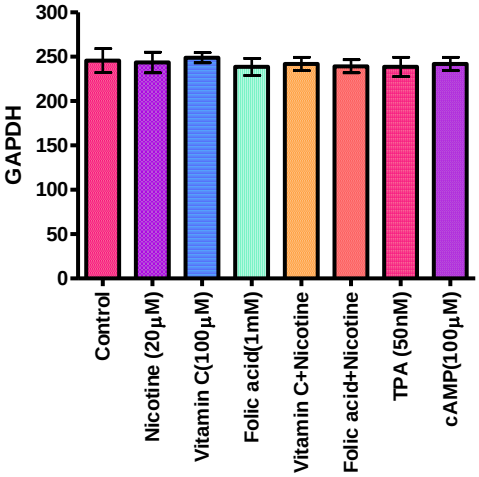


Fig. 8.15: Shows protein analysis in day 5 chick cardiomyocytes micromass cultures by Western blot (a), graphical representation of ratio of Cx43 to GAPDH (b) and GAPDH (housekeeping gene) only (c). {** ($p<0.01$) for Dunnett's post hoc test}.

8.3.4. Retinoic acid plus folic acid and vitamin C

8.3.4.1. Immunohistochemistry

In cultures treated with retinoic acid (20µM), the expression of Cx 43 is less on the surface as well as inside the cytoplasm of cells (fig 8.16 b) as compare to controls (fig 8.16 a). The addition of folic acid and vitamin C in addition to retinoic acid significantly increased the expression of connexin 43 as shown in fig 8.16 c & d respectively and are comparable to controls.

8.3.4.2. Western blotting

Retinoic acid at a concentration of 20µM significantly reduced the ratio of Cx 43 to GAPDH (<0.05) at 96 hrs post exposure in micromass cultures (fig 8.17 a & b). While in cultures treated with vitamin C (100µM) and folic acid (1mM) the ratio of Cx 43 to GAPDH is not significantly different to that of controls.

The cells treated with vitamin C and folic acid, in addition to retinoic acid were also comparable to controls (fig 8.17 a & b).

Treatment of cells with TPA (50nM) showed significant difference in the expression of Cx 43 (<0.05) to that of control cultures. While an increased expression of Cx 43 was observed in the cells which were treated with 100µM of cyclic AMP (fig 8.17 a & b).

The expression of GAPDH (house keeping gene) remained constant in all culture groups (fig 8.17 c).

8.3.4.3. Conclusion

Immunohistochemistry and western blotting results showed that the expression of connexin 43 was significantly decreased by retinoic acid. However addition of multivitamins folic acid and vitamin C reversed the effects of retinoic acid, and the expression of Cx43 in cultures treated with folic acid and vitamin C in addition to retinoic acid was comparable to control.

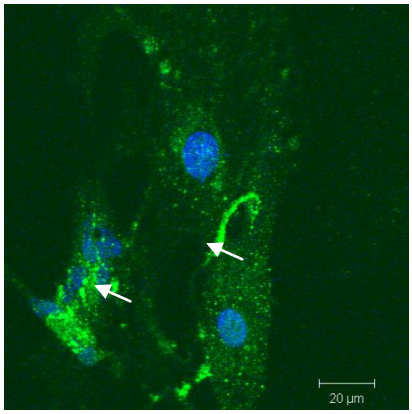


Fig 8.16 a: Day 5 control, showing expression of Cx43 (600x magnification oil immersion)

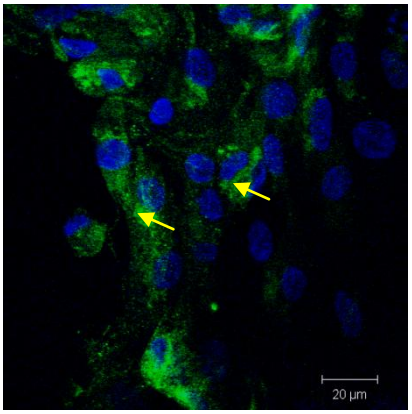


Fig 8.16 b: Day 5 retinoic acid treated cells, showing expression of Cx43 (600x magnification oil immersion)

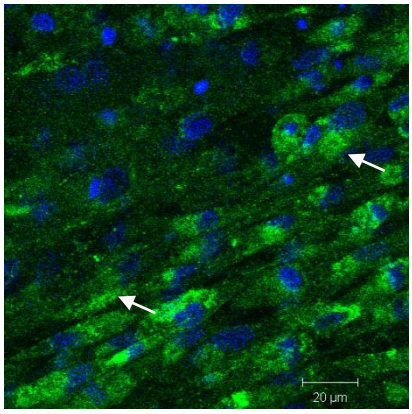


Fig 8.16 c: Day 5 retinoic acid plus vitamin C treated cells, showing expression of Cx43 (600x magnification oil immersion)

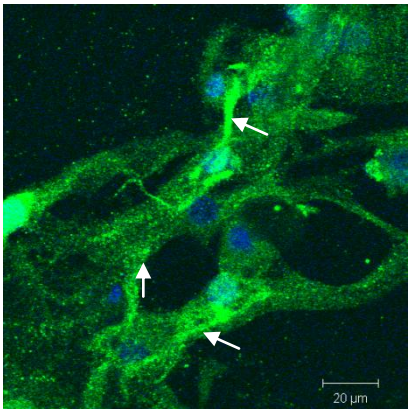
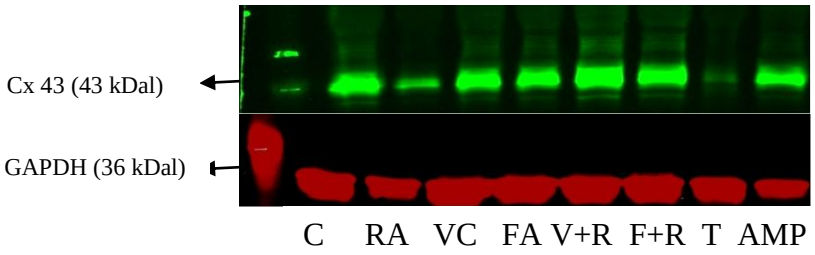


Fig 8.16 d: Day 5 retinoic acid plus folic acid treated cells, showing expression of Cx43 (600x magnification oil immersion)

Fig 8.16: Shows expression of connexin 43 (Cx43) in day 5 controls (a), retinoic acid treated (b), retinoic acid and vitamin C treated, (d) retinoic acid and folic acid treated.
White arrows indicate expression of Cx43 on cell surface; Y**ellow arrows** indicate Cx43 expression inside the cells

Fig 8.17 a



C control
RA retinoic acid
VC vitamin C
FA folic acid
V+R VC+RA
F+R FA+RA
T TPA
AMP cyclic AMP

Fig 8.17 b

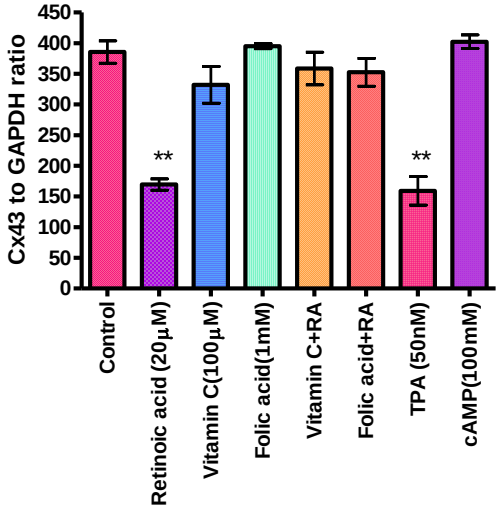


Fig 8.17 c

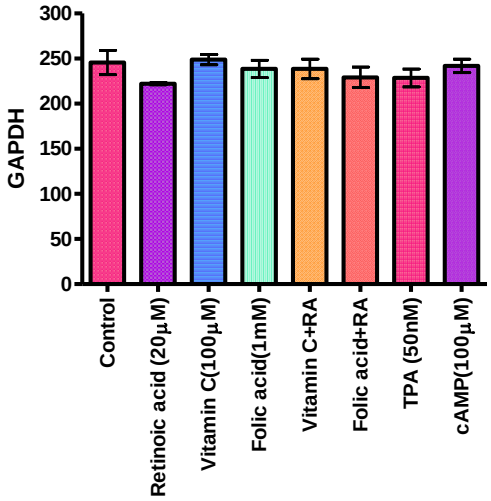


Fig. 8.17: Shows protein analysis in day 5 chick cardiomyocytes micromass cultures by Western blot (a), graphical representation of ratio of Cx43 to GAPDH (b) and GAPDH (housekeeping gene) only (c). {** ($p<0.01$) for Dunnett's post hoc test}.

8.3.5. Diabetic conditions plus folic acid and vitamin C

8.3.5.1. Immunohistochemistry

In cultures treated with diabetic conditions (20mM glucose +15mM β-hydroxybutyric acid), the expression of Cx 43 is less on the surface as well as inside the cytoplasm of cells (fig 8.18 b) as compared to controls (fig 8.18 a). The addition of folic acid and vitamin C in addition to diabetic conditions significantly increased the expression of Cx 43 as shown in fig 8.18 c and 8.18 d respectively and are comparable to controls.

8.3.5.2. Western blotting

There was a synergistic action of 20mM glucose and 15mM β hydroxybutyric acid when added to chick heart micromass cultures which results in a significantly reduced Cx43 to GAPDH ratio in comparison to controls (fig 8.19 a & b). The effect of diabetic condition on Cx 43 to GAPDH ratio is reversed with addition of folic acid and vitamin C (fig 8.19 a & b). These two vitamins themselves produce no significant changes in Cx43 to GAPDH ratio to controls (fig 8.19 a & b). The cells treated with TPA (50nM), showed significant difference in the ratio of connexin 43 to GAPDH (<0.05) compared to that of control cultures. An increased expression of Cx 43 to GAPDH was observed in the cells which were treated with 100μM of cyclic AMP (fig 8.19 a & b). The GAPDH remained constant in all culture groups (fig 8.19 c).

8.3.5.3. Conclusion

The ratio of Cx 43 to GAPDH was significantly reduced by the diabetic condition. However addition of vitamins folic acid and vitamin C reversed the effects of diabetic condition, and the expression of Cx 43 in cultures treated with folic acid and vitamin C in addition to diabetes was comparable to control.

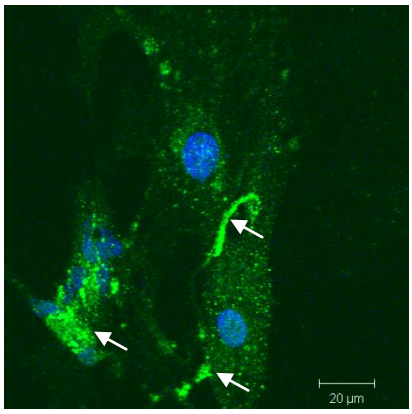


Fig 8.18 a: Day 5 control, showing expression of Cx43 (600x magnification oil immersion)

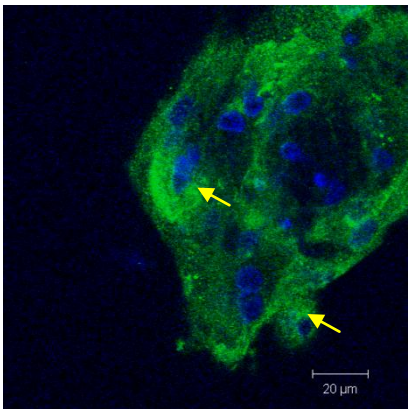


Fig 8.18 b: Day 5 DM treated cells, showing expression of Cx43 (600x magnification oil immersion)

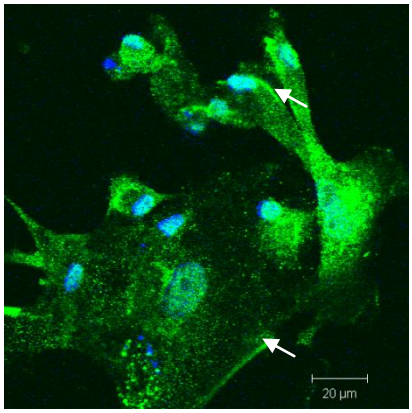


Fig 8.18 c: Day 5 DM plus vitamin C treated cells, showing expression of Cx43 (600x magnification oil immersion)

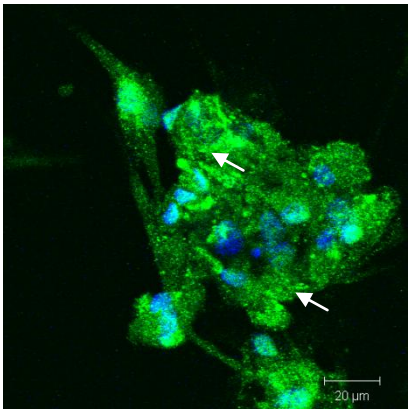


Fig 8.18 d: Day 5 DM plus folic acid treated cells, showing expression of Cx43 (600x magnification oil immersion)

Fig 8.18: Shows expression of connexin 43 (Cx43) in day 5 controls (a), DM treated (b), DM and vitamin C treated, (d) DM and folic acid treated. DM (diabetic conditions); **white arrows** indicate expression of Cx43 on cell surface, **yellow arrows** indicate Cx43 expression inside the cells

Fig 8.19 a

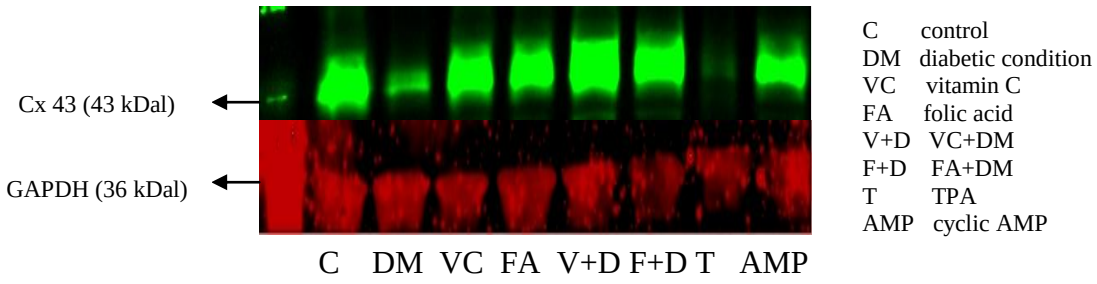


Fig 8.19 b

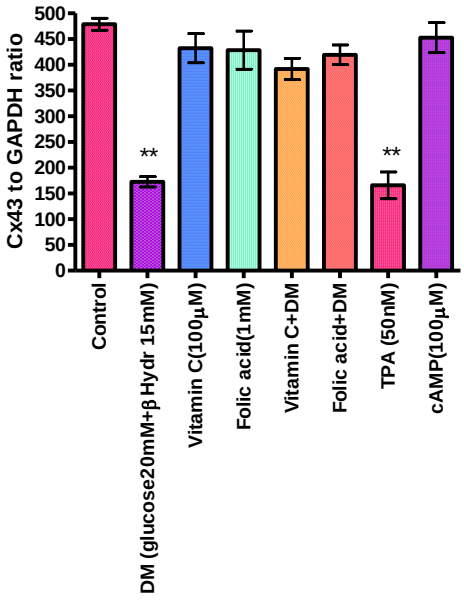


Fig 8.19 c

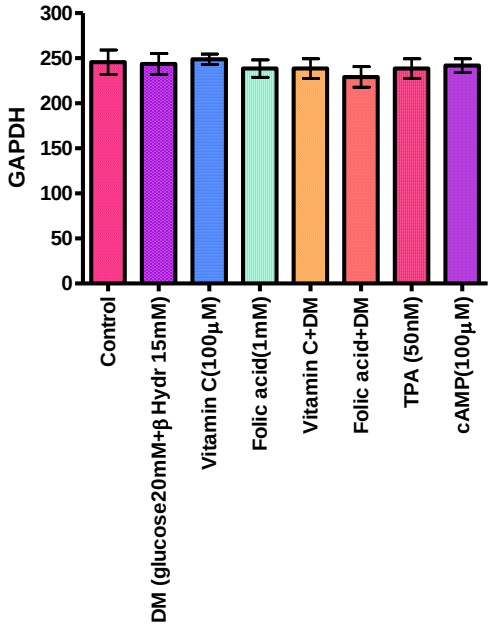


Fig. 8.19: Shows protein analysis in day 5 chick cardiomyocytes micromass cultures by Western blot (a), graphical representation of ratio of Cx43 to GAPDH (b) and GAPDH (housekeeping gene) only (c). {** ($p<0.01$) for Dunnett's post hoc test}.

8.4. Discussion

This chapter aimed to detect a possible role of connexin 43 (Cx43), in the etiology of defective contractile activity and less viability of chick cardiomyocytes when exposed to different environmental teratogens, using two molecular techniques; immunohistochemistry and western blotting. Development of the heart involves a series of molecular and cellular events including transformation of cardiogenic precursors initially into a linear heart tube, followed by a looped structure, and finally into a mature four-chambered heart. Many genetic and transgenic approaches have shown crucial roles for transcription factors, signalling molecules, cell adhesion molecules, and ion channels in development of the heart (Olson & Srivastava, 1996; Simon *et al.*, 2004a). Gap junctions are made up of a family of proteins called connexins and are responsible for coordination of intercellular communications, and maintenance of metabolic continuity between adjacent cells (Berthoud *et al.*, 2004; Zhang *et al.*, 2007). The major connexins present in the heart are Cx43, Cx40, and Cx45. Cx43 is one of the key proteins involved in embryonic heart development and is essential for normal growth and synchronous beating of the fetal heart. While Cx40 is predominately expressed in the atrium and Cx45 expressed in the ventricular conduction system, Cx43 is expressed in both atria and ventricles (Leaf *et al.*, 2008; Saito *et al.*, 2009; von Maltzahn *et al.*, 2009). The results of this chapter showed that common environmental teratogens including ethanol, nicotine and retinoic acid and diabetic conditions showed an overall decrease in the expression of Cx43 in chick

cardiomyocytes in micromass cultures. In one study conducted to detect the role of Cx43 in mouse heart, Cx43 null homozygous mice, replaced by Cx26, showed slower ventricular contractions and reproductive disorders (Winterhager *et al.*, 2007). Previous work on animals also demonstrated a vital role of Cx 43 in development of the heart and coronary vessels (Simon *et al.*, 1998; Simon *et al.*, 2004b; Liu *et al.*, 2006; Shasha *et al.*, 2006). All the teratogens also caused a decrease in contractile activity of cardiomyocytes (Chapter 5, 6, 7). The two effects can be related to each other, due to the fact that the heart cells behave as an electrical syncytium and their action potential, composed of rapid excitation followed by a delay, corresponds to intracellular transfer of current. This process occurs at gap junctions and is facilitated by connexins. Any disruption of these molecules results in asynchronous and decreased beating of excitable cardiac cells (Haussig *et al.*, 2008).

The results of immunohistochemistry illustrate that there was less expression of Cx43 on the cell surface when treated with these teratogens. However addition of vitamin C and folic acid diminished the effects of these toxins, and Cx43 was more obviously expressed at the surface rather than inside the cells and was comparable to control. These results suggest that micronutrients consumed either in the diet or as supplements might be helpful in protecting against teratogen induced insults on cardiomyocytes.

In the light of previous data which show a role of TPA and cyclic AMP (cAMP) in connexin regulation, (Rivedal & Opsahl, 2001; Faucheux *et al.*, 2002), these two chemicals were used as positive and negative controls in this study. Treatment of cardiomyocytes with TPA showed a reduced

overall expression of Cx43. These findings are in line with studies that show a decrease in production and expression of Cx 43 when treated with TPA (Rivedal & Opsahl, 2001). There is a growing body of evidence that TPA increases the phosphorylation of connexins through activation of protein kinase C pathway (PKC). On the other hand cAMP increased its expression particularly on the cell surface. The crowding of Cx channels is controlled by many factors, including hormones, which activate the production of cAMP. The activation of cAMP results in an increase in the number of membrane gap junctions in various cell types. The cAMP causes this increase via the protein kinase A pathway (Faucheux *et al.*, 2002). It is possible that teratogens used in this study utilized the protein kinase C pathway to down regulate the expression of connexin 43.

Chapter 9

Effect of various teratogens and vitamin supplementation on expression and distribution of ET-1

9.1. Introduction

Endothelins comprise a family of conserved 21-amino acid peptides that consist of three members encoded by different genes (ET1, ET2, and ET3) and a role has been suggested for them in heart development. The endothelin-1 (ET-1) isoform contains free carboxyl and amino termini with two intra molecular disulfide bonds. It is expressed in humans as well as many animal species. It is formed in many cell types including vascular endothelial cells, renal tubular endothelium, cardiac myocytes, cells in the pituitary, macrophages, mast cells etc (Inoue *et al.*, 1989). ET-1 is synthesised to its active form from its precursor pre-proendothelin by the converting enzyme ECE (Xu *et al.*, 1994). Two types of endothelin converting enzymes have been identified (ECE-1 and ECE-2). ECE-1 is membrane bound and cleaves both intra and extra cellular proendothelin-1 to endothelin-1. The ECE-2 is involved in intra cellular cleavage of endogenously synthesized proendothelin-1 to endothelin-1 at the trans-Golgi apparatus. The endothelins are secreted via the rough endoplasmic reticulum, Golgi cisternae, Golgi vesicles, and small exocytic vesicles. The secretion of endothelin can be inhibited by atrial natriuretic factor and protein kinase (Remiss *et al.*, 1993; Rubanyi *et al.*, 1994). The endothelins exert their different biological actions via the G coupled-protein endothelin receptors type A and B (ET_A and ET_B) (Yanagisawa, 1994; Yokota *et al.*, 2001). These receptors are classified according to their binding affinities to different endothelins. The first receptor shows maximum binding to ET-1 while the ET_B receptor has equal binding affinity to all types of

endothelins (Sakurai *et al.*, 1992). Endothelins seem to act mostly as local paracrine/autocrine peptides, but circulating levels of Endothelins, especially in pathological states of increased serum concentration, have great biological importance (Sakurai *et al.*, 1992).

Endothelins and their receptors were initially known for their involvement in the regulation of blood pressure (Yanagisawa *et al.*, 1988). Afterwards an unanticipated role as growth factors during embryonic development was discovered. Experimental studies on mouse embryos reveal that mice which carry homozygos mutations for Endothelin receptor type A (ET_A), ET-1 or ECE-1 were viable to term but die shortly after due to severe defects in the formation of neural crest derivatives (Clouthier *et al.*, 1998; Clouthier *et al.*, 2003). The expression and function of ET-1/ ET_A signalling appears to be crucial in all species. The pharmacological changes of either ET-1 or endothelin receptors also resulted in altered development in chick (Kempf *et al.*, 1998) and rat (Spence *et al.*, 1999) embryos. Further more mutations of ET-1 gene resulted in multiple deformities in zebra fish (Kimmel *et al.*, 2003; Miller *et al.*, 2001; Miller *et al.*, 2003). These studies have showed that the Ednra signaling is essential for late neural crest development in mice, chick and fish embryos (Bonano M. *et al.*, 2008).

9.2. Materials and Methods

Chick cardiomyocytes were seeded in 8 well chamber slides at a concentration of 3×10^6 in each well in the form of 20µl droplets. They were incubated for 2 hours to allow cellular attachment. Then 500µl of

DMEM growth medium was added and the cultures were left for 24 hours (Chapter 2, section 2.1.4.5.2). The DMEM medium was replaced by different concentrations of test chemicals after 24 hours. On day 6 cultures were fixed in paraformaldehyde solution (2% w/v in PBS) for 1 hour at room temperature. The cells were washed washed thrice with PBS each for 5 minutes, before being permeabilised with 0.2% Triton X-100 in PBS (v/v) for 10 minutes. The cells were incubated in 5% goat serum in PBS for 1 hour to block nonspecific bindings of secondary antibody. The cells were incubated with the primary antibody (ET-1) (200µl) (Table2.2) overnight at 4^{o}C. The secondary antibody (Table 2.3) diluted in 0.1% BSA in PBS was applied and incubated for 1 hour in the dark. Cells were washed in PBS (3 x 5 minutes) to remove excess unbound secondary antibody before mounting with Vectorshield™ containing DAPI. Sections were viewed under a confocal microscope (Chapter 2, section 2.3.5.1).

9.3. Results

The results of experiments performed on day 9 hearts stained for ET-1 show a bright staining (Fig 9.1), while Fig 9.2 shows negative staining with Alexa 488 in the absence of primary antibody which confirms the reactivity of ET-1 in chick hearts. Day 5 chick micromass control hearts similarly show a bright staining with ET-1 as shown in Fig 9.3, also cultures treated with folic acid and vitamin C show the same bright staining as controls (Fig 9.4 and Fig 9.5 respectively). The micromass cultures treated with ethanol 100µl/ml (Fig 9.6 b), nicotine 10µM (Fig 9.7 b), retinoic acid 20µM (Fig 9.8 b) and Diabetic condition (glucose 20mM + β-

hydroxybutyric acid 15mM) (9.9 b), showed a decreased overall expression of ET-1 inside the cells. The addition of folic acid (1mM) and vitamin C (100µM), to cultures treated with ethanol show that the expression of ET-1 is comparable to controls (Fig 9.6 c and d respectively). Similarly treatment of cardiomyocytes with folic acid and vitamin C in addition to nicotine (fig 9.6 a) shows that the level of ET-1 is comparable to controls (Fig 9.7 c and d). The same effect of folic acid and vitamin C was observed when added to retinoic acid treated cultures (Fig 9.8 c and d). Fig 9.8 c and d also illustrate the protective effects of folic acid and vitamin C on diabetic condition and no difference in ET-1 expression as compared to controls was observed.

Conclusion

The teratogenic agents used in this study cause a decrease in expression of ET-1 in chick cardiomyocytes micromass cultures. However addition of vitamin C and folic acid rescued the cells and expression of ET-1.

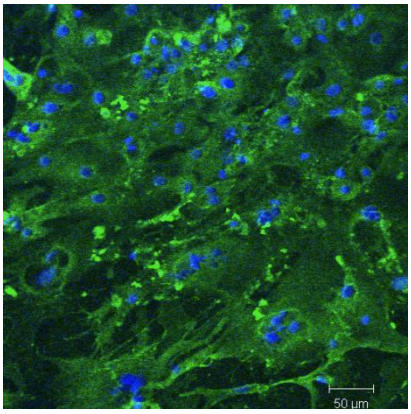


Fig 9.1: Day 9 heart expressing ET-1. 200x magnification

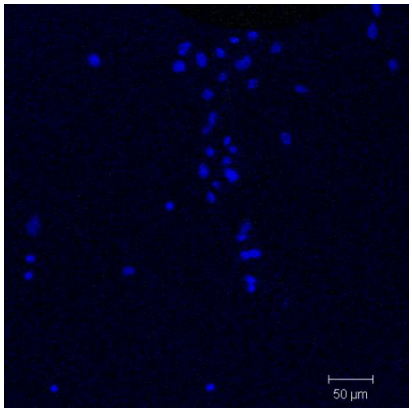


Fig 9.2: Day 9 heart –ve control Alexa 488. 200x magnification

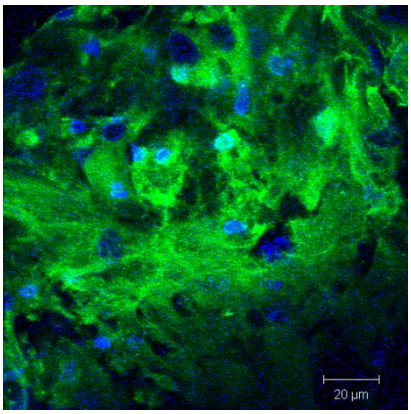


Fig 9.3: Day 5 control, showing expression of ET-1. 600x magnification (oil immersion)

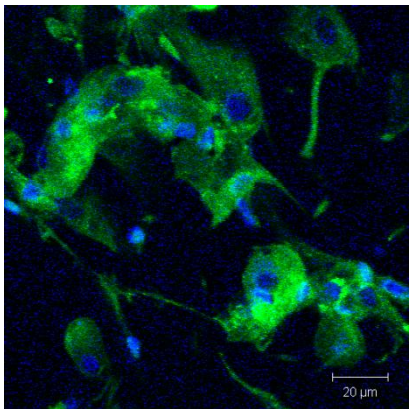


Fig 9.4: Day 5 chick heart cells treated with folic acid expressing ET-1. 600x magnification (oil immersion)

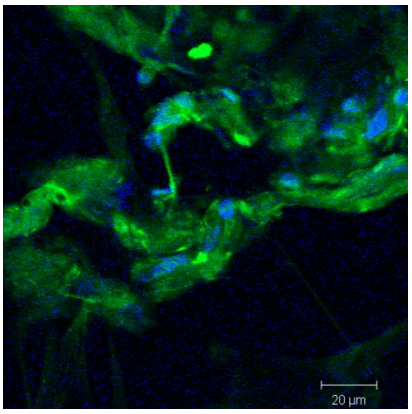


Fig 9.5: Day 5 chick heart cells treated with vitamin C expressing ET-1. 600x magnification (oil immersion)

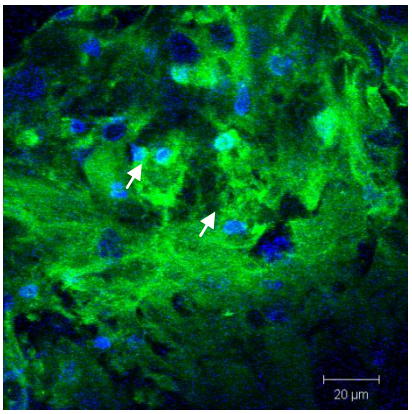


Fig 9.6 a: Day 5 control, showing expression of ET-1. 600x magnification (oil immersion)

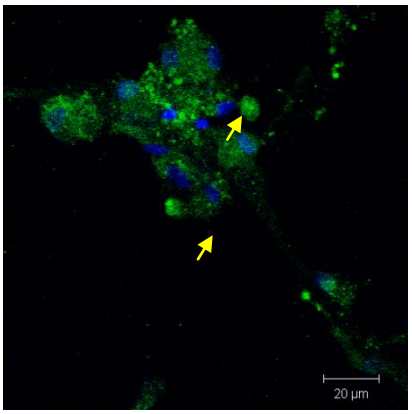


Fig 9.6 b: Day 5 ethanol treated cells, showing expression of ET-1. 600x magnification (oil immersion)

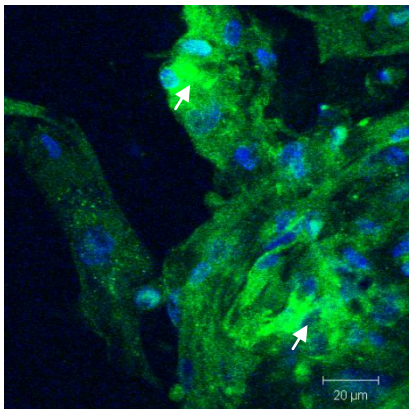


Fig 9.6 c: Day 5 ethanol + folic acid treated cells, showing expression of ET-1. 600x magnification (oil immersion)

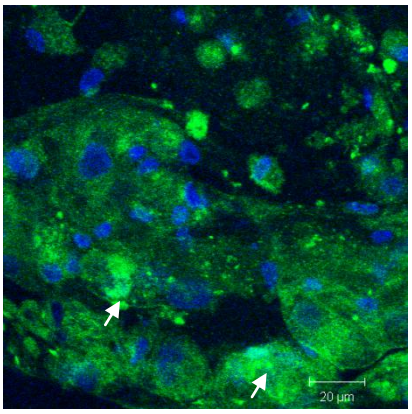


Fig 9.6 d: Day 5 ethanol +vitamin C treated cells, showing expression of ET-1. 600x magnification (oil immersion)

Fig 9.6: Shows expression of ET-1 in day 5 controls (a), ethanol treated (b), ethanol and folic acid treated (c) ethanol and vitamin C (d). White arrows indicate a brighter staining of ET-1, while yellow arrows indicate less expression of ET-1 in ethanol treated cells.

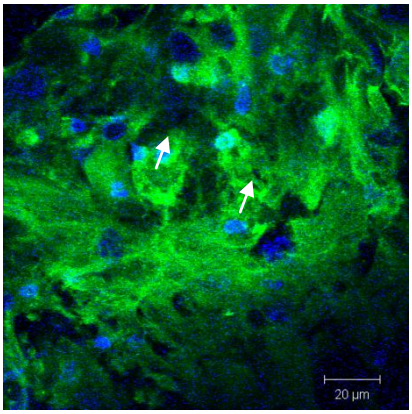


Fig 9.7 a: Day 5 control, showing expression of ET-1. 600x magnification (oil immersion)

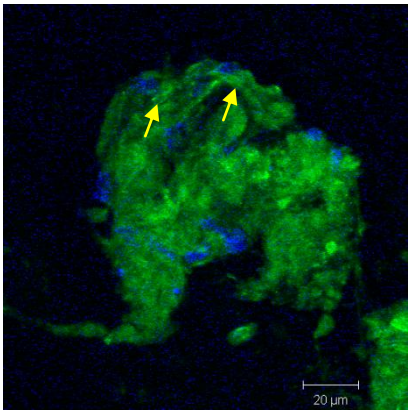


Fig 9.7 b: Day 5 nicotine treated cells, showing expression of ET-1. 600x magnification (oil immersion)

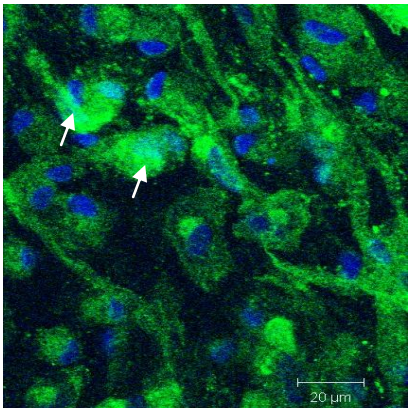


Fig 9.7 c: Day 5 nicotine + folic acid treated cells, showing expression of ET-1. 600x magnification (oil immersion)

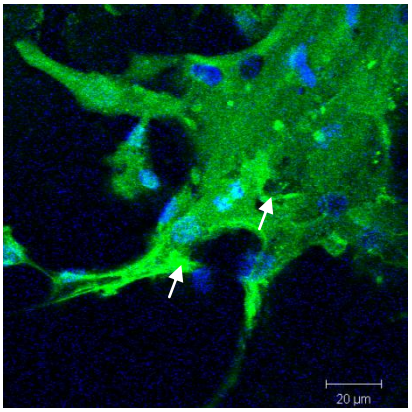


Fig 9.7 d: Day 5 nicotine + vitamin C treated cells, showing expression of ET-1. 600x magnification (oil immersion)

Fig 9.7: Shows expression of ET-1 in day 5 controls (a), nicotine treated (b), nicotine and folic acid treated (c), nicotine and vitamin C (d). White arrows indicate a brighter staining of ET-1, while yellow arrows indicate less expression of ET-1 in nicotine treated cells.

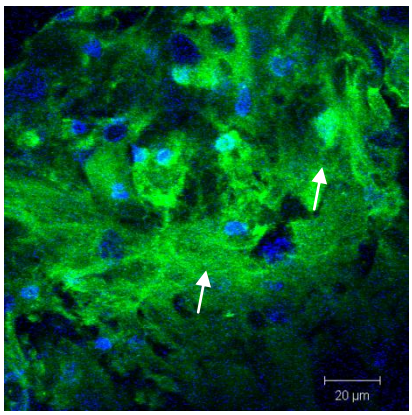


Fig 9.8 a: Day 5 control, showing expression of ET-1. 600x magnification (oil immersion)

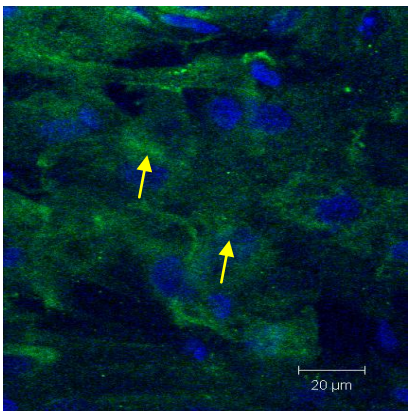


Fig 9.8 b: Day 5 retinoic acid treated cells, showing expression of ET-1. 600x magnification (oil immersion)

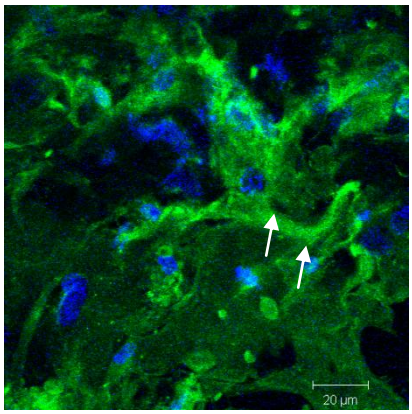


Fig 9.8 c: Day 5 retinoic acid + folic acid treated cells, showing expression of ET-1. 600x magnification (oil immersion)

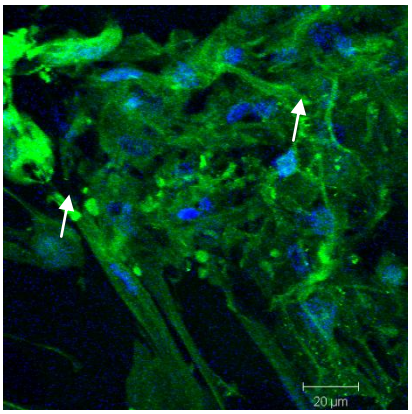


Fig 9.8 d: Day 5 retinoic acid + vitamin C acid treated cells, showing expression of Et-1. 600x magnification (oil immersion)

Fig 9.8: Shows expression of ET-1 in day 5 controls (a), retinoic acid (RA) treated (b), RA and folic acid treated (c), RA and vitamin C (d). White arrows indicate a brighter staining of ET-1, while yellow arrows indicate less expression of ET-1 in RA treated cells.

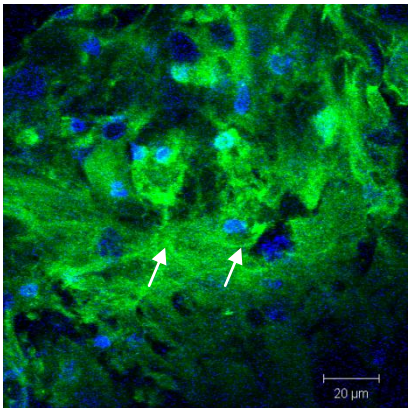


Fig 9.9 a: Day 5 control, showing expression of ET-1. 600x magnification (oil immersion)

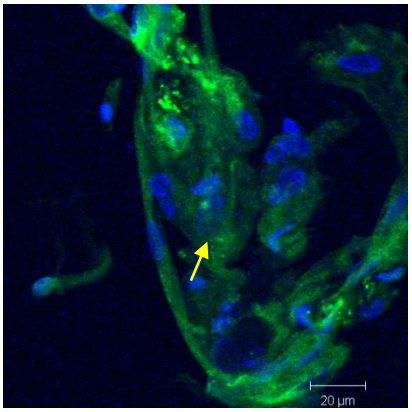


Fig 9.9 b: Day 5 Diabetic condition (20mMglucose+15mMβ hydroxybutyric acid) treated cells, showing expression of ET-1. 600x magnification (oil immersion)

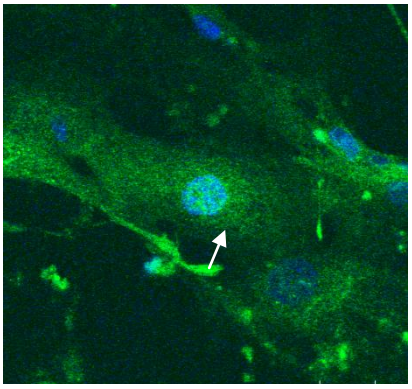


Fig 9.9 c: Day 5 Diabetic condition + folic acid treated cells, showing expression of ET-1. 600x magnification (oil immersion)

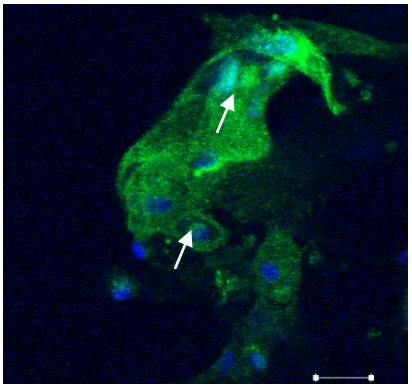


Fig 9.0 d: Day 5 Diabetic condition + Vitamin C treated cells, showing expression of ET-1. 600x magnification (oil immersion)

Fig 9.9: Shows expression of ET-1 in day 5 controls (a), Diabetic conditions (20mMglucose+15mMβ hydroxybutyric acid) treated (b), Diabetic condition and folic acid treated (c), diabetic condition and vitamin C (d). White arrows indicate a brighter staining of ET-1, while yellow arrows indicate less expression of ET-1 in diabetic conditions.

9.4. Discussion

The aim of this chapter was to determine the possibility for different teratogens {ethanol, retinoic acid, nicotine and diabetic conditions (glucose+ β hydroxybutyric acid)} to affect normal development of the heart, by interfering with ET-1. Almost all of teratogens tested, cause a decrease in immuno expression of endothelin 1 in chick cardiomyocytes. ET-1 is a 21 amino acid peptide, shown to be involved in many developmental processes including development of the heart. One study conducted on mouse embryos show that null homozygous mice for ET- exhibit cardiovascular malformations. The most affected parts were the outflow tract and endocardial cushion, and great vessels (Clouthier *et al.*, 1998; Kuriharam *et al.*, 1995). It is obvious from many animal studies that cardiac neural crest cells are involved in the formation of the outflow tract and the endothelium of the great vessels (Thomas *et al.*, 1998). In another study on mouse embryos, retinoic acid was shown to decrease the expression of ET-1 in branchial arch structures. The mice treated with retinoic acid showed craniofacial abnormalities, suggesting a role of ET-1 in retinoic acid induced malformations (Zhang *et al.*, 2006). Similar types of developmental defects were also observed in ET-1 deficient zebra fish and chickens (Groenendijk BC. *et al.*, 2008; Miller Craig T. *et al.*, 2007; Walker *et al.*, 2007). The chemicals tested in this study also show decrease in contractile activity and cell viability of chick cardiomyocytes. Ethanol and diabetic conditions also revealed developmental defects in the chick heart. It might be possible that these teratogens target ET-1 expression in

the heart and neural crest cells with a resultant decrease in cellular differentiation and heart defects.

Chapter 10

General Discussion

10.1. Introduction

This study was conducted to detect the possible role of environmental teratogens in the etiology of congenital heart defects. Also to find out the function of nutrition particularly micronutrients in the prevention of environmentally induced cardiovascular anomalies. The chick embryo was chosen as the experimental model due to the fact that chick eggs are easily available at low cost, the embryonic tissues are easy to dissect out for different micromass cultures, and no licence is necessary to conduct experiments on early embryos. Moreover the stages of chick development are very well recognized (Hamburger & Hamilton, 1951) and there are many similarities discovered between the chicken genome and human genome.

Congenital malformations are the principal cause of poor pregnancy outcome. Approximately 50% of all human concepti are lost before implantation, and of those that implant, further 15–20% are lost before term delivery. Of the fetuses which reach the end of term pregnancy, an estimated 3% are born with one or more severe congenital defects (Moore & Persaud, 1998). These figures show that errors which result in prenatal death or postnatal abnormalities are frighteningly common. Although rigorous research has been conducted on underlying mechanisms of many developmental defects, still many mechanisms are poorly understood.

Some lifestyle factors such as a diet low in nutrients, alcohol consumption, cigarette smoking, misuse of drugs available over the counter and some maternal diseases like diabetes mellitus might play a crucial role in the

etiology of congenital anomalies. Therefore identifying and adjusting these life style factors would be a step forward in detecting and preventing environmentally induced defects. Alcohol, one of two social drugs investigated in this study was shown to have direct impact on the development of chick heart cells and chick embryos cultured in ovo. The chick heart micromass cultures showed decreased cellular differentiation, cell viability and protein content. Chick embryos cultured in ovo showed other gross malformations as well, along with heart defects. However, nicotine, an active component in maternal smoking, revealed its teratogenic effects on the chick heart cells only. A decrease in contractile activity of chick cardiomyocytes was observed, but it had no cytotoxic effects on the cells. The chick embryos showed no developmental defects. This might be due to the fact that the dose used in this study was not sufficient to produce any effects on whole chick embryos. It is obvious from previous data that these social drugs are most commonly consumed by women of child bearing age from low socioeconomic status, less educated, younger age and usually jobless (Mehta, 2008). Alcohol when consumed in excess of the recommended range produces a spectrum of defects. However mild to moderate drinking might produce congenital anomalies (Henderson *et al.*, 2007). Therefore some other factors apart from these drugs, particularly diet, may also be involved in the causation of developmental defects. The results of this study also demonstrate the protective effects of folic acid and vitamin C on ethanol and nicotine induced malformations. Moreover these micronutrients were also shown to have preventive effects on teratogenesis brought about by excessive retinoic acid intake and

diabetic conditions. However these vitamins when added to cadmium chloride treated cells did not show any protective effect. This could be due to the fact that cadmium chloride exerts its effects by a different mechanism. The mechanisms involved in cadmium induced cell damage are very complex. It has been suggested that cadmium induces mitochondrial and lysosomal damage, which leads to lipid peroxidation, DNA damage (Fotakis *et al.*, 2005; Fotakis & Timbrell, 2006), chromosomal aberrations and apoptosis (Biesalski *et al.*, 1998). Some data reveals the production of reactive oxygen species in cadmium induced damage but our study shows no role for antioxidants in the prevention of cadmium induced cytotoxicity (Lampe *et al.*, 2008; Panjehpour & Bayesteh, 2008). The recognised relationship between deficiency of the micronutrient folic acid and neural tube defects is of very long standing. Low social status with poor dietary conditions are well known risk indicators for a variety of unfavourable perinatal and infant outcomes, for instance low birth weight and perinatal, neonatal and postnatal mortality. Poor maternal nutrition may not only be responsible for congenital anomalies including heart defects, orofacial defects, cleft lip etc, but also have several long term effects on the offspring. Animal studies have proved a relationship between a low nutritious maternal diet during the periconceptional period and long term consequences on the child's health. A diet which is deficient in proteins exerts several effects on the offspring. Studies on mice, and rats for detection of a possible role for a low protein diet in the periconceptional period showed behavioural and cardiovascular problems in the offspring and they were also growth retarded (Kwong *et*

al., 2000; Kwong *et al.*, 2006; Watkins *et al.*, 2008; Watkins *et al.*, 2010). Also poor maternal nutrition affects the fetus's hepatic expression of certain genes involved in glucose metabolism and blood pressure regulation and thus the body's homeostasis; these effects were observed in a gender specific manner (Kwong *et al.*, 2007).

Low nutritious diet does not only denote low caloric diet but also a diet which is low in many micronutrients, for example folic acid, vitamin A, vitamin C, E, zinc iodine, copper, and manganese etc. Deficiencies of these micronutrients result in a wide range of malformations (Shah & Ohlsson, 2009). Not only deficiency but also excess intake of some micronutrients e.g. retinoic acid can also produce craniofacial and heart defects (Zhang *et al.*, 2006).

Although the concept of suboptimal maternal nutrition is not new, still there are certain groups of people who are not fully aware of the importance of micronutrients for healthy pregnancy outcomes. Primary deficiency of nutrition is not very common in developed countries, as compared to developing countries, but there are a number of life style factors (alcohol abuse, smoking, recreational drugs, genetic defects and maternal illness), which result in secondary deficiencies.

10.1.1. Experimental Techniques used to detect possible teratogenic effects of different environmental teratogens

This study was designed to detect developmental toxicity of certain environmental teratogens in a system which was cost effective, reproducible, reduced animal killing and avoided any maternal metabolism.

Two culture techniques were employed; chick heart micromass culture and in ovo culture. The rat limb bud micromass culture has been validated for the detection of potential developmental teratogens by the ECVAM validation study which took place from 1997 to 2000 (Spielmann *et al.*, 2001). The chick cardiomyocyte culture was evaluated to detect teratogens that affect cardiomyocyte differentiation and viability and hence cardiac development. The chick micromass culture was based on a limb bud and midbrain system developed by (Atterwill *et al.*, 1992), and later used with cardiomyocytes by (L'Huillier *et al.*, 2002; Hurst *et al.*, 2007; Ahir, 2009). In a blind study conducted on paired chemicals with known embryotoxicity and non toxicity, chick cardiomyocyte micromass culture detected potential toxicity of embryotoxic chemicals while their counterparts showed no developmental toxicity (Hurst *et al.*, 2009), and results of micromass culture were compared with D3 embryonic stem cells an already validated toxicity screening method by ECVAM. Similarly in another blind study with different paired chemicals Ahir also found similar results (Ahir, 2009). These results confirm the reliability of the chick heart micromass culture for teratogenicity screening tests. For the micromass cultures used in this study it was necessary to confirm the presence of cardiomyocytes as the majority of cells, before the evaluation of teratogenic potential of different chemicals. During explantation cardiomyocytes are not the only cells which are dissected out; some other cells like fibroblasts and smooth muscle cells are also likely to be present. Conventional histological techniques; H & E staining and Masson's trichrome staining were used to detect cardiomyocytes in cultures on the basis of their morphological

characteristics. Although the results confirmed the presence of cells it did not confirm the nature of the cells in the cultures. To further confirm the presence of cardiomyocytes immunohistochemical methods were applied with some molecular markers (CH1, MF20, 12/101) which are only expressed by heart cells and not fibroblasts. The results confirmed that the majority of cells in chick cardiomyocyte micromass cultures were heart cells.

Once the presence and number of cardiomyocytes was confirmed, preliminary experiments were performed to select the optimal cell density and day for heart explantations. The cell density of 3×10^6 was considered optimal and employed in further experiments due to satisfactory attachment and differentiation of micromass cultures. Beating was observed after 24 hrs of seeding initially in individual cardiomyocytes, (Hewitt *et al.*, 2005; Hurst *et al.*, 2007) but later a synchronous beating was observed as cardiomyocytes in cultures make connections with each other. Day 5 was selected for explantation due to the fact that day 5 embryonic hearts are still in the period of organogenesis; an important point when detecting teratogenic potential of toxic substances (Sadler *et al.*, 1982). Three end points; cell viability, protein content and cellular differentiation, were used to detect teratogenicity of chemicals. Cell viability was assessed by the resazurin reduction assay. The resazurin assay was initially designed to assess bacterial and yeast contaminations in biological fluids (Erbs & Ehlers, 1950). Later it was used in cytotoxicity assays to detect the viability of cells due to the fact that it has the ability to enter the cells and itself it is not toxic, therefore the same cells can further be used for other

endpoints (Clothier, 1992; Hurst *et al.*, 2007). The other cytotoxicity assay employed was the Kenacid blue assay. This assay provides a good association in detecting the toxicity of different chemicals and is advantageous in getting repeat measures on the same cells as compared to other cytotoxicity assays (Clothier & Samson, 1996; O' Brien *et al.*, 2000). The in ovo culture technique was evaluated to detect gross and histological defects in chick embryos when teratogenic agents were injected in ovo through the air sac region. Pilot experiments were conducted to ensure that the volume of test chemical injected was enough to produce effects, PBS, neutral red dye and Parafilm had themselves no teratogenic or toxic effects on chick embryos. The results confirmed that none of above vehicles showed any toxicity.

This study was further extended to examine some of the potential molecular mechanisms involved in cardiovascular defects when cultures were exposed to different teratogens and their protection brought about by multivitamins.

Development of the heart is a complex process and involves a series of molecular events. The two genes used in this study were Cx43 and ET-1. Cx43 is abundantly expressed in adult and embryonic hearts and data regarding heterozygous null animals show a wide range of cardiac defects (Shasha *et al.*, 2006). It is required in the formation of the myocardium of atria, ventricles, ventricular septum, atrioventricular junction, outflow tract, and the conduction system of the heart (Christoffels *et al.*, 2004). Apart from Cx43, other members of the connexin family (Cx40 and Cx45) are also involved in the formation of the heart (Alcolea *et al.*, 1999). ET-1 was

initially recognized for its vasoconstrictor functions but later on it was also proved to be a growth factor involved in the development and growth of many embryonic organs (Bonano M. *et al.*, 2008). Its role in the formation of the heart is very well recognized. Many studies reveal a direct relation of ET-1 and development of neural crest cell-derived parts of the heart. Furthermore this molecule also plays an important role in the formation and growth of the interventricular septum, conduction system and ventricular myocardium (Zhang *et al.*, 2006).

The heart is composed of cardiomyocytes which act as an electrical syncytium. Efficient and well orchestrated impulse propagation and synchronized beating is achieved through cell-cell communication gap junction organization (Smyth *et al.*, 2010). The gap junctions are specialized clusters of hexameric arrays, composed of hundreds of connexins. In the heart, gap junctions are located at intercalated discs which are distinct zones of cardiomyocyte-cardiomyocyte coupling in the heart (Saez *et al.*, 2003). To date approximately 20 connexins have been discovered in the human genome. The most abundant connexin found in the adult and embryonic heart is Cx43. Alterations in the organization and expression of Cx43 are considered to be key contributors involved in arrhythmias and contractile malfunctions (Severs *et al.*, 2008). Also there is a growing body of evidence from animal studies that remodelling of gap junctions, particularly disturbances in Cx43 expression, is related to congenital defects of the heart (Ya *et al.*, 1998). The results of this study suggest that exposure of cardiomyocytes to ethanol, nicotine, retinoic acid and diabetic conditions, resulted in decrease in the expression of Cx43 both

in immunohistochemical analyses and western blotting (chapter 8). The addition of vitamins was shown to have substantial effects on the above mentioned teratogens and Cx43 was expressed as with control embryos. The mechanism by which these teratogens affect the expression of Cx43 and thus the gap junctions might be related to oxidative stress. One recent study showed a direct relation of oxidative stress and a decrease in the expression of Cx43 (Smyth *et al.*, 2010). The oxidative stress was shown to decrease the amount of Cx43 at gap junctions plaques by two means; first excessive production of reactive oxygen radicals caused an increase in Cx43 phosphorylation, which in turn resulted in excessive turnover, internalization and lateralization of Cx43 at gap junctions plaques with decreased cell-cell communication (Beardslee *et al.*, 2000). The oxidative stress induced increased phosphorylation is via the protein kinase C pathway; secondly, oxidative stress also affects forward trafficking of Cx43 to gap junction plaques by creating defects in post Golgi microtubule transfer. The microtubules are responsible for the transfer of correctly aligned connexins to the cell surface and gap junction plaques. Disturbances to this pathway result in transport of less Cx43 to the cell surface, such that there is not enough to be functional and there is a resultant decrease in intercellular communication (Smyth *et al.*, 2010). This study confirmed the role of reactive oxygen species cardiac malformations involving Cx43 distribution in both cardiac cells and chick cardiomyocytes cultured in ovo, due to the fact that addition of antioxidants folic acid and vitamin C could reverse the effects of the above teratogens. Also, connexin 43 expression was restored with the addition of

antioxidants. Furthermore this study also confirmed one possible mechanism of oxidative stress induced damage to Cx43 levels. It is well known that TPA increased phosphorylation of Cx43 and decreased the levels of Cx43 at gap junction plaques and thus reduced cell-cell communications (Rivedal & Opsahl, 2001). The teratogens used in this study produced an excessive amount of reactive oxygen species which have deleterious effects on Cx43 phosphorylation with resultantly less available Cx43 at the cell surface.

Immunohistochemical analysis showed that expression of cytoplasmic ET-1 was less in teratogen-treated cultures as compare to controls and also addition of folic acid and vitamin C restored its cytoplasmic concentration. The possible mechanism of decreased levels of active ET-1 in the cell cytoplasm could also be related to oxidative stress produced by environmental teratogens used in this study, as oxidative stress has been shown to be involved in regulation of ET-1 in other systems. A study conducted on rat pulmonary endothelial cells showed that the level of ET-1 is decreased with addition of H_2O_2 and glucose oxidase in a dose dependant manner and is increased by addition of antioxidants (Michael *et al.*, 1997). Other studies conducted on bovine carotid arteries also demonstrated the same response (Saito *et al.*, 1998 ; Masatsugu *et al.*, 2003). However there is evidence that oxidative stress produced by hypertensive and diabetic conditions also enhances the production of ET-1 from endothelial cells. The possibility that other factors including pro-inflammatory markers might be involved along with oxidative stress in the generation of higher levels of ET-1 has been suggested (Saito *et al.*, 2001; Pollock, 2005;

Manea *et al.*, 2010). However the question of how oxidative stress affects the levels of ET-1 in chick hearts has yet to be fully answered.

10.2. Conclusions

To conclude, this study has demonstrated the toxic effects of ethanol, nicotine, retinoic acid, cadmium chloride and diabetic conditions in chick heart micromass culture. The micromass culture also revealed that sodium fluoride and the traditional medicine ginseng had no teratogenic potential in chick hearts, at least at the concentrations employed. Also micronutrients used in this study were shown to have no cytotoxic effect, except retinoic acid as already mentioned. Furthermore the protective role of micronutrients, particularly vitamins, against environmental teratogen induced cardiovascular malformations was also observed for the majority of chemicals tested, except cadmium chloride. The developmental defects brought about by ethanol were also observed in chick embryos cultured in ovo. However nicotine showed no developmental defects in in ovo culture. The diabetic condition also revealed developmental defects in chick embryos. The teratogenic effects of ethanol and diabetic conditions were abrogated by supplemental vitamins. The expression of both Cx 43 and ET-1 was affected by these environmental toxins, but addition of vitamins proved to be helpful in restoring the expression of both molecules comparable with controls. In summary the present work confirmed a protective role of healthy nutrition particularly micronutrients against environmentally induced congenital anomalies.

10.3. Future Work

Future work may be extended in two different directions. Firstly, as described in fig 10.1, aggressive public awareness programmes should be started in a more wide ranging way. Although the notion of a periconceptional healthy diet, especially in micronutrient intake, and healthy pregnancy outcome is not new, still many pregnancies are unplanned, particularly in low socioeconomic groups and these are the groups where binge drinking, cigarette smoking and misuse of over the counter drugs are very common. There should be some longitudinal studies conducted at the GP surgery level, where more and more women should be studied. Also, another approach should be pre-pregnancy counselling regarding planned pregnancies and encouragement about a healthy diet and avoidance of social and recreational drugs. Public health seminars are another good approach to create awareness about intake of multivitamins among women of child bearing age.

Secondly, future work may be to further extend the experimental work to detect the role of other connexins particularly Cx40 and Cx45 in developmental defects. Also it would be worthwhile to detect the expression of Cx43 in chick heart sections and to see whether it plays a role in migration of cardiac neural crest cells, which are essential components of the secondary heart field and play a vital role in the development of many parts of the heart. Also investigations concerning

the possible role of oxidative stress in affecting the post Golgi microtubule network and forward trafficking of Cx43 to be available for gap junction plaques would be of interest.

As the chick micromass provided reliable and reproducible results it would be useful to extend the work to other organs, in particular chick brain development and to detect possible beneficial role of nutrition for its development (fig 10.1).

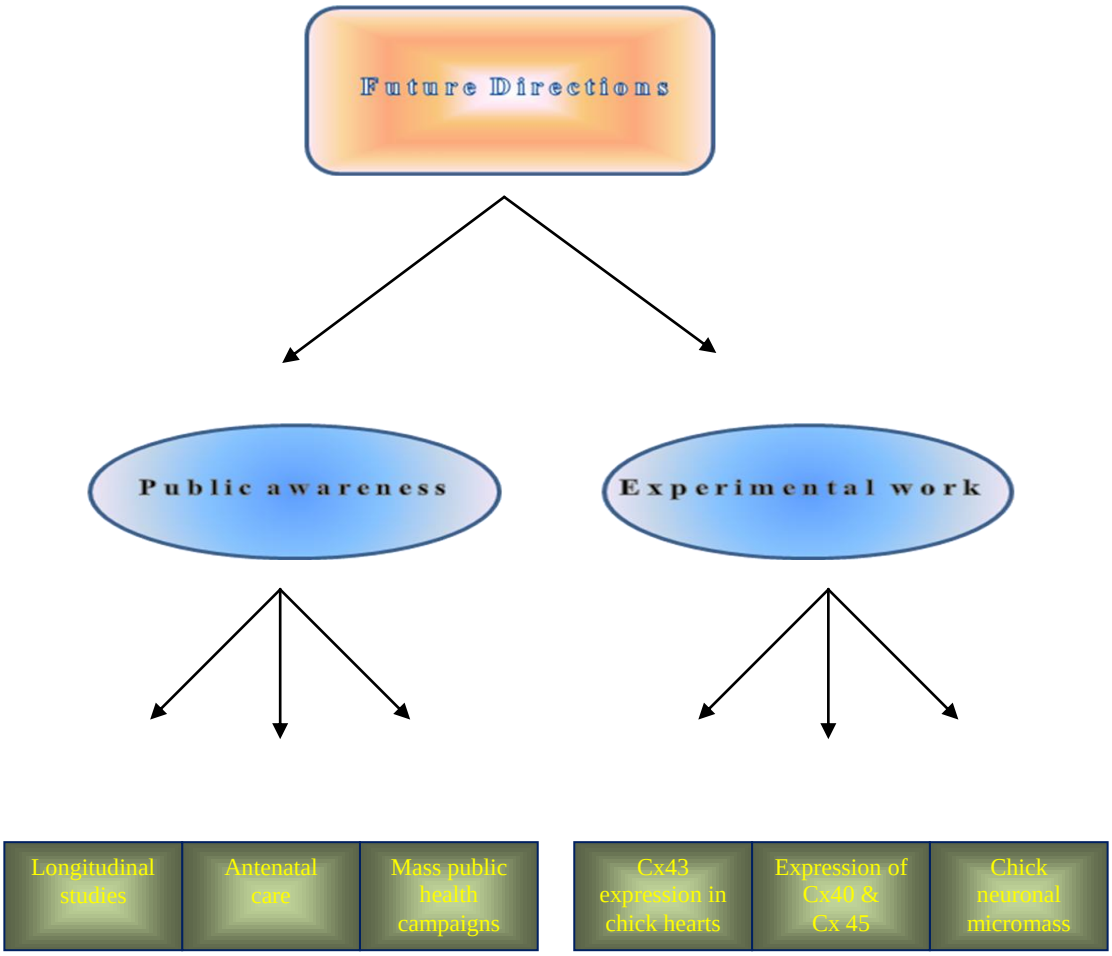


Fig 10.1: Shows directions for future work

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