The chick cardiomyocyte micromass system and stem cell differentiation along specific pathways: Prediction of embryotoxic effects and their mechanism

By

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Abstract

Malformations in humans at birth have been recorded since ancient times. These malformations are anatomical or physiological anomalies present at the time of birth that may be caused by genetic or environmental factors or a combination of both. The pathogenesis is only known in 10%, of which 1% or less are caused by drugs and medications. Certain disease states, like maternal epilepsy and depression during gestation itself, contribute to abnormal development. Further, this dilemma is augmented by the use of medications during pregnancy. The antiepileptic (AEDs) and antidepressant drugs (ADPs) with a history of producing malformed neonates are mostly classified as moderate teratogens. This study was designed to evaluate teratogenic potential at the cellular and molecular levels of AEDs and ADPs on cardiomyocytes at different stages of development and the neural stem cell derived neurons using *in vitro* systems.

In the micromass system (MM), five day old embryonic chick cardiomyocytes were cultured to form beating foci, while embryonic stem cell were differentiated into contracting cardiomyocytes (ESDC) using the hanging drop method. In a third *in vitro* system early chick Neural Stem Cells (NSC) were diverted to a neuronal lineage. Drug toxic effects were estimated on cultured cell viability and protein content. The effects on gap junctions (Cx43) in cardiomyocytes and neurofilament (NF) in NSC were also evaluated because of their important role in cell differentiation and regulation. Oxidative stress, being the potential source of xenobiotic toxicity induction, was also analysed and toxic effects were counteracted using antioxidants and other molecules.

In AEDs, valproic acid (VPA) mainly targeted the cardiomyocyte differentiation and contractile activity with reduced Cx43 turnover. In NSC the VPA effects were different and it did not inhibit the neuronal differentiation. With carbamazepine (CBZ) the low doses showed no effect on NSC compared to high doses. In ESDC, the contractile activity stops at a 200μM dose with reduced cell viability and proliferation. Cx43 phosphorylation was reduced after CBZ treatment which might have affected the contractile activity. An increase ROS production with CBZ

treatment was recorded, which was protected either by the addition of Ascorbic acid (AA) or superoxide dismutase (SOD). The other AEDs, Phenytoin (PHT) and Primidone (PRM), mainly affected the cardiomyocyte contractile activity with some chronic exposure effects. In ADP, bupropion (BPN) severely affects cell proliferation in all systems. The NF-L was not statistically reduced in neurons but Cx43 expression in cardiomyocytes declined which might result in reduced contraction. The other ADP, lithium carbonate showed developmental stage dependent effect on cardiogenesis, where contractile activity ceased completely at higher dose in the ESDC with increased cell proliferation. Lithium mimics the Wnt/β-catenin pathway and also inhibits the PI cycle, effects which were reversed by the addition of myo-inositol in the ESDC system. In NSC the lithium showed no significant inhibitory effects on neural differentiation at and above drug serum therapeutic concentrations. The active constituents of the herbal antidepressant drug St. John's wort, hypericin and hyperforin, showed synergistic inhibition of contractile activity with reduced proliferation at higher doses in the MM system.

Drug interference at the molecular level during development may induce modification at the gene and protein levels with altered signalling. The tissue specific effects depend on the drug mechanism, while increased oxidative stress in part has a contribution in initiating the embryopathies. By identifying the exact mechanism of toxicity induction, the molecular mechanism can be protected against and thus abnormal development be avoided.

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Table of Contents

Abbreviations

5-Fu	5-Fluorouracil
5-MTHF	5-Methyltetrahydrofolate
6-Mu	6-Methyluracil
AA	Ascorbic acid
ADP	Antidepressant
AED	Antiepileptic
ANOVA	Analysis of variance
Apgar	Appearance, Pulse, Grimace, Activity, Respiration
BPN	Bupropion
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBZ	Carbamazepine
Cx43	Connexin43
DAG	Diacyl glycerol
DAPI	4’,6-diamido-2-phenlyindole
DCF	2’,7’ Dichlorofluorescin
DCFH-DA	2’,7’ dichlorofluorescein diacetate
DMEM	Dulbecco’s modified eagles medium
EBs	Embryoid bodies
ECVAM	European Centre for the Validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetra acetic acid
EINECS	European Inventory of Existing Chemical Substances
ESC	Embryonic stem cell
ESDC	Embryonic stem cell derived cardiomyocytes
EST	Embryonic stem cell test
EU	European Union
FA	Folic acid

FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FSG	Fish skin gelatin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GJ	Gap junction
GJIC	Gap junction intercellular communication
GSK3-β	Glycogen synthase kinase 3-beta
H&E	Haematoxylin and Eosin
H&H	Hamburger and Hamilton
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
HDAC	Histone deacetylase
HF	Hyperforin
HP	Hypericin
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IMPase	Inositol monophosphatase
INVITTOX	*In vitro* Techniques in Toxicology
IP_3	Inositol triphosphate or Inositol 1,4,5-trisphosphate
Li_2CO_3	Lithium carbonate
LIF	Leukemia inhibitory factor
MAO	Mono amine oxidase
MAPK	Mitogen Activated Protein Kinase
MHC	Myosin heavy chain
MM	Micromass
NEP	Neuroepithelial cells
NGS	Normal goat serum

NS	Neurospheres
NSC	Neural stem cell
NTD	Neural tube defects
PBS	Phosphate buffer saline
PCx43	Phospho-Connexin43
PDGF	Platelets derived growth factor
PG	Penicillin G
PHT	Phenytoin
PI	Phosphoinositol
PKA	Protein kinase A
PKC	Protein kinase C
PRM	Primidone
PtdIns	Phosphatidylinositol
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
ROS	Reactive oxygen species
SDS	Sodium docedyl sulfate
SHH	Sonic hedgehog
SJW	Saint John's wort
SOD	Superoxide dismutase
STAT	Signal Transducer and Activator of Transcription
TBST	Tris Buffered Saline with Tween20
TPA	12-O-tetradecanoylphorbol-13-acetate
TRIS	1,3-dicholoro-2-propyl phosphate
VPA	Valproic acid
WB	Western Blot
WEC	Whole embryo culture
ZO-1	Zonula occludens-1

Chapter 1

Introduction

1.1 Chemical testing (Chemical regulation)

With advancements in research and technology, the production and use of chemicals has been dramatically increased and their related toxicological hazard led to the establishment of various chemical regulation policies. In the white papers entitled "*Strategy for the future chemical policy*" the European commission proposed comprehensive chemical regulation policies REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) (Scialli, 2008). These policies aim to generate relevant risk assessment and management knowledge for the classification and precautions for chemicals (Rudén & Hansson, 2008). REACH aims to harmonise testing requirements for chemicals already in use with those already in place for new chemical substances. Hence all chemicals should have the same portfolio of hazard data, i.e. have completed testing using the same single system that is now adopted for new chemicals since 1981 (Scialli, 2008). As a consequence, in February 2001 the EU released a white paper stating approximately 30,000 chemicals already in use would require some additional hazard toxicity testing assessment by 2012. The EU regulations, apart from other requirements, demanded further information about the chemicals in terms of reproductive and developmental toxicology. Furthermore, the EU has plans for a future chemical policy with regulation of new and existing chemicals. Registration of chemical substances which are already on the EINECS (European Inventory of Existing Chemical Substances) will undergo phased hazard toxicity assessment between 2010 and 2018, depending upon the substance volume and whether it is being manufactured in or imported into the EU (Lilienblum *et al.*, 2008). The major issue raised within this policy was the chemical testing method for completing the potential risk portfolio. The traditional methods using *in vivo* animal testing are not practically feasible in the time frame and require a very large number of animals. This also totally contradicts EU and national government aims to reduce the number of animals used in research, thus alternative embryotoxicity/teratogenicity assays were in strong demand at least for the initial evaluation phase (Lilienblum *et al.*, 2008). This thesis evaluates and examines the

teratogenic effects of selected drugs on the developing heart and the molecular mechanisms of related toxic effects using *in vitro* systems.

1.1.1 Chemical testing in animals

Chemical testing on humans solely for hazard identification is not permissible (Höfer *et al.*, 2004), but the ultimate proof of a human teratogen can only come from human exposure (Brown & Fabro, 1983). Extrapolation of animal data for chemical and drug safety evaluation is the initial basis prior to human exposure (Schardein *et al.*, 1985). Animals are considered as an alternative to humans for chemical testing - virtually all the chemicals which induce toxic effects in humans are expected to produce toxic effects in animals (Brown & Fabro, 1983). Animal use in chemical screening prior to marketing provides basic information regarding drug induced effects and has certainly improved our knowledge and understanding of diseases, diagnosis and treatment (Carere *et al.*, 2002; Piersma, 2004). Development toxicity testing in animals involves chemical exposure followed by assessment of toxic effects in dams and fetuses (Piersma, 2004). Animal use in teratogenicity testing for already identified teratogens and for drug screening before marketing has also improved our understanding of drug related effects, especially at the molecular level, including the role and importance of micronutrients and disease states during development. Some disadvantages of interspecies difference, difference to humans and high dose exposure compared to humans may affect the outcome (Brown & Fabro, 1983). The European commission animal welfare guideline 1986 required any person planning to undertake animal testing to consider alternatives prior to beginning their research (Lilienblum *et al.*, 2008). Teratogenicity testing in animals requires large number of embryos with the mother also sacrificed in most *in vivo* systems (Piersma, 2004). Home Office licences and project reviews have certainly reduced experimental animal usage (Balls & Fentem, 1999; Fuscoe, 2007; Walmond *et al.*, 2002).

1.1.2 Reduction, Refinement and Replacement (The Three R's)

William Russell and Rex Burch in their book "humane experimental techniques" (1959) described the three alternative principles to minimize the number of animals use in research (Lilienblum *et al.*, 2008). The 3R's stands for reduction, refinement and replacement; it encourages the reduction in number of animals used in general by improved experimental design and minimizing the volume of testing (Fuscoe, 2007; Lilienblum *et al.*, 2008), the use of available alternative methods and where possible development of early biomarkers to make results more reliable and quick thereby replacing the use of animals (Fuscoe, 2007; Lilienblum *et al.*, 2008), and refinement to minimise animal suffering and distress during experimental procedures (Fuscoe, 2007). The ECVAM (European Centre for the Validation of Alternative Methods) and NICEATM-ICCVAM (National Toxicology Programme Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)) organisations were initiated in Europe and the USA for this purpose and they support the development of alternative methods to animal use, their evaluation, validation and regulatory acceptance for new chemicals (Scialli, 2008; Walmod *et al.*, 2004). Also the UK based FRAME (Fund for the Replacement of Animals in Medical Experiments) principally focuses on the principles of 3R's because of the high requirement for funding and animals (Scialli, 2008). The 3R principles include alternative methods for example using *in vitro* cell lines, lower organism embryos, *ex vivo* or *in vivo* with reduced and refined methods. With the advancement of technology non-testing methods are applicable using computer systems or referring to the effects of structurally related compounds (Lilienblum *et al.*, 2008; Scialli, 2008). Early biomarkers using trans proteomics, proteomics and metabolic studies may provide more insight into drug related effects on development (Fuscoe, 2007).

1.2 Teratology and teratogens

The word teratology has Greek roots "Teras" meaning monstrosity or prodigy, and was originally referred as the study of monsters (Kalter, 2002). Simply teratology is the study of abnormal prenatal physiological and anatomical development, which

results in congenital defects. These malformations can be induced by exogenous chemicals or by physicals agents (Bailey *et al.*, 2005; Chung, 2004).

Teratogens have been defined as agents or factors that produce physical defects in the developing embryo/fetus. In a broader sense it not only includes physical defects with in utero exposure but deficits in organ function; hormonal, behavioural or immunological changes or growth deficiencies (Brown & Fabro, 1983), although only some 10% of all congenital defects have known pathogenesis. These malformations affect the quality of life and are associated with enormous emotional and fiscal costs (Bailey *et al.*, 2005; Brent, 1995).

1.2.1 Congenital malformations

The word congenital is derived from 'L. congenitus' meaning "born with" (Moore & Persaud, 1998). A congenital malformation is an anatomical or physiological anomaly present at the time of birth that may be caused by genetic or environmental factors or a combination of both during prenatal development (Chung, 2004; De Santis *et al.*, 2001). Congenital malformations affect 5% of all births (De Santis *et al.*, 2001) and account for approximately 20% of deaths in the perinatal period. An average of 3% of new-born infants has major malformations and another 3% malformations are detected later in life (Chung, 2004). The cause is only detected in 10% of the new born while the remaining 90% remain unknown; among this known group, 1% or less are due to known drugs and medications (De Santis *et al.*, 2001). These malformations are the leading cause of infant mortality. The majority of congenital malformation affected infants die during the first year of their life. An estimated of 495,000 deaths are recorded worldwide every year (Rosano *et al.*, 2000). Prenatal malformation diagnosis followed by prenatal surgeries, pregnancy termination or susceptible factors consideration in highly pre-disposed patients may provide the solution to these problems (Rosano *et al.*, 2000).

1.2.2 Background, history and principles

Teratology is the science of abnormal development induced by exogenous agents (Bailey *et al.*, 2005). Through artistic interpretations and written scriptures abnormal

development has been recorded over centuries in legend (Warkany, 1977). An earlier explanation of abnormal development was that it was solely the result of genetic defects (Warkany, 1977) but maternal nutrition, life style, disease states and exposure to toxic chemicals refuted the earlier ideas and broadened the term teratogen (Keen *et al.*, 2003). The science of teratology was born between 1920s and 1930s when Hale confirmed malformed piglets were born to a mother with retinol deficiency during pregnancy (Bailey *et al.*, 2005; Kalter, 2003). Subsequent evidence after a decade came to light in 1941 with the identification of congenital cataract in children born to mothers who had suffered from rubella virus infection, and mercury poisoning effects in 1956 (Igata, 1993; Kalter, 2003). These were followed by many other studies which confirm many chemicals and physical agents like nitrogen mustard, hormones, antimetabolites, alkylating agents have a role in producing anomalies in mammalian species (Warkany, 1965). The Dutch wartime famine (Hunger winter) during the Second World War further highlighted the importance of micronutrients during pregnancy (Smith, 1947).

However, the thalidomide catastrophe brought immediate attention to modern studies on developmental toxicology. Thalidomide was developed as mild sedative in 1950s. It was used to prevent nausea during pregnancy. The drug produces children with deformed limbs including phocomelia and approximately 8000 neonates worldwide were affected with thalidomide before its identification as teratogen. Thalidomide was not tested comprehensively for its teratogenic effects before marketing. After the catastrophe it was shown to induce similar pattern of malformation in the rabbit and non-human primates. However in rodents no malformation was seen. This highlighted the complexity and requirements of teratogenicity testing (Bailey *et al.*, 2005; Jergil, 2009). It was following the thalidomide epidemic that studies and research on teratogens were given importance and new guidelines were set regarding introduction and safe exposure of pharmaceuticals and chemicals. This resulted in identification of many teratogens including infectious diseases, drugs, radiations and maternal factors by various studies (Moore & Persaud, 1998).

In 1959 Wilson, based on a firm groundwork of surveys and experimental work, laid the formulation of principles to underlie teratological susceptibility to environmental agents (Kalter, 2003).

1. The susceptibility to teratogenesis depends on the fetus genotype and the manner in which it interacts genetically with adverse factors.

2. The susceptibility to teratogenesis depends on adverse influence at the particular stage of development.

3. Teratogens act via specific toxic mechanism on developing cells and tissue to initiate abnormal development.

4. The extent of teratogenesis depends on teratogens nature of influence on developing tissue.

5. The manifestation of adverse effects results in death, malformation, growth retardation and functional deficit.

6. The manifestation of teratogenesis depends on adverse agents' dose and duration of exposure.

1.3 Teratogenicity testing and developmental biology

Teratogens are agents that increase the incidence of congenital malformations (Chung, 2004). The mechanism of induction of teratogenesis involves either alteration of the genotype or the adverse effect of an environmental agent or factor during gestation (Beckman & Brent, 1984). In developmental toxicology the effects are observed from the zygote stage through organogenesis and the fetal period to the term of pregnancy (Jergil, 2009). Gestation in the human involves three major development stages: pre-organogenesis, active organogenesis and the fetal period. The pre-organogenesis period is generally not as susceptible to teratogens as the active organogenesis period, due to the formation of the organs, but some organs like teeth, external genitalia and the brain continue to develop even after this period. That is why teratogens like antiepileptic drugs (AEDs) continue to affect the brain beyond the first trimester of pregnancy (Ornoy, 2009). At the earlier stages totipotent stem cells have a great capacity to grow and repair. Teratogenic insult at this stage either results in embryo lethality or the alteration is repaired. The adverse

effect during the time of organogenesis results in congenital malformation (Beckman & Brent, 1984). The exposure at late stages results in cell depletion and growth retardation (Beckman & Brent, 1984). Apart from developmental stage, the drugs' dose and the exposure time critically interferes with normal development (Beckman & Brent, 1984). The knowledge of the teratogenic potential of substances would minimize the hazards (Bailey *et al.*, 2005). Identification of teratogenic potential of chemicals that come into contact with pregnant women every day is crucial (Brent, 1995). Cohort studies and surveys in humans may provide the basic preliminary information, but it is necessary to screen and prepare comprehensive teratogenic profiles of agents before being marketed. A number of *in vivo* and *in vitro* testing procedures in animals are currently in regulation for teratogenicity testing. Beside this, the US food and drug administration agency (FDA) has categorized the chemicals according to their teratogenic potentials.

Pregnancy Category	**Description**
A	Adequate and well controlled studies have failed to demonstrate risk to the fetus.
B	Animal studies have failed to demonstrate a risk to fetus and there are no adequate and control studies in humans.
C	Animal studies have shown adverse effects on fetus and there are no adequate and well controlled studies in humans.
D	There is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience or studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks.
X	Studies in animals or humans have demonstrated fetal abnormalities and/or there is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience, and the risks involved in use of the drug in pregnant women clearly outweigh potential benefits.

Table 1.1 FDA pregnancy category of chemicals (De Santis *et al.*, 2001).

1.3.1 *In vivo* teratogenicity testing

The preliminary teratogenic profile of a drug or chemical is usually obtained by *in vivo* teratogenicity testing (Pratt *et al.*, 1982), including the type of developmental defects and the basic information about site of toxic action, time of administration during development and organs affected. In humans the basis for this information is mostly reported cases. *In vivo* teratogenicity testing in humans is unethical, because of the mother and fetus involvement and the risk to their life (Steele & Copping, 1993). Normally mothers are asked to complete a survey during pregnancy for the analysis of drug and disease related effects (Brown & Fabro, 1983; Hanson *et al.*, 1978). The data obtained is statistically analysed to reach some conclusion. Normally these types of studies involved various factors along with particular medicine like combination therapy, depression, disease states, smoking, genetic carriers, socioeconomic conditions, the number of patients and the extent of their information sharing and cooperation (Grisso *et al.*, 1984; Hanson *et al.*, 1978). Alternatively rodents like rat, mice and rabbit or some higher animals are most frequently used for *in vivo* teratogenicity testing, none of these animals, even their strains, possess the same metabolic system or the same placental transport as the human (Pratt *et al.*, 1982). The standard requirement for an *in vivo* testing system is at least 20 pregnant animals per dose ranging from low to highly toxic (Pratt *et al.*, 1982). This requires a large number of animals for each study, which is totally against the 3R's principles (Fuscoe, 2007; Lilienblum *et al.*, 2008). In *in vivo* studies the biggest advantage is the use of natural process for estimating the toxic effects. Most of the drugs act through their toxic metabolic products (van Gelder *et al.*, 2010), which in the case of *in vitro* testing is difficult to produce even after administration of liver S9 fractions, the effects observed are indistinguishable between drugs or metabolites. Some *in vitro* teratogenicity testing methods have been developed and validated to overcome the shortcomings of *in vivo* procedures and to study the mechanisms at the molecular level (Schwetz *et al.*, 1991).

1.3.2 *In vitro* teratogenicity testing

In vitro teratogenicity testing provides an alternative to *in vivo* animal testing. The *in vitro* methods overcome the intrinsic problems and differences in *in vivo* animal teratogenicity testing (Brown *et al.*, 1995) and provide quick, cheap and reproducible chemical teratogenicity screening. The biggest advantage of *in vitro* tests is the requirement for a lower number of animals compared to its *in vivo* counterpart (Bournias-Vardiabasis & Teplitz, 1982; Walmod *et al.*, 2004). Due to the increased demand of research in drug discovery and treatment; hundreds of drugs are marketed every year and are exposed during pregnancy. *In vivo* methods for teratogenicity testing requires a lot of animals for each drug screening before marketing. The use of *in vitro* methods is less time and animal consuming and the results are often comparable to *in vivo* testing (Bournias-Vardiabasis & Teplitz, 1982). *In vitro* methods indicate potential teratogenic hazards at an early stage of screening. These methods primarily focus on specific mechanisms such as cell adhesion and proliferation (Bacon *et al.*, 1990), but by adding different end points and molecular markers to the *in vitro* system, reliability and efficiency can be improved (Seiler *et al.*, 2004). Three *in vitro* teratogenicity tests have been validated by ECVAM these include the embryonic stem cell test, limb bud micromass and whole embryo culture. Beside these a number of other tests have also been developed which are in the process of refinement or validation (Scialli, 2008). These tests depend upon organ, tissue or embryo exposure to drugs. Embryonic tissue micromass has been adopted for teratogenicity testing decades ago. In limb bud micromass, rat gestation day 14 limb bud cells are isolated and cultured; the limb bud mesenchymal cells differentiate into chondrocytes, the extent of which is estimated using Alcian blue staining (Scialli, 2008). Flint using the same culturing method exposed embryonic brain cells to chemicals (Invittox Protocol n^{o} 112). Beside these, micromass of heart tissue has also been developed for teratogenicity testing (L'Huillier *et al.*, 2002), which is discussed in detail in section 1.3.2.1. Whole embryo culture (WEC) was the first validated procedure to undergo industrial application (Schmid *et al.*, 1993). The WEC relies on rat embryo culture at an early

somite 1-5 stage. Embryos are exposed to chemicals with growth medium for 48h and substantial differentiation examined (Scialli, 2008). The third ECVAM validated method is the embryonic stem cell test (EST). The EST uses *in vitro* D3 mouse embryonic cell line differentiation into cardiomyocytes, along with the 3T3 adult fibroblast cell line for comparison. The test chemical effects on cardiomyocyte differentiation and cell viability is estimated and toxicity is predicted (see section 1.3.2.2) (Scholz *et al.*, 1999). Using lower organisms such as Hydra, Johnson and colleagues proposed a teratogenic substance detection assay (Johnson & Gabel, 1983). The Hydra is a fresh or salt water lower animal with radial symmetry. Hydra reproduces asexually by producing buds, which simply break away when they are mature. Using their regeneration ability the teratogenicity of water soluble compounds can be detected. But this assay is not reliable due to its single end point (Wilby & Tesh, 1990). The fruit fly drosophila has also been utilized in teratogenicity testing. The adult flies are anesthetized and exposed in vials to distilled water containing instant drosophila medium with test chemicals. The flies are allowed to mate and lay eggs. The eggs are exposed to test chemicals and take 9-10 days for hatching. The adult flies are morphologically examined and scored (Bournais-Vardiabasis, 1994; Bournias-Vardiabasis & Teplitz, 1982; Bournias-Vardiabasis *et al.*, 1983). The South African clawed frog (Xenopus laevis) embryo is another source of detecting teratogenicity testing. ECVAM has approved The Frog Embryo Teratogenesis Assay (FETAX) as a non-mammalian assay because of its resemblance to early major human development. The endpoints of embryo mortality, malformation and growth inhibition are used in FETAX (Douglas *et al.*, 1989).

In this study two *in vitro* systems for teratogenicity testing were utilized - the chick cardiomyocyte micromass system and the embryonic stem cell test with some modification.

1.3.2.1 The micromass system

Umansky in 1966 devised a method to study the development and differentiation processes of chick mesenchyme limb bud cells into chondrocytes, which became the basis of the micromass test. Later on in 1983 Flint, using rat embryo midbrain,

detected the effects of teratogenic agents on differentiating neurons (Invittox Protocol n^{o} 112). The micromass system involves the primary cell culture of different organs (Spielmann *et al.*, 2001). The basis of this test is the chemical's disruption of embryonic cell differentiation and re-establishment of the system (Flint & Orton, 1984). The micromass culture system detects chemical interference in the normal process of cell differentiation, development, cell proliferation, cell-cell communication and cell matrix interactions (L'Huillier *et al.*, 2002). Using the chick micromass system has advantages over that of rats and mice. Firstly, because it obviates the step of sacrifice of the mother in order to culture the embryo. There is rapid development of the embryo on the fifth day of incubation and heart, brain and cartilages are developed to considerable size. It is easy to handle, with no need for animal house or mating procedures, and easy to predict the exact development stage. Up to day 9 the embryo can be used without a Home Office license (L'Huillier *et al.*, 2002) and chick limb bud micromass shows similar responses to chemicals as rat micromass (Brown & Wiger, 1992). In chick embryonic cardiomyocyte micromass culture, 5 day old cardiomyocytes are cultured at a very high cell density, so they proliferate and differentiate to form foci and in the case of cardiomyocytes these foci shows phases of contraction and relaxation (Hurst *et al.*, 2009). The test chemicals were exposed and cytotoxicity is observed using relevant endpoints (Atterwill *et al.*, 1992).

1.3.2.2 Embryonic stem cell test (EST)

The blastocyst derived pluripotent mouse embryonic stem cells (Murabe *et al.*, 2007a) have the ability to differentiate into all three germ layers, including spontaneous differentiation into contracting cardiomyocytes. These cardiomyocytes exhibit the appropriate proteins, receptors and ion channels that closely resemble *in vivo* cardiogenesis (Wei *et al.*, 2005). Therefore ES cells are used as a tool to predict tissue specific mutagenic, cytotoxic and embryotoxic effects of chemicals using *in vitro* systems (Kusakawa *et al.*, 2008; Murabe *et al.*, 2007a). The D3 embryonic stem cells can be grown in an undifferentiated state in the presence of Leukemia inhibitory factor (LIF), which controls STAT signalling via the membrane

bound gp130 signalling complex (Wei *et al.*, 2005; Yamanaka *et al.*, 2008). When the cells are allowed to differentiate in hanging drops (without LIF), which provides an excellent three dimensional environment for development (Banerjee & Bhonde, 2006), they form embryoid bodies which resemble the blastocyst exhibiting all three germ layers (Koike *et al.*, 2007; Murabe *et al.*, 2007b). EBs are formed in hanging drops as a result of the highly expressed Ca^{2+} dependent adhesion molecule E-cadherin homophilic binding in the ES cells (Kurosawa, 2007; Larue *et al.*, 1996; Yoon *et al.*, 2006). Upon plating of these embryoid bodies, the cardiomyocyte lineage becomes predominant and spontaneously differentiates into contractile areas (Anneelieke *et al.*, 2007; Wei *et al.*, 2005). This differentiation represents three stages; the pace maker like cells, the intermediate and terminal like atrial, sinus nodal, ventricular and Purkinje-fibres (Boheler *et al.*, 2002). A number of improvisations have been made in the embryonic stem cell test (EST) using different molecular markers and end points. The fibroblast cell line is used to mimic maternal toxicity, but inhibition of fibroblast proliferation does not represent the *in vivo* situation and is omitted in number of studies (Anneelieke *et al.*, 2007; Marx-Stoelting *et al.*, 2009).

The EST was designed to test drug and chemical embryotoxicity. The previously validated *in vitro* teratogenicity testing assays required the use of embryos or their tissues while in EST immortal D3 ES cells eliminate this factor and reduce time, cost and animals (Anneelieke *et al.*, 2007; Scholz *et al.*, 1999). The European Centre for the Validation of Alternative Methods (ECVAM) has approved the EST as a valuable tool to investigate the embryotoxic potential of chemicals on the differentiation process as an alternate to animal use (Rohwedel *et al.*, 2001). According to the ECVAM validation study the accuracy of EST is 78% for all experiments, and 100% for strongly embryotoxic chemicals. It can easily differentiate between strong, weak and non-teratogen chemicals (Buesen *et al.*, 2009; Scholz *et al.*, 1999) and provides an opportunity to study the cellular and molecular mechanism of early development and the effects of teratogens (Kitazawa *et al.*, 2005; Takahashi *et al.*, 2003).

1.4 Stem cells

Functionally stem cells are defined by their ability to produce identical daughter cells (self-renew) and to generate progeny with new morphological and functional characteristics (differentiation) (Davila *et al.*, 2004). Stem cells are undifferentiated or unspecialized cells with unlimited selfrenewal capacity without undergoing senescence, which is clearly distinct from progenitors and precursor cells (Lanza, 2006). These characteristics are critically involved in development and tissue regeneration (Li & Xie, 2005).

1.4.1 Stem cell classification

Stem cells are primitive cells present in all organisms, generally classified into embryonic and adult types according to their origin (Davila *et al.*, 2004). The morula stage derived embryonic stem (ES) cells are totipotent (differentiate into all embryonic and extra embryonic tissues), as development proceeds the ES cells at blastula stage are restricted to pluripotent potentials (differentiate into all embryonic tissues only) (Davila *et al.*, 2004; Friel *et al.*, 2005). The adult stem cells differentiate into different cell types (multipotent) and have limited selfrenewal abilities or become further lineage committed with unipotent differentiation potentials (Friel *et al.*, 2005). The adult stem cells and progenitors are essential component of cell homeostasis and repair (Davila *et al.*, 2004; Filipczyk *et al.*, 2007).

1.4.2 Stem cells research-Potential benefits and drawbacks

Embryonic stem cells isolated from blastocysts can be expanded immortally without losing their pluripotency and can be differentiated into several tissue types. This provides their biggest advantages over primary cell cultures which have limited proliferation capacity. The differentiation process in many respects resembles normal *in vivo* development which provides an easy tool to investigate the basic processes that are difficult to explore *in vivo* (Lanza, 2006; Lanza, 2004). Pluripotent stem cells are difficult to differentiate into a uniform cell type and mostly they represent a heterogeneous population of cells. In the case of ES cell derived cardiomyocyte the

atrial, ventricular and SA node cell types were observed, alongside endodermal and ectodermal markers positive staining (Boheler *et al.*, 2002). Due to their pluripotent potentials when the undifferentiated stem cells are transplanted into tissue they may form teratomas (Lanza, 2006; Lanza, 2004). Apart from forming teratomas they may cause immune reactions in the recipient. For the isolation of human stem cells the human embryo at a very early stage is used which poses some ethical problems. The derivation of stem cells from adult tissue is another option but these cells are mostly lineage committed and exhibit low differentiation capacities (Lanza, 2006; Lanza, 2004). Induced pluripotent stem cells may provide the solution in which stem cells are produced using fibroblast cells which are then differentiated into cardiomyocytes (Zwi *et al.*, 2009).

1.4.3 Embryonic stem cells isolation and characterization

Mouse ES (mES) cells are the *in vitro* counterparts of *in vivo* epiblast cells (Burdon *et al.*, 2002). The pluripotent embryonic stem cells are derived from the blastocyst inner cell mass of the pre-implantation embryo. The blastocyst is a ball shape structure (fig 1.1) with an outer covering trophoblast layer, the hollow space blastocoel and inner cell mass (the group of cells) (Friel *et al.*, 2005; Li & Xie, 2005). Embryonic stem cells were first isolated from mouse by Evan and Kaufman and independently by Martin in 1981, while in 1998 from humans. *In vitro* the ES cells are maintained in an undifferentiated state by growing on mitotically inactivated mouse embryonic fibroblasts (feeder layers) or by addition of a differentiation inhibition factor (Doetschman *et al.*, 1985; Filipczyk *et al.*, 2007). The cells under these conditions are believed to maintain the normal karyotype and unlimited self-renewal (Boheler *et al.*, 2002). Upon differentiation inhibitor removal ES cells form cystic embryoid bodies, and that is believed to initiate signalling and spontaneous differentiation into germ layers (Doetschman *et al.*, 1985; Sachinidis *et al.*, 2002), which resembles the blastocyst stage. Their developmental characteristics were confirmed: when ES cells were implanted into blastocysts, they develop into a complete embryo (Gajovic & Gruss, 1998). Various transcription factors and proteins in ES cells contribute toward 'stemness' such as STAT3, OCT4, SOX2, Nanong

(Yamanaka *et al.*, 2008), SSEA1 and alkaline phosphatase activity (Zandstra *et al.*, 2000), and these are used to characterize the stem cells presence.

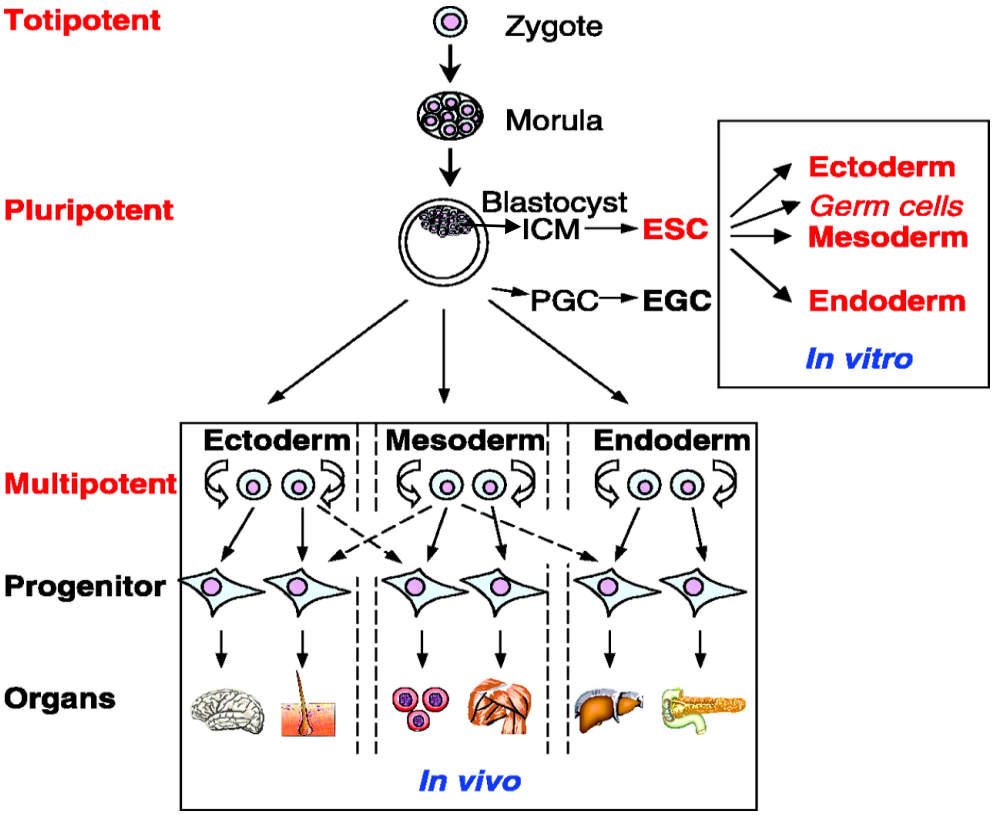


Fig 1.1 Stem cells hierarchy. Zygote and early stage development of stem cells to the generation of complex organism (Wobus & Boheler, 2005).

1.4.4 Molecular facets of self-renewal and pluripotency (stemness)

The stem cell microenvironment (niche) is thought to play the major role in the self-renewal or differentiation decision making (Lanza, 2006). *In vitro* mES cells are maintained in undifferentiated state by the addition of cytokines from the interleukin (IL)-6 families (Zandstra *et al.*, 2000). Leukemia inhibitory factor (LIF), an IL-6 family cytokine (Bader *et al.*, 2000), is either provided by feeder layers or as the recombinant protein (Burdon *et al.*, 2002). Upon inhibitor withdrawal the mES cells spontaneously differentiate into all three germ layers (Pandur, 2005). In human ES cells the cytokine LIF is not sufficient to retain pluripotency, mainly due to the decreased number of LIF receptors (Aghajanova *et al.*, 2006). Fibroblast growth factor is used as alternative in human ES cells to maintain pluripotency (Vallier *et*

al., 2005). LIF acts via a LIF-specific receptor and signal transducer gp130 complex (Bader *et al.*, 2000; Zandstra *et al.*, 2000). This complex signals activates JAK/STAT and MAPK pathways (Bader *et al.*, 2000). This finally stimulates STAT3 which is necessary to maintain the ES self-renewal, while MAPK has a role in cell proliferation and differentiation (fig 1.2) (Burdon *et al.*, 2002). The balance between JAK/STAT cascade and MAPK cascade is crucial for determining cell fate. LIF signalling is poorly understood post STAT3 activation. LIF is considered an important supplement in the medium to support cell establishment and self-renewal (Lee *et al.*, 2009). LIF over expression in mice during gastrulation inhibits mesodermal differentiation *(Bader et al., 2000).* The OCT4, SOX2 and Nanong transcription factors control pluripotency, self-renewal and cell fate determination pathways (fig 1.2). In the absence of serum, LIF is insufficient to maintain pluripotency or block neuronal differentiation (Yamanaka *et al.*, 2008).

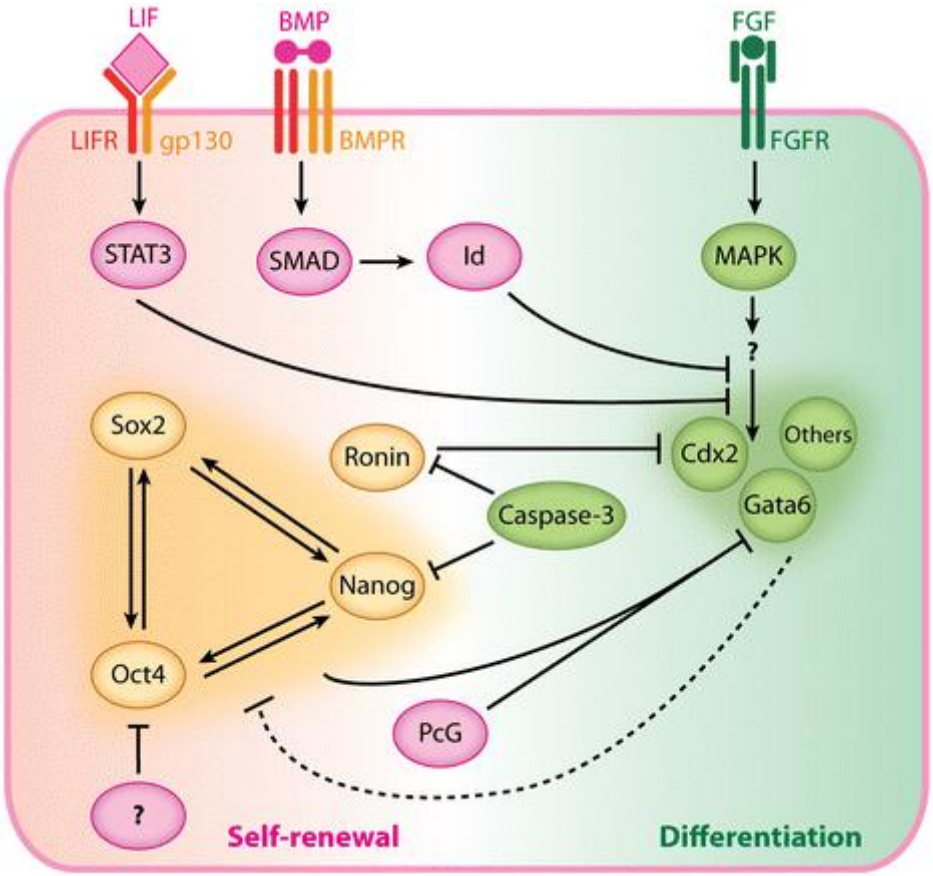


Fig 1.2 The regulation of stem cell pluripotency (Shenghui *et al.*, 2009).

1.4.5 Embryonic stem cell differentiation into cardiac lineage

The role of signalling molecules and transcription factors in cardiogenesis has already been identified (Sachinidis *et al.*, 2003), but still the molecular mechanism that drives stem cells toward the cardiac lineage is nebulous (Pandur, 2005). Cardiac transcription factors control the expression of various genes that regulate cardiac-specific differentiation (Akazawa & Komuro, 2003). These includes the GATA family transcription factors, cardiac specific homeobox transcription factor (Csx/Nkx2.5), MEF2 and T-box transcription factor (Akazawa & Komuro, 2003). GATA transcription factors are the key regulator molecules in development. GATA-4, 5 and 6 is detected and persists later in the developing heart. GATA promotes various cardiac specific genes ANF, BNF, cTnl, α-MHC, MLC (Akazawa & Komuro, 2003; Sachinidis *et al.*, 2003). GATA null embryos showed various cardiac developmental defects. The transcription factors like Nkx2.5 and GATA clearly have a role in cardiogenesis but the exact mechanism is not fully understood (Akazawa & Komuro, 2003; Sachinidis *et al.*, 2003). Nkx2.5 is one of the earliest markers of the heart field mesoderm in vertebrates. Loss of function of Nkx2.5 is associated with congenital heart defects (Sachinidis *et al.*, 2002). Over expression of Nkx2.5 causes myocardium hyperplasia (Cleaver *et al.*, 1996). On the other hand inhibition of Nkx2.5 resulted in complete absence of heart formation (Fu *et al.*, 1998; Grow & Krieg, 1998). Nkx2.5 interacts with other transcription factors including GATA transcription factor, T-box factors and serum response factor. Nkx2.5 is also involved in the roles of cardiogenic extracellular signalling proteins including BMPs and Wnts (Akazawa & Komuro, 2003). The T-box proteins belong to a family of transcription factors that are essential for early cardiac lineage determination, chamber specification and valvuloseptal development (Naiche *et al.*, 2005). At the receptor level many molecules like Wnt, BMP have been shown to play important role in stem cells and stem cell differentiation (Filipczyk *et al.*, 2007). Signalling downstream of various classical signalling pathways including PI3K, PKC, MAPK and others have been implicated to have roles to play in stem cell differentiation into cardiomyocytes, but the exact molecular mechanism by which these may mediate the stem cell

cardiomyocyte lineage commitment and differentiation is not fully understood (Brand, 2003; Sachinidis *et al.*, 2003; Sachinidis *et al.*, 2002; Sauer *et al.*, 2000).

Extracellular signalling factors are central for stem cells differentiation or for remaining in the undifferentiated state. Stem cell lineage commitment involves complex networks of extracellular signalling molecules that activate various signalling pathways. These activated signal transduction pathways in turn exert complex positive or negative controls on this process. A number of growth factors or extracellular signalling molecules have been identified that are involved in stem cell differentiation into cardiomyocytes including bone morphogenetic proteins (BMP), sonic hedgehog (SHH), fibroblast growth factors (FGF), Wnt and Notch proteins (Filipczyk *et al.*, 2007). BMP and its family members play crucial role during embryonic development, cell division or proliferation, differentiation, organisation, migration, adhesion, extracellular matrix production and apoptosis (Pardali & Dijke, 2012). BMP signalling occurs via two pathways MAPK and Smad (Monzen *et al.*, 1999; Zaffran & Frasch, 2002). BMP has an important role in *in vivo* cardiogenesis and the differentiation of stem cells into cardiomyocytes (Zaffran & Frasch, 2002). BMP are expressed very early in the endoderm and ectoderm and play critical roles in the induction of heart formation in the vertebrate embryo (Andrée *et al.*, 1998; Schlange *et al.*, 2000; Schultheiss *et al.*, 1997; Tzahor & Lassar, 2001). In addition to Smad pathways BMP signalling also occurs via MAPK signalling pathways. This is very important since phosphorylation is a key post-transcription mode of gene control. The MAPK signalling when activated could control many other targets including transcription factors and other kinases (Monzen *et al.*, 1999). Application of BMP-2 *in vivo* elicited ectopic expression of the cardiac transcription factors Nkx2.5, transcription factors of the GATA family, and ventricular myosin heavy chain (Sachinidis *et al.*, 2002). Insulin like growth factor and FGF are essential for the regulation of early cardiac development (Sachinidis *et al.*, 2003). Mutation in FGF results in decrease expression of cardiac markers (Sachinidis *et al.*, 2002). Wnt/β-catenin signalling negatively regulates cardiogenesis and the activation of pathways

that inhibits Wnt/β-catenin signalling is required for the induction of cardiogenesis (Sachinidis *et al.*, 2003).

In vitro ES differentiation into cardiomyocytes depends on number of factors like the number of cells forming EBs, serum, media, growth factors, time of EBs plating. The *in vitro* differentiated cells represent three stages - early (pacemaker like cells), intermediate and terminal (atrial, ventricular, nodal-His, and Purkinje like cells) (Boheler *et al.*, 2002; Sachinidis *et al.*, 2002).

1.5 Aims and objectives

The aims and objectives of current study are

- To utilize *in vitro* techniques for teratology screening of selected antiepileptic and antidepressant drugs.
- To develop and evaluate the chick embryonic cardiomyocyte micromass system and ES cell differentiation into cardiomyocytes for teratogenicity testing.
- To estimate and compare the teratogenic potential of antiepileptic and antidepressant drugs in micromass and ES derived cardiomyocytes using different end points.
- To determine the potential teratogenic mechanism of drugs at the molecular level and to counteract any toxic effects using different molecules.
- To determine the possible role of gap junction Connexin 43 in drug induced toxic effects.
- To develop and utilize a chick neural stem cells system for the neurodevelopment teratogenicity testing.

Chapter 2

Materials and Methods

2.1 Cells source

2.1.1 Chick embryonic cardiomyocytes

For the micromass culture system chick embryonic cardiomyocytes were obtained from five day old white fertile Leghorn chicken eggs (Henry Stewart & Co.UK).

2.1.2 D3 mouse embryonic stem cell line

The D3 mouse embryonic stem cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, USA; cat no. CRL-1934). The ES D3 cell line is derived from day 3.5 old mouse blastocyst inner cell mass of strain 129S2/SvPas (Doetschman *et al.*, 1985).

2.2 Materials

For the list of materials see appendix 1.

2.3 Design of Experiment

This study utilizes two *in vitro* systems, the chick micromass and D3 ES cell derived cardiomyocytes, for the evaluation of cardiotoxic effects of teratogens at the tissue specific and molecular levels. The study was primarily based on embryonic cardiomyocyte treatment with drugs both when the undifferentiated cells (stem cells) are not lineage (cardiac) committed and once they become cardiac lineage committed (micromass). For each drug dose at least three repeats were performed and analysed statistically. The drug doses used in both systems ranged from well below the blood serum therapeutic levels to higher levels as used in some other studies. In MM and D3 ESCs derived cardiomyocytes a well-known teratogen valproic acid (L'Huillier *et al.*, 2002) was used as a reference drug because of its known effects in these systems. Beside that some drugs with known effects at particular doses were also tested to confirm system reliability. For the micromass system the day of heart explantation and cell seeding density were evaluated before starting the experiments with teratogens. For D3 ESCs derived cardiomyocytes no changes were made in the cell differentiation protocol, as it is already ECVAM validated (Buesen *et al.*, 2009). The 24 well plates used in both systems were divided in columns of

blank, control and drug treated groups for easy comparison (fig 2.1) (Ahir & Pratten, 2011).

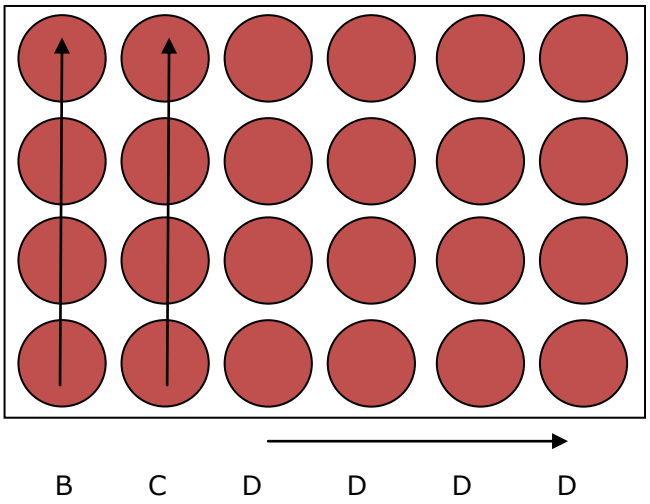


Fig 2.1 Schematic diagrams showing the cell culture lay out in 24 well plates. B=Blank, C=Control, D= Drug (arrow indicates increase in drug dose).

The cardiomyocytes with drugs were first analysed for contractile activity then subjected to cell viability and total protein content endpoints for toxicity estimation in comparison to control. The drugs were also evaluated for the production of free radicals as a potential mechanism to produce toxicity. At the molecular level the effect on cell communication was targeted by estimating connexin43 gap junction proteins using western blots and immunohistochemistry for protein localization in the cells.

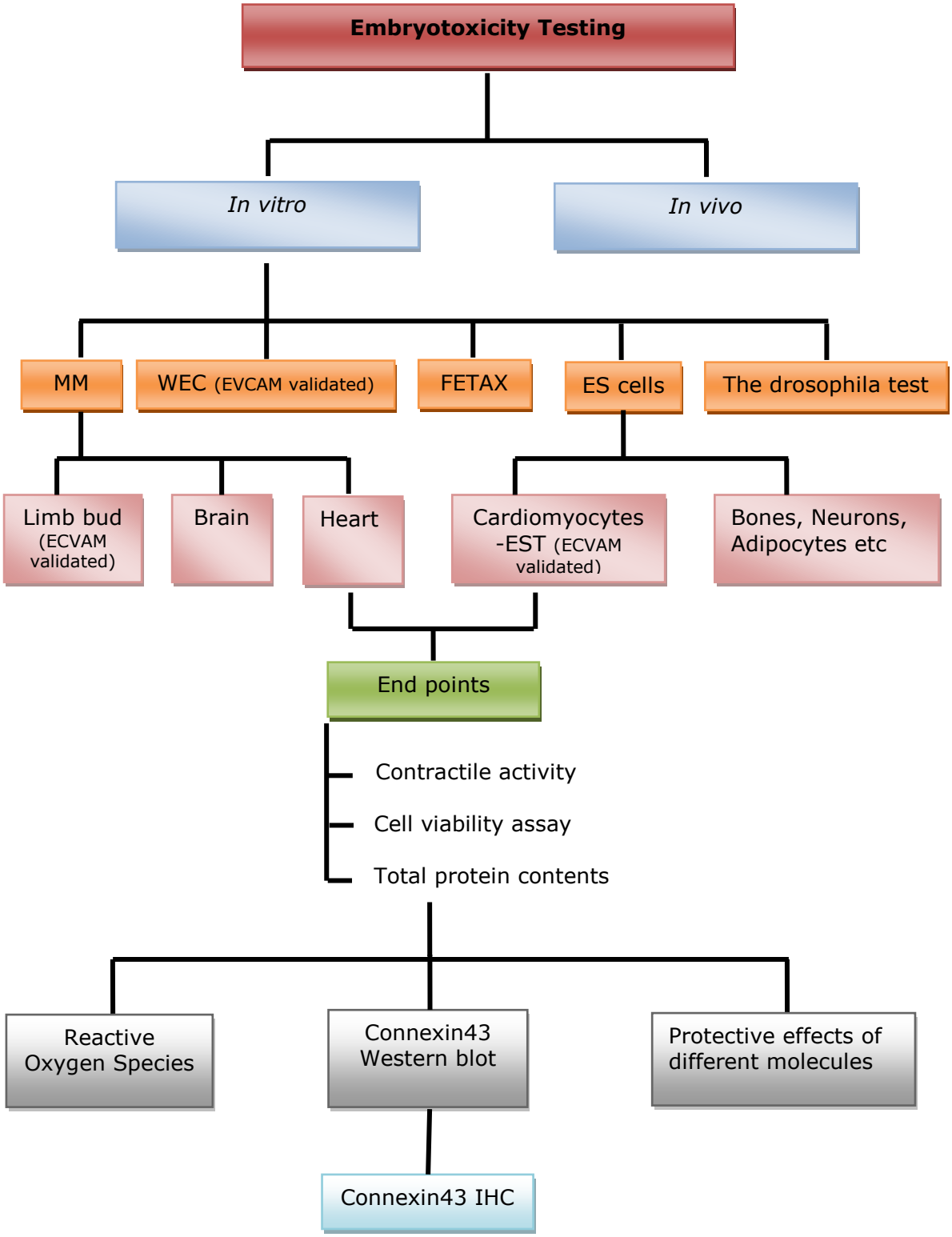


Fig 2.2 Schematic flow chart showing the design of the experiments for MM and ESDC systems. MM=micromass, WEC=whole embryo culture, ECVAM=European commission for the validation of alternative methods, FETAX=The Frog Embryo Teratogenesis Assay Xenopus, ES cells=embryonic stem cells, EST=embryonic stem cell test, IHC= Immunohistochemistry.

2.4 Methods

2.4.1 Micromass chick cardiomyocytes culture

2.4.1.1 Eggs storage and incubation

White fertile leghorn chicken eggs (labelled with the date of delivery) were stored in a cooled incubator at 12°C, during which embryonic development remains suspended (Slack, 2006). The eggs were used within two weeks of the day being laid. A minimum of 24 eggs were incubated by placing on automatic egg turner at 38°C, 100% relative humidity for five days. The day eggs were incubated was marked as day zero.

2.4.1.2 Cardiomyocytes isolation and culture

On the fifth day of eggs incubation (Hamburger and Hamilton chick development stage 26) (Hamburger & Hamilton, 1992), the class I Laminar flow hood was prepared by swabbing first with Trigene™ (Medichem international, UK), followed by sterilization with 70% ethanol. Curved and straight forceps were cleaned with 70% ethanol. Six eggs were removed at a time from the incubator, swabbed with 70% ethanol and transferred into the hood. Using the broader end of the curved forceps the egg shell (near the air sac region) was broken by gentle striking. The shell was peeled off and the vitelline membrane was gently removed to reveal the chorioallantoic membrane with blood vessels. The membrane was pierced; the embryo was lifted out and transferred to a 90mm Petri dish containing cold Hank's Balanced Salt Solution (HBBS). The embryo was killed by decapitation under the dissecting microscope; the hearts were removed and placed in 50% v/v horse serum/HBSS. When all the eggs were transplanted, the hearts were washed twice with HBSS to remove serum. 2ml (for 24 hearts) of warm trypsin 0.05%/EDTA 0.02% was added and incubated at 37^0C, 5% CO_2 for 20 min, with frequent shaking (after every 5 min). Trypsin action was neutralized by adding 8ml culture medium and the suspension was centrifuged at 1500rpm for 5 min. The supernatant was removed and pellets were resuspended in 1ml warm culture medium.

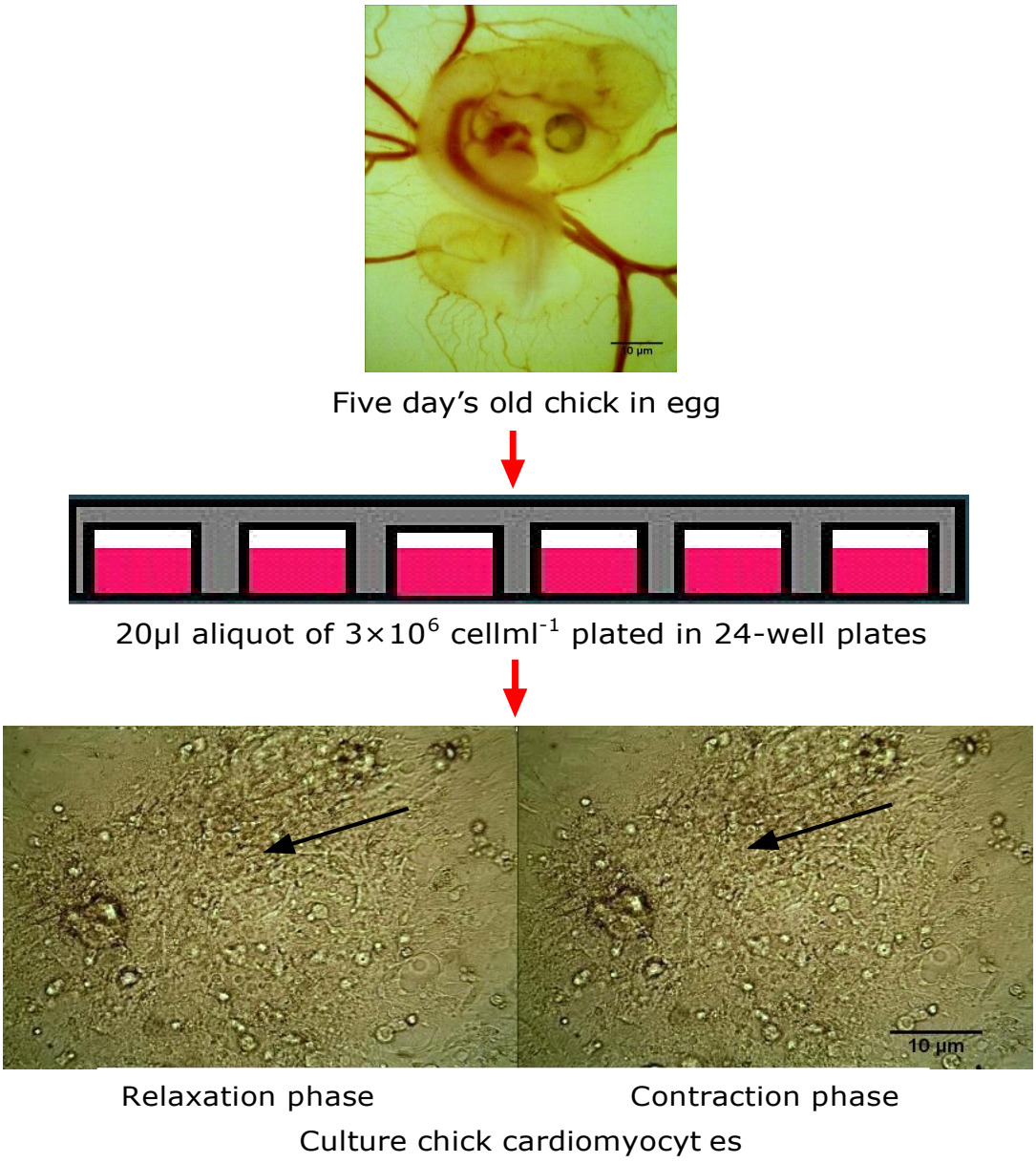


Fig 2.3 Diagram showing the micromass culture system. Cultured chick cardiomyocytes showing phases of contraction and relaxation. Arrows indicating the contracting foci's.

Using a haemocytometer to count the cells, the cell density was kept at 3×10^6 cells ml^{-1} (see appendix 5). A 20µl aliquot of cell suspension was pipetted at the centre of each well of the 24 well plates, and cells were allowed to attach to the surface in an incubator. After 2h, cells were flooded with 500µl of culture medium, and placed again in the incubator. The drugs (500µl) at double the required concentration in

medium were added after 24h. The contractile activity was recorded at 24h, 48h and 144h of cell seeding (see section 2.5.1).

2.4.2 D3 mouse embryonic stem cells culture and differentiation

2.4.2.1 Cryopreservation of D3 ES cells

D3 embryonic stem cells were cryopreserved using 10% DMSO as cryopreservation agent in a liquid nitrogen (British oxygen company, UK) Dewar. $1\text{-}5\times10^6$ cellsml^{-1} were cryopreserved using medium containing 50% of D3 ES culture medium with 40% FBS (see appendix 2, section 2.2) and 10% DMSO. The cryovials were stored in Mr Frosty™ at -80°C, for long term storage cryovials were transferred to a liquid nitrogen Dewar.

2.4.2.2 Resuscitation and routine culture of D3 ES cells

Cryovials removed from the liquid nitrogen Dewar were quickly thawed. The cryovials' contents were suspended in 10ml pre-warmed D3 ES culture medium and centrifuged at 900rpm for 5 min. The pellets were suspended in 5ml D3 ES culture medium (with LIF) in 25cm^2 tissue culture flasks (0.1% gelatin coated flasks were used for the first few passages) and kept in the incubator at 37°C with 5%CO_2. The culture medium was changed at every 24-48hr and the cells passaged once the flask was 70-80% confluent. Before performing cytotoxicity testing ES cells were passaged 2 to 3 times.

2.4.2.3 Subculture of D3 ES cells

Once the flask was 70-80% confluent with ES cells it was either passaged or differentiated. The culture medium in the flask was aspirated and the cells were washed twice using phosphate buffer saline (PBS) without calcium and magnesium. Cells were dissociated using trypsin 0.05%/EDTA 0.02% solution by placing in incubator for a few seconds. Trypsin action was neutralized by adding ES culture medium with FBS. The ES cell suspension was centrifuged (900rpm for 5 min) and pellets were suspended in pre warmed culture medium with LIF at a cell density of 5×10^4 cellsml^{-1} in a tissue culture flask.

2.4.2.4 D3 ES cells differentiation into cardiomyocytes

Day 0

A D3 ES cell suspension of 3.75×10^4 cellml^{-1} (without LIF) with experimental chemicals was prepared for the hanging drop method as described in 2.4.2.3. Using a multichannel pipette the 20µl aliquots were pipetted on the inner side of a 100mm square Petri dish lid. The lid was carefully inverted over the Petri dish containing 10ml PBS. The drops in the hanging position were incubated for the next 3 days in the incubator.

Day 3

5ml the D3 stem cell culture medium with appropriate concentration of the test chemicals was placed in a 90mm bacteriological Petri dish and this was used to harvest the embryoid bodies (EBs) by keeping the lid slightly tilted at a 45^o angle. The EBs were further incubated for 2 days at 37^0C in the incubator.

Day 5

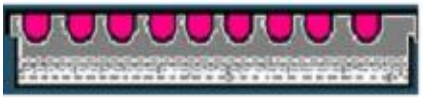
The same range of test chemicals was prepared as on day zero. 1ml culture medium with or without test chemicals was pipette into each well of the 24 well plates. The end portion of yellow pipette tip was cut with sterilized scissors and one EB was transferred to the centre of each well. The plates were labelled and kept in the incubator for another five days for proliferation, attachment and differentiation of ES cells into contracting cardiomyocytes.

Day 10

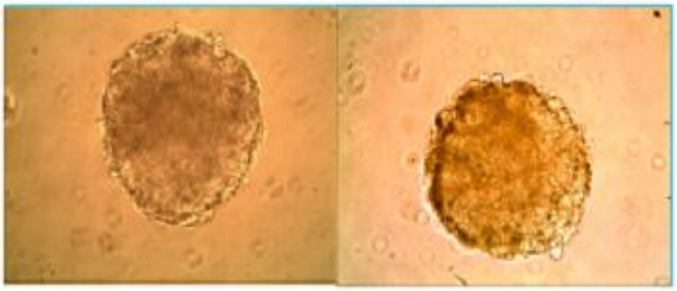
On the 10^{th} day EBs differentiated into cardiomyocytes were observed under the light microscope to determine their contractile activity. Day 10, 11 and 12 contractile activities of cardiomyocytes were recorded as described in section 2.5.1.

D3 ES cultured in T-25 Flask with LIF

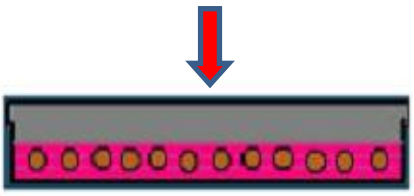
Undifferentiated mouse D3 ES cells



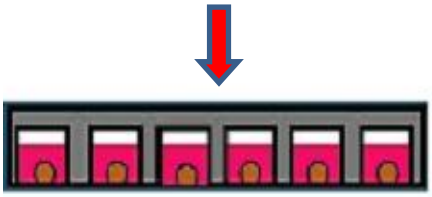
ESD3 in hanging drop without LIF



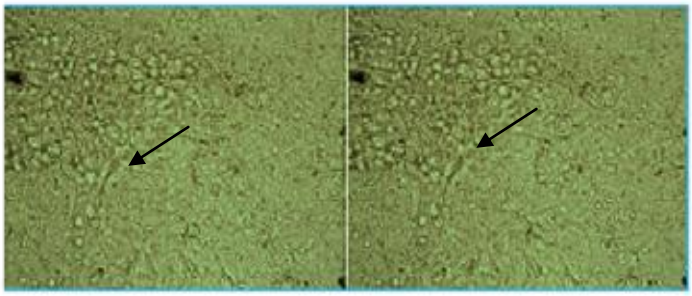
Three day old EBs Five day old EBs



EBs cultured in Petri dish



EBs plated in 24-well plates for differentiation



Relaxation Phase Contraction Phase

Fig 2.4 Schematic diagram of ES D3 differentiation into Cardiomyocytes. Arrows indicating the contracting foci (magnification x25).

2.5 End points

For the estimation drug produced toxic effects on cardiomyocytes, different end points were included in the study.

2.5.1 Contractile activity

The heart is the first mesodermal organ that starts to function (Sachinidis *et al.*, 2003), because a blood supply is necessary for the continuous supply of nutrients and oxygen to the developing tissues. Contractile activity is an intrinsic property of heart cells. Different cardiomyopathies affect the cardiomyocyte ability to show synchronized rhythmical phases of contraction. Morphogens may affect the ability of

differentiating cardiomyocytes to establish a contractile unit during development. Aberrant heart development and function indirectly affects the other organs development. For these reason cardiomyocyte contractile activity was chosen as an endpoint to evaluate the effects of different teratogens on differentiation and establishment of contractile unit in both the systems.

2.5.1.2 Cardiomyocytes contractile activity scoring

The cardiomyocytes contractile activity was manually recorded using inverted microscope. The contractile activity scoring system depends on the number of the foci contracting in a well and their pace of contraction. If all the foci were contracting a score 3 was given, which decreased with decreased number of foci contracting and became zero for no contraction.

Morphological Score	Contractile Activity of Cardiomyocytes
3	Entire well contracting
2	Numerous foci contracting
1	Few foci contracting
0	No contractile activity

Table 2.1 Morphological scoring system for cardiomyocytes contractile activity.

2.5.2 Resazurin Assay

Resazurin, a 7-Hydroxy-3H-phenoxazin-3-one 10-oxide redox dye (Bueno *et al.*, 2002), is used in a sensitive non-radioactive one step cell viability measurement assay. It is also called the Alamar blue assay (Nakayama *et al.*, 1997). It involves the reduction of resazurin to resorufin which is commonly used as a tool to identify cell proliferation and cytotoxic potential of chemicals (Anoopkumar-Dukie *et al.*, 2005). This assay is non-destructive to cells, allows the continuous proliferation of cells (Anoopkumar-Dukie *et al.*, 2005) and indicates the presence of viable and metabolically active cells that reduce the blue non-fluorescent resazurin dye to pink fluorescent resorufin intracellularly (Anoopkumar-Dukie *et al.*, 2005). Upon further

reduction it forms colourless dihydroxyresorufin (O'Brien *et al.*, 2000). This reduction can be quantified by measuring optical density (Nakayama *et al.*, 1997). This redox indicator conversion is facilitated by mitochondrial, microsomal and cytosolic oxidoreductases present within the cells (Anoopkumar-Dukie *et al.*, 2005). The Resazurin assay is very sensitive, only 80 cells can give a reading (O'Brien *et al.*, 2000).

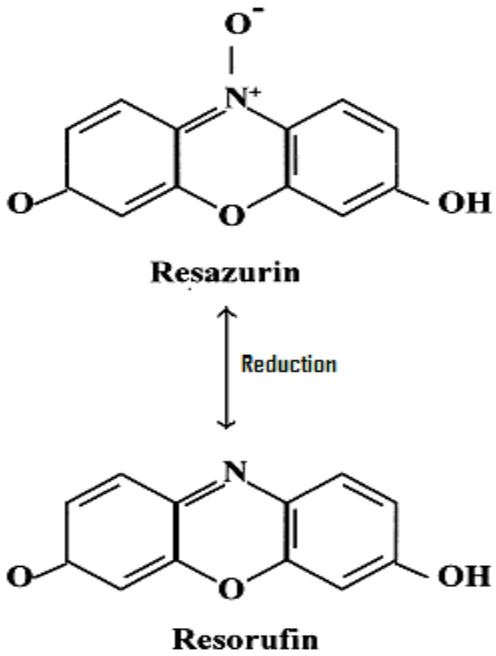


Fig 2.5 Resazurin reduction to Resorufin (O'Brien *et al.*, 2000).

2.5.2.1 Resazurin assay-Cell viability measurement

100µgml^{-1} of resazurin stock solution was prepared in HBSS (with calcium and magnesium), filtered, aliquoted, wrapped in aluminium foil and stored at -20^{o}C. On 6th day of micromass and 12th day of ESDC, the resazurin stock solution was diluted in HBSS to 10µgml^{-1}. The culture medium in each well was replaced with 500µl of pre-warmed resazurin solution; the cells were then incubated at 37^{o}C with 5% CO_2 for one hour. Using FLUORstar plate reader, the optical density was determined, with an excitation filter wavelength of 530±10nm and emission filter wavelength of 590±12.5nm, and a gain of 60. The numerical data indicates the amount of resorufin produced which in turn determines the cell activity. Once the optical activity was recorded the resazurin solution was replaced with 300µl of kenacid blue fixative and plates were incubated overnight at 4^{o}C before the kenacid blue assay.

2.5.2.2 Resazurin standard curve

The resazurin standard curve was performed for the interpretation of data. The optical density values obtained from the plate reader were converted to the amount of resorufin produced by plotting the resorufin standard curve. Resorufin solutions of different concentration 125, 250, 500, 1000, 2000ngml^{-1} in HBSS were prepared. A 500µl aliquot of each concentration was added into the wells of a 24-well plate, keeping the blank only with HBSS. The optical density was determined, with an excitation filter wavelength of 530±10nm and emission filter wavelength of 590±12.5, with a gain of 60. The results were plotted on a scatter graph and values were linked in order to get the best fit straight line.

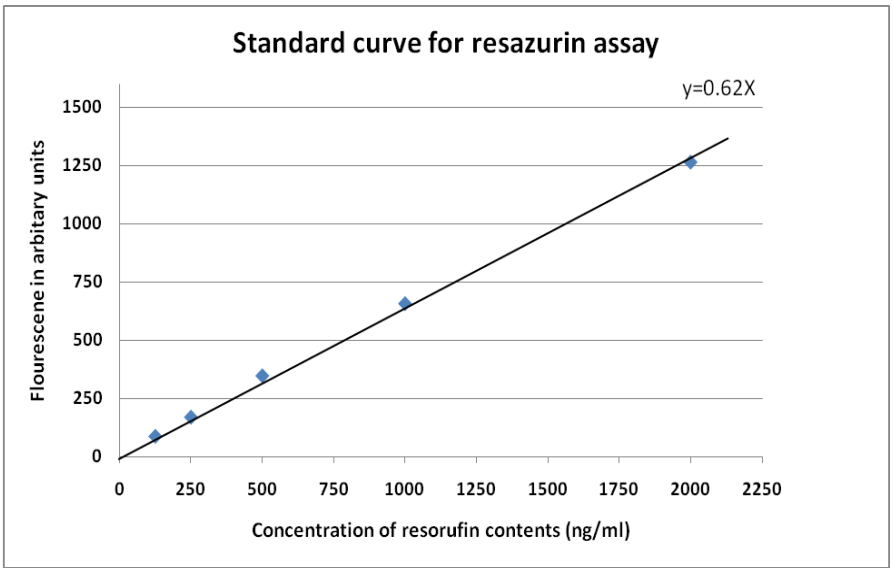


Fig 2.6 Resazurin reduction assay standard curves. For linear section; y = 0.62x where y is emission at 590±12.5nm and x is resorufin concentration (ngml^{-1}).

2.5.3 Kenacid Blue Assay

The protein content of cells can be used as a tool to determine their proliferation and differentiation and chemicals which are cytotoxic or alter the growth of cells decrease the production of protein (Memon & Pratten, 2009). The protein content of cells can be determined by using kenacid blue dye, which in turn indicates the cellular toxicity of chemicals (Clothier *et al.*, 2002). It acts by measuring the binding of dye by cellular proteins: the kenacid blue dye binds to the $-NH_3^+$ terminal of the amino acid irrespective of active or non-active protein (De St. Groth *et al.*, 1963).

The kenacid blue method gives a direct relationship between protein content, cell number and binding of the dye (Knox *et al.*, 1986).

2.5.3.1 Kenacid blue-Protein content measurement

After the resazurin assay the cells were fixed with 300µl of kenacid blue fixative (ethanol 500ml, distilled water 490ml and glacial acetic acid 10ml) overnight at 4^0C. Fresh kenacid blue working solution was prepared by adding 6ml glacial acetic acid in 44ml kenacid blue stock solution (kenacid blue dye 400mg, ethanol 250ml and distilled water 630ml). An aliquot of 500µl of kenacid blue working solution was added to each well and the plate kept on a plate shaker for 2h. During this time the dye reacts with the cell protein. Excess dye was removed by rinsing first then agitating with 500µl washing solution (acetic acid 50ml, ethanol 100ml and distilled water 850ml) for 20 min. Washing solution was replaced by 400µl kenacid blue desorb (ethanol 700ml, potassium acetate 98.1g and distilled water 300ml), which takes off the dye and cells from the surface, and the plate was agitated for 1 hour. Optical density was recorded using ASYS HITEC Expert 96 well plate reader with reading filter 570nm and reference filter 405nm.

2.5.3.2 Kenacid blue standard curve

In order to calculate the amount of protein, a kenacid blue standard curve using bovine serum albumin was plotted. For this 2.4mg BSA was dissolved in 3ml distilled water followed by addition of 7ml ethanol to produce 70% ethanol solution. 80µgml^{-1} albumin concentration is produced by dissolving 3ml albumin solution in 6ml 70% ethanol. This was then serially diluted to produce 80µgml^{-1}, 70µgml^{-1}, 60µgml^{-1}, 50µgml^{-1}, 40µgml^{-1}, 30µgml^{-1}, 20µgml^{-1} and 10µgml^{-1}. The plate was kept in refrigerator overnight, the ethanol was evaporated and the protein adhered to the surface. On the next day the plate was fixed with 300µl kenacid blue fixative overnight. The kenacid blue assay was performed as described in section 2.5.3.1. The results were plotted on a scatter graph and the best fit line was drawn to interpret the results.

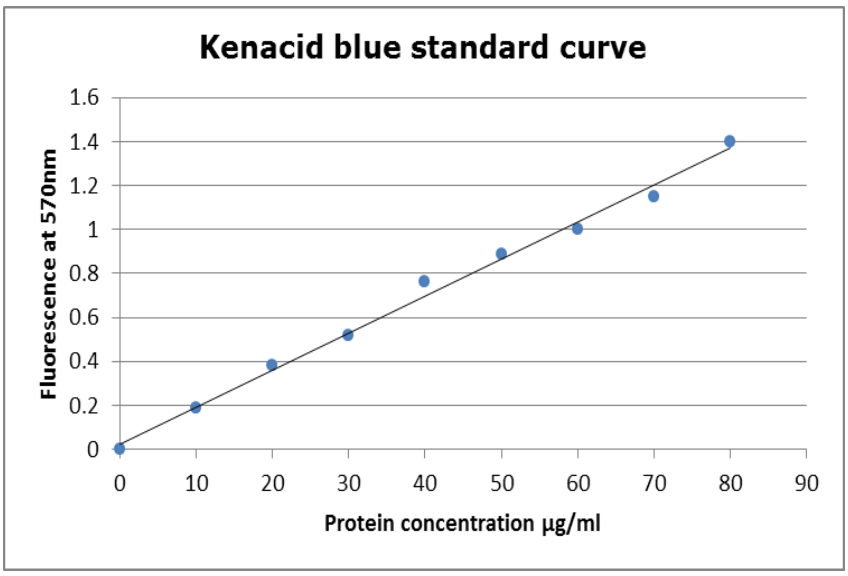


Fig 2.7 Kenacid blue assay standard curve. For linear section; y=0.016x where y is absorbance at 570nm and x is protein content (μgml^{-1}).

2.5.4 Reactive oxygen species assay

Reactive oxygen species (ROS) are produced as a by-product of aerobic metabolism. Aerobic organisms possess a passive defence mechanism against ROS and actively use ROS in signal transduction pathways for cell survival and proliferation regulation (Kobayashi & Suda, 2012). Reactive oxygen species (ROS) accumulation is coupled with an increase in oxidative stress, which has been implicated in the pathogenesis of several disease states (Guo *et al.*, 2010).

The assay employs the cell permeable fluorogenic probe 2', 7' dichlorofluorescein diacetate (DCFH-DA). The non-ionic non-polar DCFH-DA (non-fluorescent) passively enters the cells; the diacetate group is cleaved by cellular esterase. The reactive oxygen species (ROS) in the cell oxidise the DCFH to produce highly fluorescent dichlorofluorescein (DCF). The fluorescence measured is proportional to the ROS levels within the cell. The effects of antioxidants or free radical compounds on DCFH-DA can be measured against the fluorescence of the DCF standard. H_2O_2 was used as a positive control because it is the major oxygen species responsible for oxidative stress and has been implicated as a cellular toxin (Lee *et al.*, 2008).

DCF-DA esterase cleavage results in charged molecule productions which are better retained by cells than is the parent compound. In earlier studies it was observed that some cells do not retain the dye and it was leaked out of the cells. To counteract this

problem the same approach was applied as by Garle *et al.,* in 2000. The DCF formation was measured with the continuous presence of DCFH-DA in the medium. This represent the total DCF produced in intracellular and extracellular spaces. Culture medium readily oxidises DCFH-DA to DCF but this is slow in the case of HBSS (Garle *et al.*, 2000).

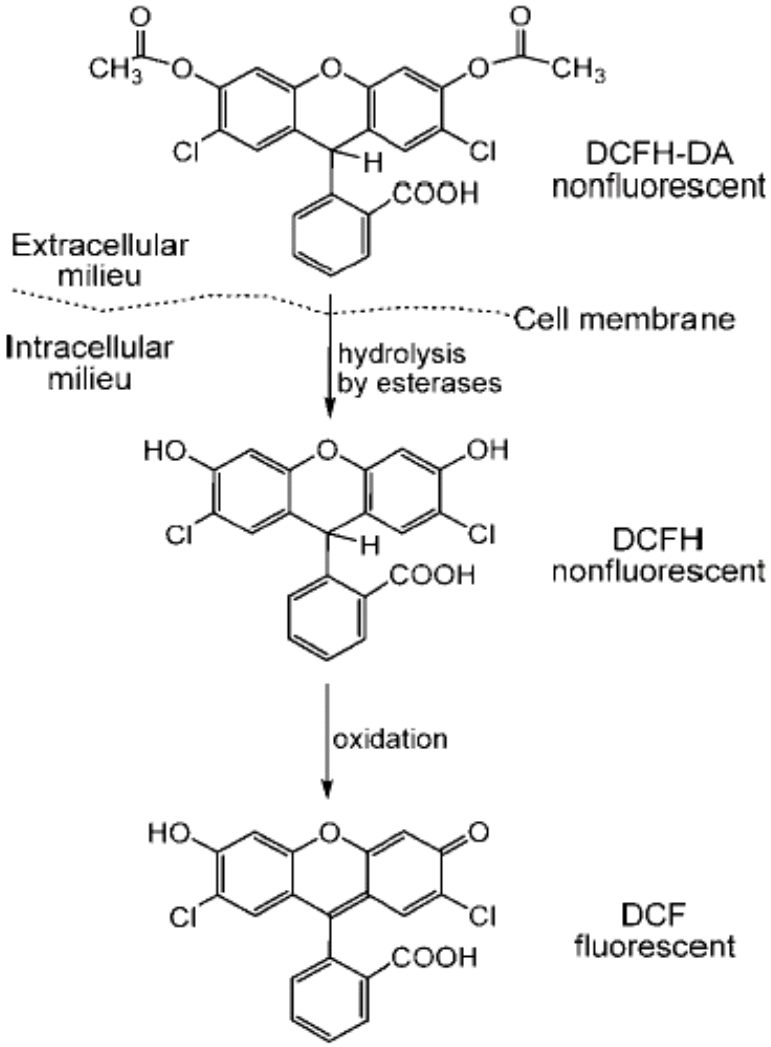


Fig 2.8 Uptake of non-fluorescent 2',7' dichlorofluorescein diacetate (DCF-DA) into the cell followed by removal of acetate groups and oxidation to form fluorescent DCF (Shen *et al.*, 2006).

2.5.4.1 Reactive oxygen species measurement

For reactive oxygen species (ROS) analysis the cells were grown in black walled clear bottom 96-well plates. For micromass (6000cellswell^{-1}) the assay was performed after 24h of cell seeding, while in ES ESCD five days old EBs (3 EBswell^{-1}) were plated with dye and drug. 4mM fresh stock solution of 2',7'-dicholorofluorescin diacetate (DCFH-DA) dye was prepared by dissolving in methanol. This was further diluted to 200 folds in HBSS to give 20μM final concentration. The cells were washed with HBSS twice. 100μl of drug solution prepared in HBSS along with dye was added to each well of 96 wells plate keeping the blank without cells. The fluorescence was recorded using excitation filter 485nm and emission filter 520nm at time points 0h, 1h, 2h and 4h. The conversion of DCFH-DA to DCF (Dicholorofluorescein) was determined by reference to a DCF standard (5 μM). The fluorescence of DCF was linear over the concentration of 0.3-5μM.

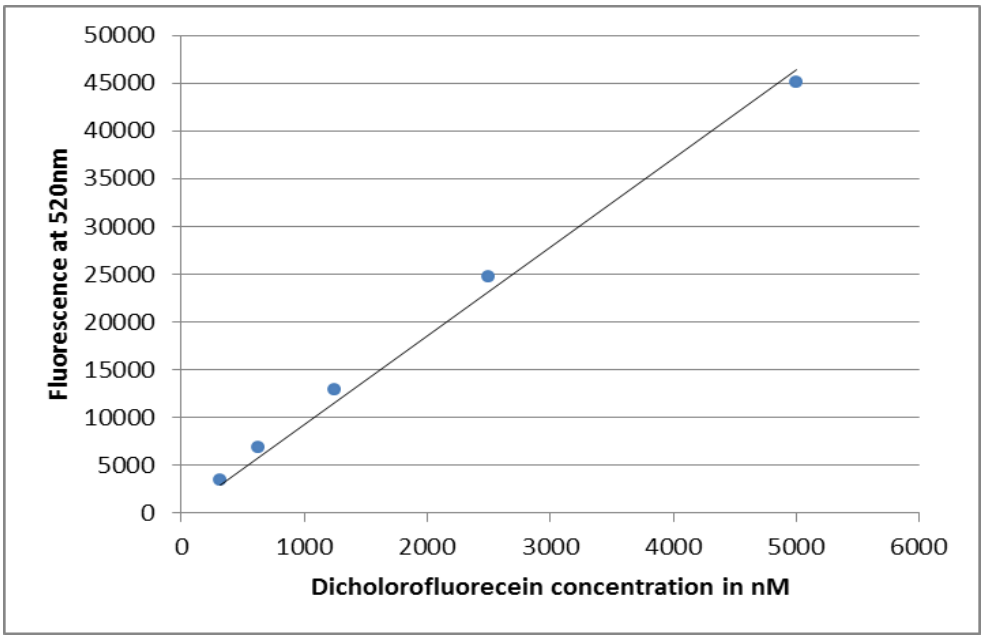


Fig 2.9 DCF standard curve showing the linear relation with increase in dose.

2.6 Preparation of Drug Solutions

The drug stock solutions were prepared either by dissolving directly in the culture medium or by dissolving first in DMSO, methanol or β-Hydroxypropyl cyclodextrin then adding culture medium depending on drug's solubility. The appropriate concentrations of drugs required for the experiment were prepared by diluting the stock solutions. The final concentration of solvents in Controls and Blanks were kept constant.

Drug	Solvent
Ascorbic acid	Culture medium
Bupropion HCl	Culture medium
Carbamazepine	0.16% DMSO
Folic acid	Culture medium
Hyperforin	0.16% Methanol
Hypericin (Calbiochem, UK)	0.008% DMSO
Lithium carbonate	Culture medium
Myo-inositol	Culture medium
Phenytoin	45% w/v 2–Hydroypropyl-β-Cyclodextrin
Primidone	0.10% DMSO
Sodium valproate	Culture medium
Super oxide dismutase (SOD)	Culture medium

2.7 Immunohistochemistry

2.7.1 Connexin43 Immunohistochemistry

On the 6th day of Micromass and 12th day of ESDC cultured cardiomyocytes in 8 well chamber slides were washed twice with PBS at room temperature (RT). The connexin43 (Cx43) was stained following the method as described previously by Le et al (Le & Musil, 1998). In brief the cells were fixed with 2% PFA [w/v] solution (diluted from 4% PFA stock) for 30 min at RT followed by 30 min rinse with PBS at RT. For Cx43 staining cell were post fixed with -20^{o}C acetone for 5 min followed by 30 min PBS rinse. Cardiomyocytes were permeabilized for 30 min with PBS containing 0.1% Triton X100, 0.2% bovine serum albumin, and 5% normal goat serum (PTBN). Cells were incubated with Cx43 antibody diluted in PTBN (1:500) overnight at 4^{o}C. Three PTBN rinses of 10 min each were given before incubating the cardiomyocytes with secondary antibody (1:1000) diluted in PTBN. This is followed by secondary antibody washing; slides were then mounted with Vectashield containing DAPI stain (Vector laboratories, UK) and viewed under confocal microscope.

2.7.2 SOX2, MHC and Troponin Immunohistochemistry

For SOX2 and Myosin heavy chain (MHC) immunohistochemistry the cells were fixed with 4% PFA for 20 min followed by two PBS washes. The cells were then permeabilized with 0.3% triton X-100 for 1h followed by 1h block with 5% NGS solution. Cardiomyocytes were incubated over night with primary antibodies diluted in 5% NGS. Three 5 min and three 15 min washes were given with 0.1% BSA solution in PBS. The cells were then incubated with secondary antibody diluted in NGS for 1h in dark followed by washing with BSA solution. The slides were mounted with Vectashield containing DAPI stain and observed under fluorescence microscope.

2.8. Western blot

2.8.1 Preparing cell lysate

The embryonic chick cardiomyocytes (see section 2.4.1) and D3 ES cells derived cardiomyocytes (see section 2.4.2) were grown and treated in 24-well plates. The cells were washed twice with ice cold PBS on ice. Freshly prepared lysis buffer with protease inhibitor tablet was mixed with 1X solubilisation buffer 1:1; 100µl of this solution was added to each well and pipetted up and down thoroughly to take off all the cells. The contents of each well was transferred to a separate labelled Eppendorf tube, heated at 95^{o}C for 5 min, vortexed, centrifuged and stored at -20^{o}C for future use.

2.8.2 Preparation of tissue lysate (Lowry test)

For normalizing the protein contents, tissues (100mgml^{-1}) were dissolved in lysis buffer with protease inhibitor tablet. BSA standard curve was prepared using 1mgml^{-1} BSA. 1ml Lowry solution A+B 1:1 was added to all samples and standards. After 10 min incubation with Lowry solutions 100µl of Folin reagent in water 1:1 was added and incubated at RT for 45 min. 200µl of each sample and BSA standards were pipetted in triplicate in 96 well plates (Fig 2. 10). The plate was read at 750nm. The samples were normalized to 1mgml^{-1} by adding lysis buffer with Solubilisation buffer.

B1	→	→	S8	→	→	T8	→	→			
S1	→	→	T1	→	→	T9	→	→			
S2	→	→	T2	→	→						
S3	→	→	T3	→	→						
S4	→	→	T4	→	→						
S5	→	→	T5	→	→						
S6	→	→	T6	→	→						
S7	→	→	T7	→	→						

Fig 2.10 Showing triplet arrangement of BSA standard and samples in 96 well plate (B=blank, S=standard, T=tissue sample).

2.8.3 Western blotting

For Western blot analysis of protein, proteins were first denatured by heating the Eppendorf tube at 95^{o}C for 5 min, followed by vortex and centrifuged at 13000rpm for 1 min. 15µl each sample was placed in a well of precast gel including 1µl molecular weight marker (Lonza, UK) in the first well. The gel was run in 1× electrophoresis buffer at 200V for 45 min. The gel was then transferred onto a nitrocellulose membrane and run again in cold transfer buffer for 90 min at 100V. Once the protein transfer was completed, the nitrocellulose membrane was collected, few drops of Ponceau solution was placed on the blot, to confirm protein transfer. The appropriate section on the blot was cut and a few quick washes were given to remove the Ponceau stains. 3% fish skin gelatine (FSG) solution in TBST was used as blocking reagent for 1h on the plate shaker. The appropriate dilutions of the primary antibody in 3% FSG solution were prepared and sealed in a bag with the blot for overnight incubation at 4^{o}C on the plate shaker. On the next day non-specific primary antibody binding was removed by giving 3X quick washes, 3X 5 min and 3X 10 min with TBST. The blot was incubated for one hour at 37^{o}C on the plate shaker with the appropriate secondary antibody dilution in 3% FSG solution. After secondary antibody incubation the blot was washed again with TBST and scanned using an Odyssey scanner at resolution of 84µm. For Cx43 and phospho-Cx43, GAPDH was performed afterwards. Blot was incubated for 1h with GAPDH 1:10000, followed by washing, secondary antibody incubation for 1h and again washing with TBST. The blot was scanned and analysed using Odyssey3 software.

2.9 Statistical analysis

For MM and D3 ES derived cardiomyocytes three independent repeats were performed for each experiment. The data was analysed using Prism 5 statistics software. For Parametric tests (Resazurin assay and Kenacid blue assay) one-way ANOVA followed by Dunnett's post hoc test was performed. For Contractile Activity scoring (non-parametric data) Kruskal-Wallis test with Dunn's post hoc test was used to determine significant values. $P<0.05$ was considered as significant difference from control.

Chapter 3

Evaluation of chick cardiomyocyte micromass and embryonic stem cell derived cardiomyocyte systems

3.1 Introduction

For any scientific study it is necessary to thoroughly investigate the experimental system. The experimental design, working conditions, materials, methods and the experimental models are normally optimized prior to use, to have a more effective, efficient and reproducible outcome. The MM and ESDC systems used in this study were examined for different experimental conditions as a part of a fuller evaluation. The chick cardiomyocytes micromass system is relatively new to the world of *in vitro* micromass teratogen insult estimation, compared to the limb bud micromass, which is ECVAM validated (Spielmann *et al.*, 2004). In the MM system the exact stage of heart explantation, cell seeding density, identification of cardiomyocytes and the known effect of the previously tested drugs were evaluated and compared. The chick as a model was chosen because of its easy handling compared to rat or mice and chick limb bud results are comparable to rat micromass (Hurst *et al.*, 2009; L'Huillier *et al.*, 2002).

For the D3 mouse embryonic stem cell differentiation into contracting cardiomyocytes, no alteration or improvisations were made to the ECVAM validated protocol of ES differentiation (Paquette *et al.*, 2008; Scholz *et al.*, 1999). Some experiments with known teratogens and non-teratogens were performed as a part of the study to confirm the reliability and reproducibility of the system in our laboratory. The differentiated contracting cardiomyocytes were tagged using cardiac protein markers and subjected to H&E staining for cellular morphology.

3.2 Development of the chick

The oviparous development of the chick closely resembles the mammalian viviparous system, and distinguishes it from lower vertebrates, but at the molecular level it has the same basic processes as in all vertebrates (Slack, 2006). The development of the chick is divided into three phases: blastoderm formation inside the oviduct lasts for one day, embryogenesis inside the egg shell continues for three days and then growth for remaining 18 days (Perry, 1988).

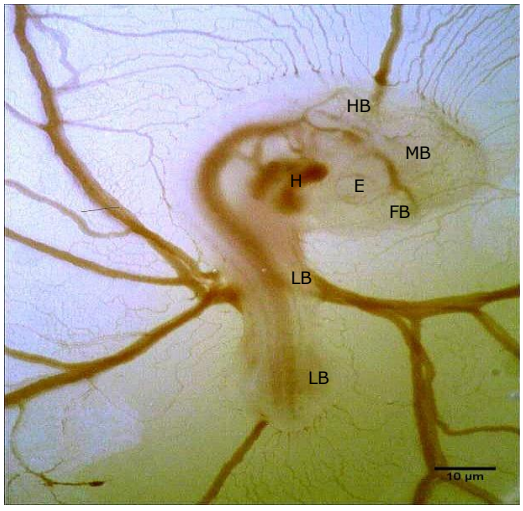
3.2.1 Development before egg laying

After fertilization in the infundibulum, the eggs pass downward to the oviduct where they are first covered with albumin then shell membrane followed by shell (Bellairs & Osmond, 1998). The fertilized egg during this process undergoes cleavage which is highly meroblastic, and produces a circular blastoderm. When the blastoderm divides into a few hundred cells, a subgerminal cavity appears beneath it. Now the central translucent region of the blastoderm is called the area pellucida, the outer region is called the area opaca and the junction is called marginal zone. At this time primary and secondary hypoblast develops in the subgerminal cavity and Koller's sickle, a thickening of cells, appears in posterior marginal zone. At the time of laying the blastoderm consists of approximately 60,000 cells (Slack, 2006). After laying, when the appropriate developmental conditions are provided, development restarts (Bellairs & Osmond, 1998).

3.2.2 Development after egg laying

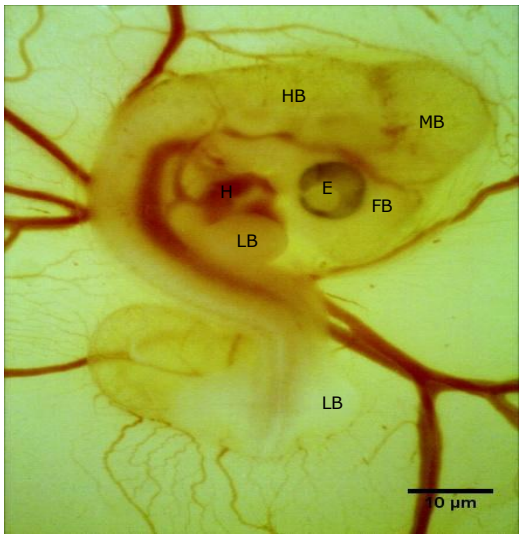
The gastrulation period is initiated by cells in the Koller's sickle condensing to form the primitive streak (Bellairs & Osmond, 1998). The primitive streak elongates both anteriorly and posteriorly into the area opaca. Epiblast cells migrated through the primitive streak to become mesoderm and the definitive endoderm part of the lower layer. Now the area pellucida changes to a pear shape and further condensation at the anterior end of primitive streak forms Hensen's node. Cells are present in Hensen's node, some of which migrate anteriorly to form the head process or posteriorly to form the principal structures of the body consisting of notochord in the middle, somite on both sides and neural plate on the anterior edge of the area pellucida (Slack, 2006). After 48h the blood islands start to appear and link to form the vascular system. The vascular system joins the heart which begins to beat. Two distinct vascular systems are essential for chick development, the vitelline system, which transports nutrients from the yolk to embryo; the other is the allantoic system, which is involved in respiration and transport of waste material from the embryo to the allantois. By the end of the 3rd day the beak begins to grow and fore and hind limb buds are visible and the embryo acquires a C shape. The embryo

continues to grow. The incubation period is normally 21 days for the chick after which it hatches (Bellairs & Osmond, 1998).



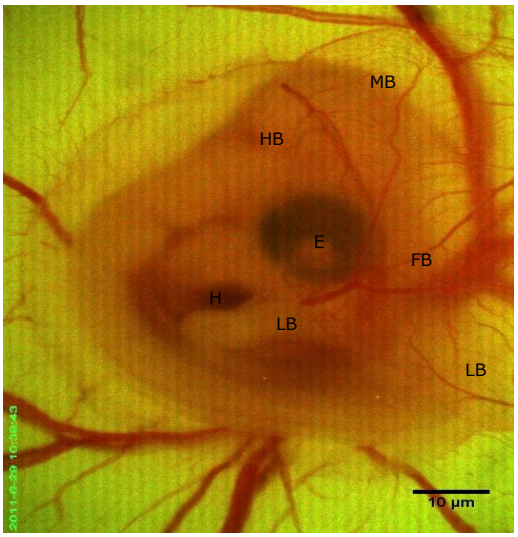
A) Day 4 of incubation (HH 24)

- Longer limb buds than wide.
- Longer somites than wide.
- No demarcation of digital plate.
- Toe plate in leg but although distinct toes are not demarcated.



B) Day 5 of incubation (HH 26)

- Limb buds are considerably longer and contour of digital plate is round.
- There is faint groove between the second and third digits.
- Beak is barely prominent.
- Demarcation of first three toes is distinct.



C) Day 6 of incubation (HH 29)

- Wings are bent at elbow.
- Second digit is larger than the other digits.
- Grooves between digits are prominent.
- Beak is more prominent.
- There is no egg tooth visible at this stage.

Fig 3.1 Development of chick on day 4, 5 and 6 of incubation. FB=fore brain, MB=mid brain, HD=hind brain, E=eye, H= heart and LB=limb bud.

3.2.3 Development of the Heart

The developing heart is a highly modified muscular vessel (Harvey, 2002) and the first functional organ, required to support development (Lyons, 1996). According to the Hamburger and Hamilton (H&H) stages of chick development, the heart begins to develop at about stage 9-10 (Bellairs & Osmond, 1998), but the cardiac progenitor cells were identified even before gastrulation (Martinsen, 2005). The mesodermal plate on both side of the neural plate splits into upper somatic and lower splanchic layers. The space between these layers (which will form the future coelomic cavity) provides amniocardiac vesicles for the formation of amnion and the precardial cavity (Bellairs & Osmond, 1998).

The splanchnic mesoderm on both side of the neural tube folds and joins to form the foregut and the precardiac cells from both sides move to the midline. The developing foregut loses contact with yolk sac and the endodermal tube and precardiac cells join to form a single tube below the developing V-shaped pharynx. In the tube the thin inner endocardium and outer thick epimyocardium are visible, which later on become the three layered endocardium, myocardium and epicardium (Bellairs & Osmond, 1998).

Heart looping begins when the right ventricular wall bulges outward and becomes convex, while the left becomes concave; the biophysical mechanism that controls the looping is not well understood (Bellairs & Osmond, 1998; Taber, 2006). The developing heart loops first into a C shape then into an S shape. This process of looping continues from stage 9-34 (Martinsen, 2005).

Initially the heart divides into the bulboventricle, atrium and sinus venosus, which later on, by the development of the bulboventricular and atrioventricular grooves divides into right and left channels. By day 4 the atrium and ventricle divide into left and right and bands of muscle begin to form (Bellairs & Osmond, 1998). The first action potential in the cardiac mesoderm appears in the ventricle by stage 9 even before the atrium and sinus venosus are formed. Contractility can be seen at stage 11 and contraction waves spread through the muscles and cause muscle contraction by stage 12 and at this stage blood starts to circulate. By day 3 circulatory systems,

vitelline, allantoic and intraembryonic, are well developed. The AV node and AV bundles develop by day 5.5-6 of incubation. During embryonic development the size of the heart increases 1000 fold (Bellairs & Osmond, 1998).

3.3 Parallel between cardiogenesis and ESCs derived cardiomyocytes

The process of cardiogenesis is very complicated. The signalling molecules and various transcription factors play an important role to drive undifferentiated stem cells towards the cardiac lineage (Sachinidis *et al.*, 2003). Embryonic stem cell differentiation into cardiomyocytes closely resembles the *in vivo* process of cardiogenesis (Wei *et al.*, 2005). The formation of ES cell aggregates resembles the early post implantation embryo, which initiates signalling and spontaneous differentiation into three germ layers (Sachinidis *et al.*, 2003).

The developing embryonic mesodermal lineage gives rise to cardiomyocytes (Sachinidis *et al.*, 2003) and this is true for the D3 mouse ES derived cardiomyocytes. The differentiating ES cells express the mesoderm lineage commitment factor brachyury T, which is necessary for mesoderm establishment in vertebrates (Mauritz *et al.*, 2008). NKx2.5 is one the earliest markers expressed during cardiac differentiation. In ESDC, Nkx2.5 is also expressed early around day 3 of ES differentiation (Rohwedel *et al.*, 2001; Rolletschek *et al.*, 2004). Inhibition of Nkx2.5 expression results in incomplete cardiac gene expression or absence of heart formation (Jamali *et al.*, 2001). The differentiating ES cells, like *in vivo* cardiomyocytes, express cardiac specific transcription factors and structural proteins like NKx2.5, GATA-4, MLC-2a, MLC-2v, ANF, MHC and troponin (Murabe *et al.*, 2007a; Murabe *et al.*, 2007b; Rolletschek *et al.*, 2004; Seiler *et al.*, 2004). This suggests the ESDC follows the same programme as its *in vivo* counterpart.

The signalling molecules like BMP, FGF and insulin like growth factors play an important role in cardiac differentiation (Sachinidis *et al.*, 2003), while noggin and Wnt over expression inhibits cardiac differentiation (Sachinidis *et al.*, 2003; Schultheiss *et al.*, 1997). Beside these differentiated cardiomyocytes show evidence of A and I bands (Boheler *et al.*, 2002) and represent atrial, ventricular and pace maker like cells with specific ion channels and gap junctions (Rohwedel *et al.*, 2001;

Rolletschek *et al.*, 2004; Wobus *et al.*, 1997). There are some molecules like DMSO, retinoic acid and ascorbic acid which promote ES cell differentiation into cardiomyocytes (Heng *et al.*, 2004; Sachinidis *et al.*, 2003; Takahashi *et al.*, 2003; Wobus *et al.*, 1997).

3.4 Effects of chick heart development stages on micromass culture system

The micromass system was designed to study the cytotoxic effects of teratogens at the cellular level. It includes primary culture of different embryonic tissue like brain, limb bud (ECVAM validated method) or cardiomyocytes (Memon & Pratten, 2009; Reinhardt, 1993; Spielmann *et al.*, 2004). Chick development stages and development speed varies a lot from other experimental animals like mouse or rat. The appropriate chick developmental stage for cell culture is important for accurate evaluation of embryotoxic effects of chemicals. If the isolated cell are not mature enough they cannot accurately mimic *in vivo* organogenesis, or if cells are isolated at an early stage they may not cope with the experimental conditions and may not provide the required number of cells to start the experiment.

3.4.1 Materials and Methods

For the identification of exact development day for micromass culture the same method of cell culture was followed as described by Memon S (Memon & Pratten, 2009). In brief the eggs were incubated for 4, 5 and 6 days at 38^{o}C, under humid conditions. 20µl of isolated heart cells suspension (see section 2.4.1) was alliquoted at the centre of the 24 well plates with one row for blank. The cells were seeded at 3×10^{6} cellml^{-1} density as described previously by Hurst (Hurst *et al.*, 2009). The seeded cells were flooded with medium after 2h. The contractile activity (see section 2.5.1) of the cardiomyoyctes was recorded at 24h, 48h and 144h of seeding, followed by the Resazurin cell viability (see section 2.5.2) and kenacid assay (see section 2.5.3).

3.4.2 Results

The chick developmental day 5 and 6 seeded cardiomyocytes behaved very differently compared to day 4 at all the endpoints evaluated. The cardiomyocytes'

contractile activity was comparable between day 5 and 6, while with day 4, decreased number of foci formation and contractile activity was recorded (fig 3.2A). The day 6 cultured cells mostly formed a flat sheet and the foci were indistinguishable compared to day 5, where number of foci and the contractile activity was maximum at all the days evaluated (fig 3.2A). The cell viability (fig 3.2B) and the protein content (fig 3.2C) of day 5 and 6 were comparable, while for day 4 cells, both end points drop down to significantly different values ($P<0.05$). The day 4 cells were poorly attached to the surface and frequently came off the surface during the kenacid blue tapping and the medium replacements. The day 6 cell were attached well, not detaching during medium replacement, but the cells showed poor attachment during the kenacid blue assay compared to day 5, which were not detaching during medium replacment or in the kenacid blue assay.

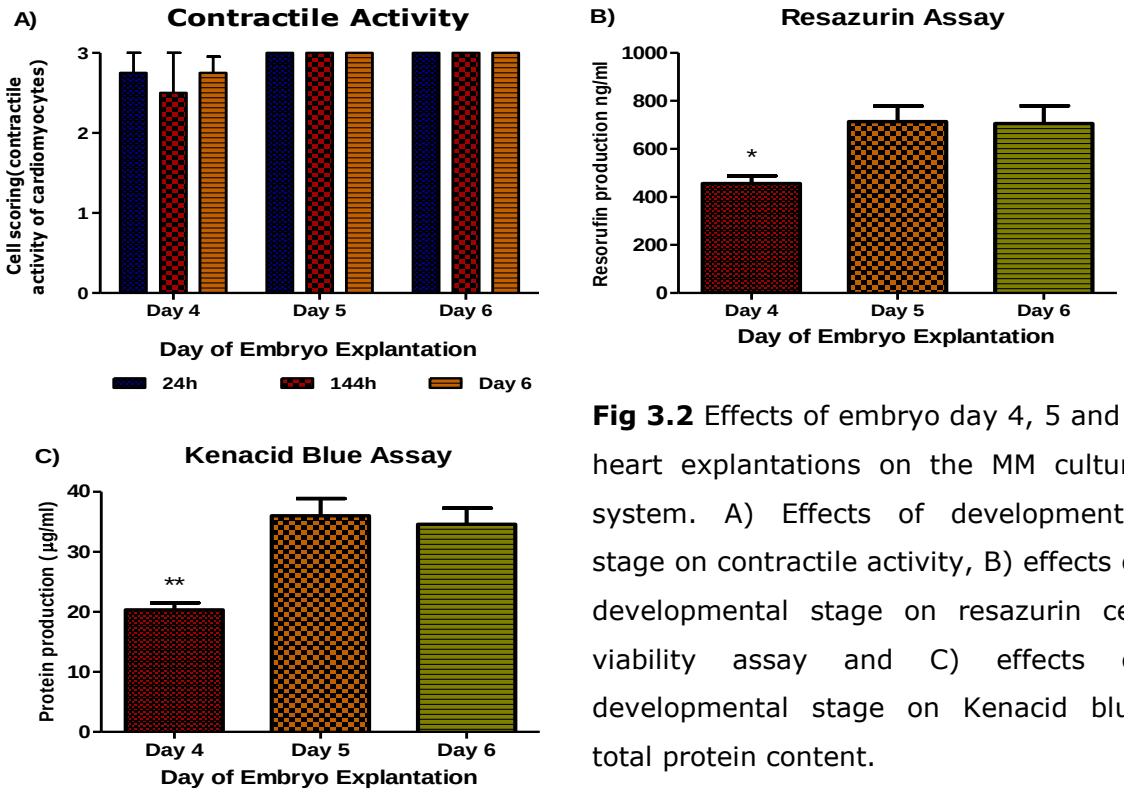


Fig 3.2 Effects of embryo day 4, 5 and 6 heart explantations on the MM culture system. A) Effects of developmental stage on contractile activity, B) effects of developmental stage on resazurin cell viability assay and C) effects of developmental stage on Kenacid blue total protein content.

3.4.3 Conclusion

During the evaluation of the optimum chick developmental day for micromass culture it was observed that day 5 chick cardiomyocytes successfully attached to the surface compared to day 4 which showed poor attachment. This might be because

cardiomyocytes were not sufficiently developed for culture, while day 6 were too differentiated for the micromass system. The day 4 cultured cardiomyocytes also showed decreased cellular viability compared to other two days with the same cell number, while protein content also decreased mainly because of low attachments.

3.5 Determination of cardiomyocytes optimal seeding density in micromass system

For optimal culture conditions, the cell seeding density is a very critical step to determine. The appropriate number of seeding cells is important for proper cell communication, which in turn forms the beating foci in the case of cardiomyocyte micromass. The cell density was evaluated using the same end points of contractile activity, cell viability and total protein content.

3.5.1 Materials and methods

Cells derived from day 5 chick hearts were used for the cell density determination. 20µl cardiomyocytes suspensions ranging from 0.5×10^6 to 5×10^6 cells were aliquoted at the centre of 24 well plates keeping the blank empty. The cells were incubated at 37^{o}C and 5% CO_2 for 2h for the cell attachment followed by flooding with more medium for cell growth and differentiation (see section 2.4.1). The contractile activity was observed (see section 2.5.1) followed by the resazurin cell viability assay (see section 2.5.2) and total protein estimation using the Kenacid blue assay (section 2.5.3) on day 6 of MM.

3.5.2 Results

The cell seeding density in the micromass system showed increased resorufin production as the cell number increases. 0.5 million cellsml^{-1} converted the resazurin to nearly 50ngml^{-1} of resorufin, which increased with cell number. The 3 million cellsml^{-1} increased conversion about 5 fold compared to 0.5 million cells. The 5 million cellsml^{-1} showed greater conversion compared to 3 million (fig 3.3A). The protein content estimation using Kenacid blue assay also followed a similar pattern of increased protein with increase in cell number from 0.5-5 million cellsml^{-1} (fig 3.3B). The contractile activity showed different effects at different cell seeding

densities. The 0.5 million cellsml^{-1} did not form beating foci and the cells were apart from each other. Similar results were observed when 1 million cellsml^{-1} was seeded. The spaces between the cells were reduced but still the cells were unable to form beating foci. 2 million cellsml^{-1} showed different results with the cells forming desynchronised beating foci but the numbers of beating foci were few and the spaces between the foci were very large. The 3 million cells ml^{-1} was found to form a very good number of foci with less space between. The contractile activity was synchronised and better than low density wells. The beating continued to be of same magnitude for up to six days of culture. The 5 million cellsml^{-1} instead of forming separate foci formed flat synchronised contractile sheets.

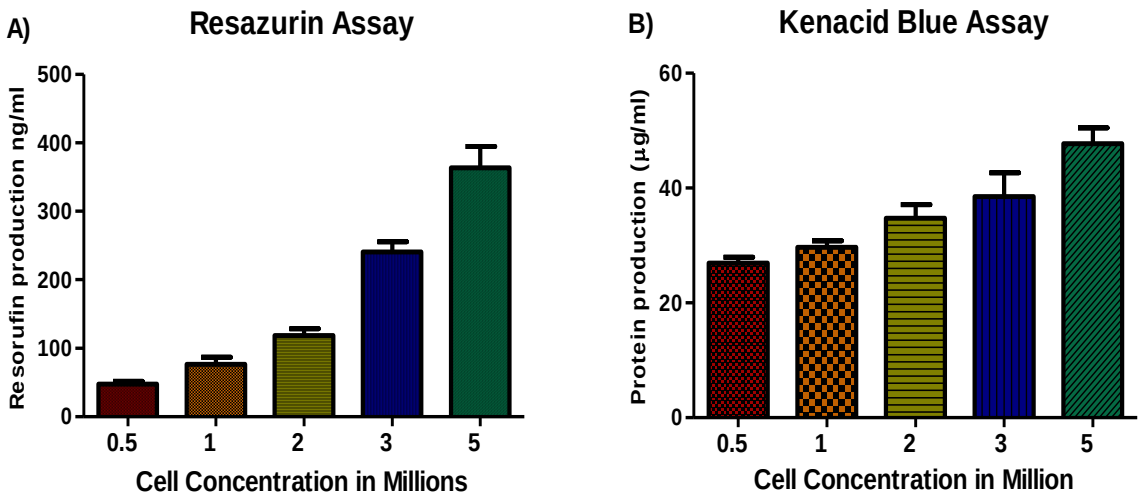


Fig 3.3 Effects of different cell seeding density in MM system. A) Resazurin cell viability assay B) Kenacid blue assay for total protein content.

3.5.3 Conclusion

The end points of cell viability and total cellular protein content were found to be directly proportional to cell seeding density. But the morphological examination of seeded cells at different time intervals revealed the appropriate cell seeding density is important for the contractile system to become established. The communication and the signalling between the cells and the distance between the seeded cells were found to be important to produce a synchronised contractile unit. Cell density below 2 million cellsml^{-1} failed to form the contractile system while 2 million cellsml^{-1} established it, but due to large spaces between the cells the beating foci were not

synchronised. The 3 million cells showed improved values by forming synchronised contractile activity. The communication between cells was definitely improved compared 2 million cellsml^{-1}. The last cell density (5 million cellsml^{-1}) tested was able to make a synchronised contractile unit with indistinguishable foci which made contractile activity scoring difficult since it depended on the number of foci beating.

3.6 Identification and characterization of cells in the MM and ESDC system

In the micromass system the cardiomyocytes were isolated from five day old chick hearts. The isolation of hearts was performed under the dissecting microscope using fine forceps. But there always remains a doubt about the isolation of some other tissues, especially fibroblasts and endothelial cells attached with the heart. The cardiomyocyte primary culture when seeded at a high density exhibits the phases of contraction and relaxation, which is their intrinsic property. But there remains a need to prove the tissue type present by tagging cardiac-specific proteins or genes. In the case of stem cells the differentiation to cardiomyocytes remains to be identified using markers to render any tissue specific effects more reliable. To further remove the doubt about the cells isolated and produced during cell proliferation different cardiac specific markers and histological techniques were employed which may provide the answer to these questions.

3.6.1 Histological staining

Traditional histological staining was used to demonstrate the tissue and cell morphology. Using different staining techniques certain specific cells and protein types were identified. Haematoxylin and eosin stain, commonly called H&E, is a popular tissue staining method in histological studies (Steven, 1977). It is the widely used stain in medical diagnosis; for example for biopsy of a suspected cancer. The staining involves application of haemalum (Aluminium ions and oxidized haematoxylin complex). It stains nuclei of cells blue. The nuclear staining is followed by counterstaining with eosin Y, which stains other, eosinophilic structures in various shades of red, pink and orange (Avwioro, 2011).

3.6.2 Immunofluorescent staining

Using immunofluorescence the ES cells and the cardiomyocytes in both the systems were stained with specific markers to characterize the type of cells present. In undifferentiated stem cells SOX2 was tagged. The SOX2 along with OCT4 and Nanong transcriptional factors maintain the undifferentiated stem cells pluripotency (Shenghui *et al.*, 2009). For the cardiomyocytes cardiac MHC and Troponin T specific antibodies were used to tag the proteins (Antin *et al.*, 2002; Garriock *et al.*, 2005).

3.6.3 Positive control

Day 9 chick heart was used as a positive control for the differentiated cardiomyocytes. At day 9 the chick heart exhibits the presence of functional cardiomyocytes with rhythmical phases of contraction and relaxation (Martinsen, 2005).

3.6.4 Methodology

3.6.4.1 Cryopreservation and cryosectioning

The day 9 chick hearts were isolated under the microscope using fine forceps. The hearts were cryopreserved in OCT. The heart, suspended in an OCT mould, was immersed in isobutanol in a beaker and briefly exposed to liquid nitrogen. The liquid nitrogen -196^{o}C temperature quickly freezes the tissue, which was then stored at -80^{o}C for future use. For cryosectioning the moulds were kept in the cryostat for 20 min before cutting the sections of required thickness.

3.6.4.2 H&E staining

The H&E staining method was adapted from the Stevens, 1977. The cryosectioned hearts or cells grown in chamber slides were first hydrated with tap water followed by haematoxylin incubation for 5 min. The slides were then washed with tap water several times until the water became clear. The slides were then differentiated using acid alcohol to remove the excessive stain before differentiating the nuclei in a saturated solution of lithium carbonate. This was followed by a tap water wash and the slides were placed in 1% eosin solution for 1 minute. The excessive eosin stain was washed using the running tap water and the sections were dehydrated through graded alcohol. A xylene dip was given before mounting with DPX.

3.6.4.3 Immunohistochemistry

The fluorescent immunohistochemical staining method is discussed in section 2.7.2.

3.6.5 Results

3.6.5.1 Day 9 heart, MM and ES derived cardiomyocytes H&E staining

The H&E demonstrates the cardiomyocytes general morphology. The day 9 heart staining confirms the presence of other endothelial and fibroblast cells along with cardiomyocytes. Fig 3.4A shows the chick day 9 complete sectioned heart with different regions, fig 3.4B is a view of the day 9 right auricle at greater magnification, showing the presence of irregular size myocytes. With H&E staining of chick cardiomyocytes in MM system the foci show deep red staining with large irregular cells. The numerous processes visible at the periphery may be involved in cell attachment (fig 3.4C). The ESDC H&E stain shows the different cell types with intensely stained foci in the centre where beating was observed. The cells were of different size with some visible processes that may support the cells in attachment (fig3.4D).

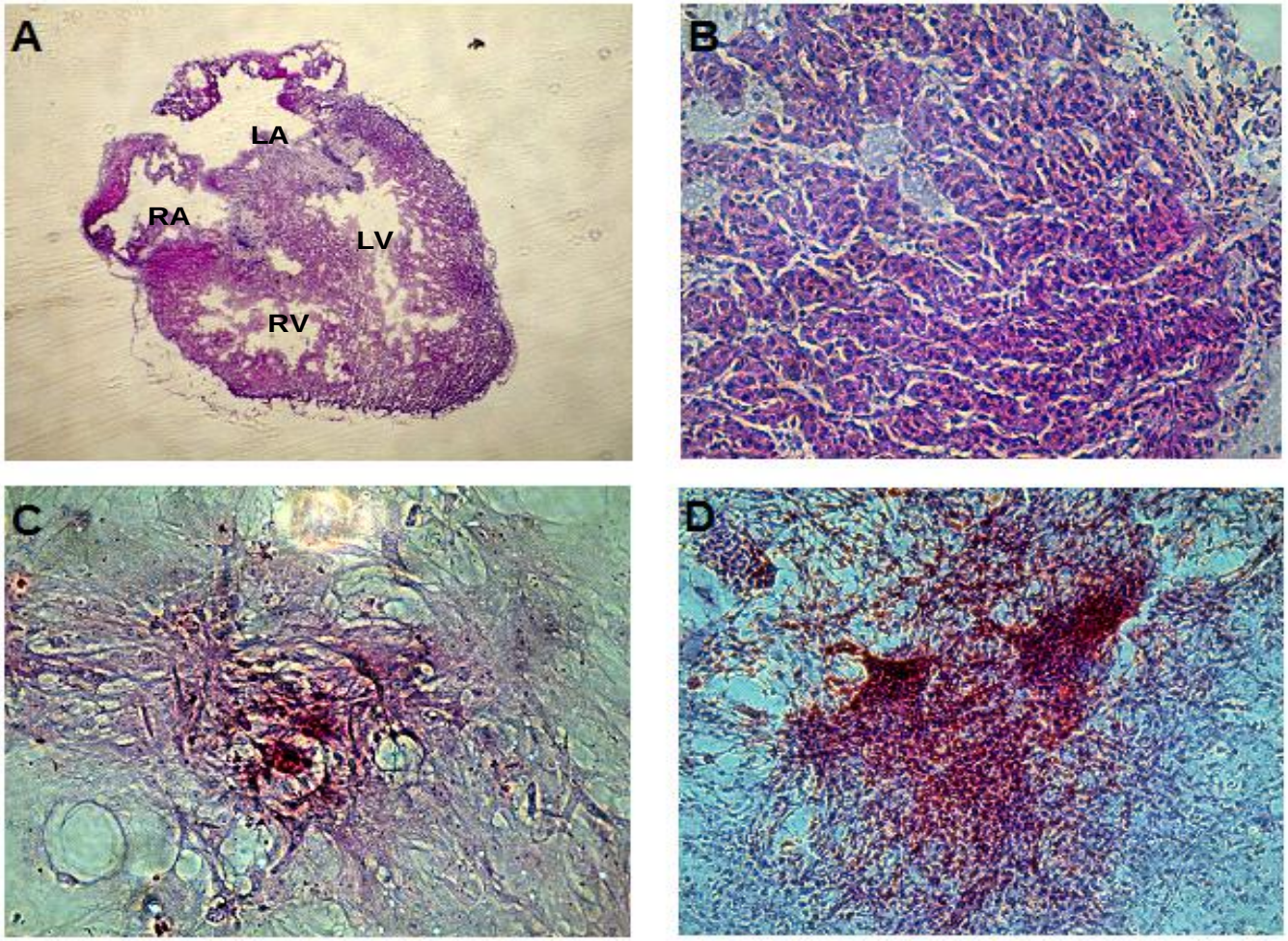


Fig 3.4 H&E staining of cardiomyocytes. A) Day 9 chick heart . RA=right auricle, LA=left auricle, RV=right ventricle and LV=left ventricle (magnification ×10), B) Day 9 chick heart auricle, C) chick cardiomyocytes in MM system and D) ES cells derived cardiomyocytes (magnification ×20).

3.6.5.2 Immunohistochemistry for undifferentiated ES cells

The undifferentiated cardiomyocytes were cultured in chamber slides in the presence of LIF. The cells were fixed and stained with SOX2 antibody as described in section 2.7.2. SOX2 is the marker of pluripotent stem cells. SOX2 is necessary for the maintenance of pluripotency along with Nanong and OCT4 transcriptional factor (Shenghui *et al.*, 2009). Being a transcription factor it is expected to be mostly expressed in the ES cell nucleus. In fig 3.5A it is clearly observed that SOX2 is expressed in the nucleus, the blue staining indicates nucleus staining with DAPI (fig 3.5B). This confirms the presence of pluripotent embryonic stem cells.

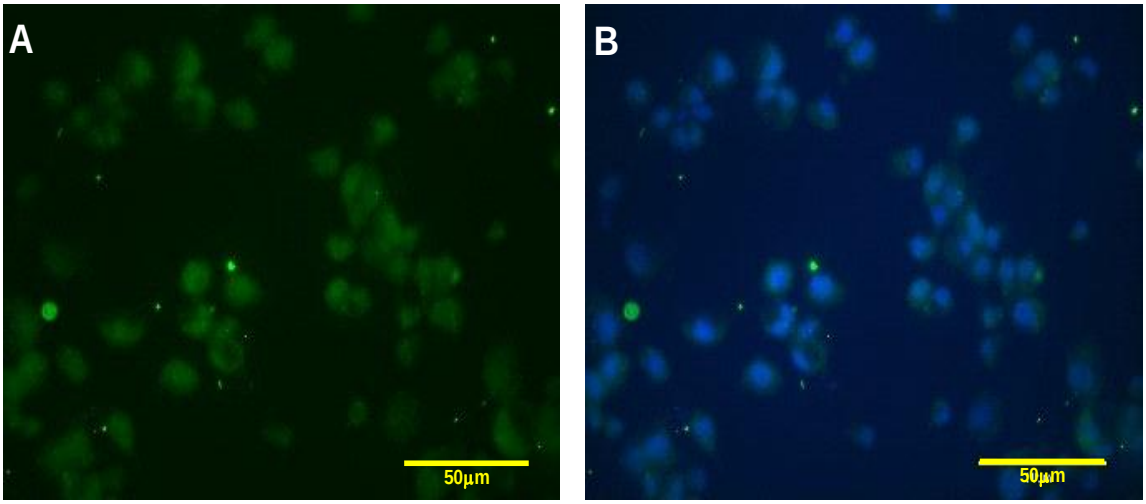


Fig 3.5 SOX2 staining of undifferentiated ES cells. A) **SOX2** staining B) nucleus staining with **DAPI** and **SOX2** merge.

3.6.5.3 Day 9 heart, MM and ESDC Immunohistochemistry

The day 9 chick heart showed positive staining for cardiac myosin heavy chain along with cardiac troponin T (fig3.6A and B). The MM cardiomyocytes showed a striated appearance of MHC and troponin T because of cell monolayers (fig 3.6C and D). As the ESDC forms large foci the striated appearance of MHC and Troponin T stains was not clearly visible (fig 3.6E and F).

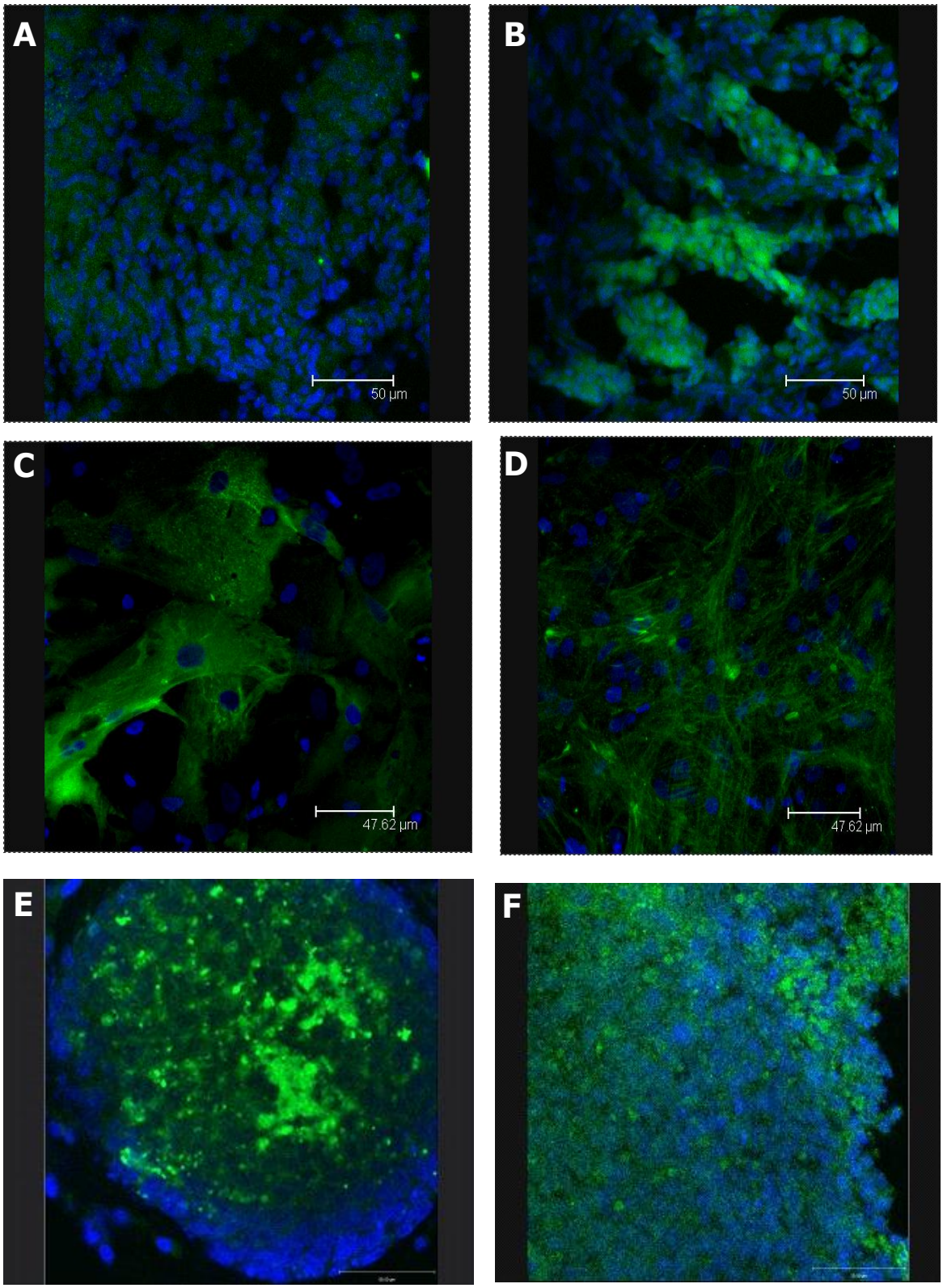


Fig 3.6 Chick day 9, MM and ESDC cardiomyocytes staining with **MHC** and **Troponin T** markers. A) Day 9 chick heart cardiac MHC staining, B) Day 9 chick heart cardiac troponin T staining, C) Chick MM cardiac MHC staining, D) Chick MM cardiac troponin T staining, E) ESDC cardiac MHC staining and F) ESDC cardiac troponin T staining. (Blue staining represents the **DAPI** nuclear stain).

3.6.6 Conclusion

The histological staining with haematoxylin and eosin shows the cellular morphology of cardiac cells and the presence of relatively low number of other cells with cardiomyocytes. The presence of other cell type supports the cardiomyocytes attachment. The ESDC shows the presence of irregular shape and different size cells. With immunostaining of MHC and troponin the presence of cardiac cells were confirmed in both the systems.

3.7 Evaluation of MM and ESDC systems reliability

Known strong, moderate teratogens and non-teratogens were selected from previous studies and evaluated in the MM and ESDC system, to compare and predict the reproducibility of results in our lab. Penicillin G (PG) is a non-teratogen compound even at high doses, mostly used as a positive control in teratological studies (Anneelieke *et al.*, 2007). A pair of uracil derivatives was selected - 5-Fluorouracil (5Fu), which is commonly used a chemotherapeutic agent, with a number of studies confirming its strong teratogenic potential at very low doses (Anneelieke *et al.*, 2007), while its analogue 6-Methyluracil (6-Mu) is a non-teratogenic compound (Cumberland *et al.*, 1994; Flint & Orton, 1984). Beside these teratogenic and non teratogenic compounds the reference drug sodium valproate (moderately teratogenic) was also included in this study to support the system reliability.

The same method of MM and ESDC was used as described earlier in chapter 2. In the MM system 1000μM PG, which is a very high dose, does not affect the cardiomyocytes. Cells were contracting like the control (fig 3.7A) with no effect on the other two end points (fig 3.7B and C) as shown previously (Ahir & Pratten, 2011). The 6-Mu as expected showed the same non-teratogenic effects in the chick MM system (fig 3.7A, B and C). While 5-Fu maintained its teratogenic attributes with statistically significant contractile activity inhibition at low doses (fig 3.7A). The VPA 1000μM showed comparable inhibition over contractile activity (fig 3.7A) with earlier studies, while other end points remained unaffected (fig 3.7B and C) (Ahir & Pratten, 2011). In ESDC the PG proved to be an ideal positive control with no effects

(fig3.7D, E and F) (Ahir & Pratten, 2011; Seiler *et al.*, 2004). With 5-Fu when same dose of 5μM was used there was no formation of EBs, a reduced dose was used as described by Seiler et al, in 2004. 5-Fu 0.25μM reduced the contractile activity (fig 3.7D) with no significant effect on cell viability (fig 3.7E) and protein contents (fig 3.7F) (INVITTOX protocol no 113). For 6-Mu at the same dose of 0.25μM all end points were comparable to the control (fig 3.7D, E and F). While VPA at high doses inhibited cell contraction (fig 3.7D) and viability (fig 3.7E) with no effects on proteins (fig3.7F) (Ahir & Pratten, 2011).

The results observed in our lab were comparable with the previous results. The use of the MM and ESDC method is reliable. This further supports the use of the method and techniques in this study.

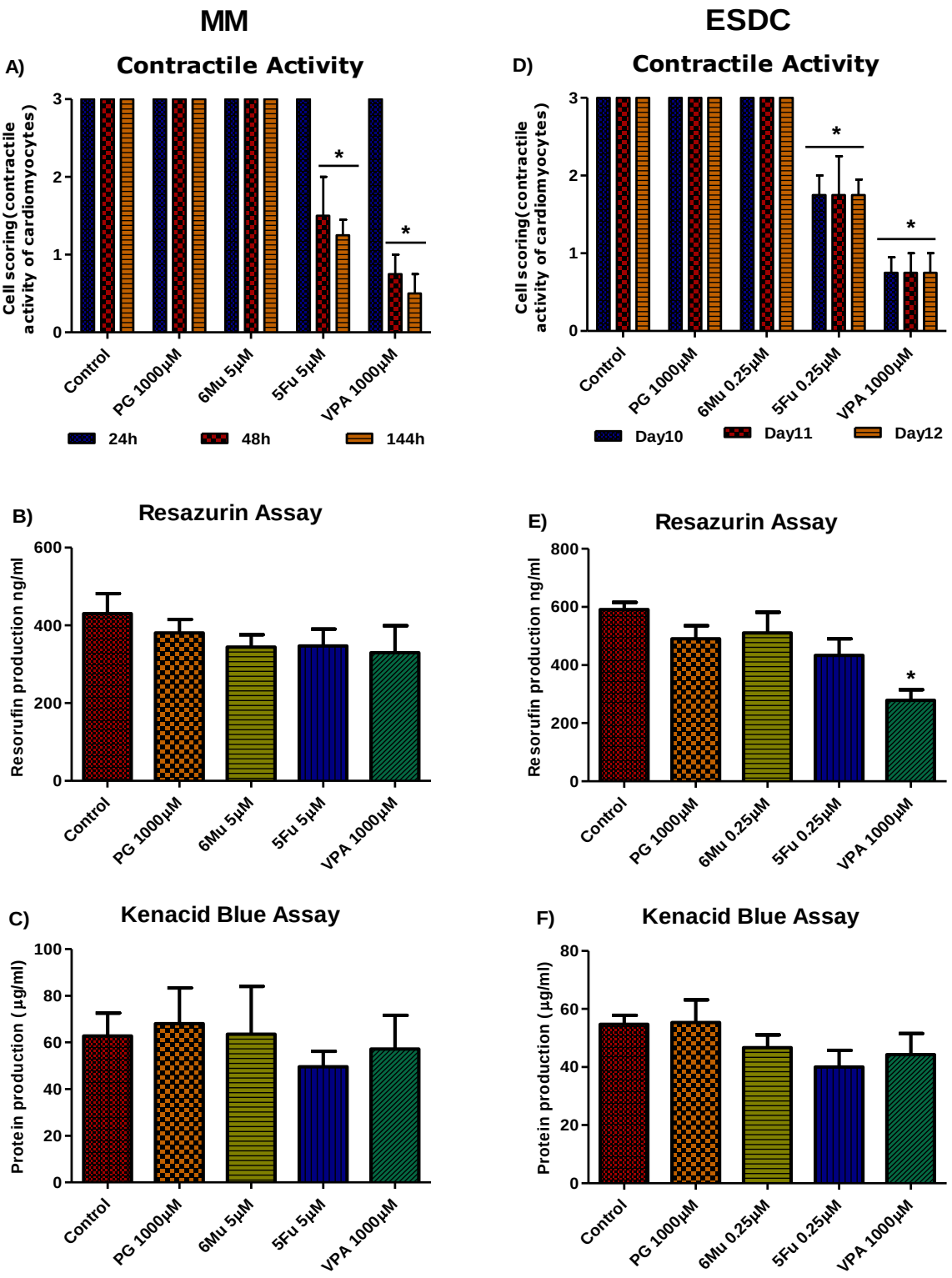


Fig 3.7 The effects of drugs on MM and ESDC systems. A) Effects on contractile activity in the MM system, B) effects on cell viability (resazurin assay) in MM system, C) effects on total protein content (kenacid blue assay) in MM system, D) effects on contractile activity in ESDC, E) effects on cell viability (resazurin assay) in ESDC, F) effects on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control.

3.8 Discussion

For the evaluation of selected teratogenic effects using these *in vitro* systems it was necessary to adopt a system that is reliable, reproducible and represents normal development. The chick cardiomyocyte micromass system is relatively recent and still not validated. The ESDC system is already validated in the EVCAM procedures and comes under the name of EST with additional use of fibroblast cells (Invittox Protocol no. 113). In this study the EST protocol of cardiomyocyte differentiation and drug treatment was adopted with no modification. Both the systems were thoroughly evaluated before starting the actual experiments with drugs. Cardiac cell markers were used to confirm the cells isolated and differentiated represent the correct tissue type. Beside these known drugs effects in these systems further support the reproducibility of results observed in other studies.

Chapter 4

Evaluation and comparison of antiepileptic drugs teratogenic effects in chick cardiomyocytes MM system and ES cells derived cardiomyocytes

4.1 Epilepsy and Teratogenesis

Epilepsy is a collection of neurological disorders exhibiting disturbance in brain function, characterized by abnormal electrical activity which results in seizure disorder (Tunnicliff, 1996). Nearly 1 million women of child bearing age in the US alone have been diagnosed with epilepsy. Mounting evidence suggests an association between epilepsy and increased perinatal risks. The use of antiepileptic drugs (AEDs) in epilepsy further increases the susceptibility to drug related teratogenicity (Fried *et al.*, 2004).

Fetal malformations occur in about 10% of pregnant women with epilepsy, of which 2% are severe. In pregnant epileptic women the seizure disorder complicates less than 1% of the pregnancies. Depending on seizure frequency and duration, in some pregnancies it worsens, in some it improves and in other remains unaffected (Brewer *et al.*, 2003). 33% women with epilepsy experienced an increase in seizure frequency during gestation, so continuing the drug therapy is necessary to avoid maternal and fetal life risks (Prakash *et al.*, 2008). The congenital malformation rate increases in maternal epilepsy, and is complicated by uncontrolled seizures, especially if they occur in the first and second trimester of pregnancy, as at this time the fetus is most vulnerable to malformation induced by epilepsy or medications. Seizure activity in the third trimester puts the fetus at a risk of hypoxic episodes (Brewer *et al.*, 2003).

4.2 Teratology and Antiepileptic Drugs

The pregnant epileptic woman who stops taking AEDs is at more risk of developing status epilepticus, which has a high mortality rate for mother and baby. Convulsive seizures in pregnancy cause fetal intracranial haemorrhage and heart rate changes (Fountain, 2009), so maintaining the AED serum therapeutic level during pregnancy is necessary to avoid maternal and fetal life risks (Prakash *et al.*, 2008). It is always controversial to find out whether the malformation is caused by the epilepsy itself or the exposure to AEDs; however AEDs are usually the major contributor toward the development of malformations (Brewer *et al.*, 2003). Earlier studies have indicated teratogenicity as an attribute of most AEDs (Kaneko *et al.*, 1999) and although no

antiepileptic drug in the world is safe or ideal (Prakash *et al.*, 2008), a few have less teratogenic effects (Pizzi *et al.*, 1996).

The congenital malformation rate found in AED exposed offspring is 9.0% as compared to unexposed offspring's 3.1% (Kaneko *et al.*, 1999). This malformation risk is increased two fold with AEDs polytherapy (Matalon *et al.*, 2002). In another study, a reduction in daily dose of AEDs may be shown to decrease the malformation rates from 13.5 to 6.2% (Kondo *et al.*, 1996).

AEDs' teratogenic effects on the embryo and fetus are evident in three major areas:

1. Increased major congenital anomalies (MCA). Usually congenital heart defects, cleft lip and cleft palate, anomalies of the urinary tract, of the limbs and brain, especially neural tube defects (NTD).
2. A specific syndrome of the particular AED, mainly affecting the cranio-facial complex causing facial dysmorphism, but often also affecting other organs such as external genitalia (i.e. Phenytoin syndrome) and neural tube (Carbamazepine and Valproate syndrome).
3. Developmental disorders, mainly affecting cognitive function and behaviour. These changes may affect language development, learning abilities and even cause Autism (Ornoy, 2009).

4.2.1 Sodium Valproate (Sodium 2-Propyl Pentanoate)

The anticonvulsant properties of Valproic acid (VPA) were serendipitously discovered in 1962 (Gurvich *et al.*, 2004). It was first marketed in 1974 for the treatment of epilepsy; later on its mood stabilizing properties were also added to its therapeutic profile (Ornoy, 2009). Sodium valproate, an analogue of VPA is found to be effective against seizures. VPA and sodium valproate ionize to the valproate ion at body pH (Katzung, 2000). The mechanism of action of VPA is not fully understood, but it may act by potentialising GABA inhibitory activity by several mechanisms, attenuating aspartate levels or by blocking Na^+, Ca^{2+} and K^+ channels in the brain (Chateauvieux *et al.*, 2010; Umka *et al.*, 2010). A therapeutic dose of 300-2000 mg/day controls seizures and may achieve plasma levels of 50-100$\mu g ml^{-1}$. Lower doses are normally recommended for bipolar disorder, mania and migraine (Ornoy, 2009). The drug

crosses the placenta and its levels in umbilical cord blood are found to be five times higher than maternal serum, which imposes increased risk of embryotoxicity (Ornoy, 2009).

VPA use during early pregnancy carries a 3-fold increased risk of congenital malformation (Kostrouchová *et al.*, 2007), with 1-2% increase in NTD (including anencephaly and exencephaly) and that elevates to 5.4% with a higher daily dose of 1000mg/day of VPA. The dominant teratogenic characteristic is lumbosacral meningomyelocele (spina bifida aperta), it also causes cardiovascular, genital and limb abnormalities, hypospadia, developmental delay, autism and the specific "Fetal Valproate Syndrome"(Ornoy, 2009; Ornoy *et al.*, 1998).

"Fetal Valproate Syndrome" was first described by DiLiberti in 1984, with characteristic intrauterine growth retardation (IUGR), a long and thin upper lip, shallow philtrum, epicanthal folds and mid face hypoplasia manifested by a flat nasal bridge, small up turned nose and the down turned angles of the mouth (DiLiberti *et al.*, 1984).

Valproic acid exposure in pregnancy brings about developmental delay that reduces cognitive function and produces attention deficit disorder and learning difficulties in the new born. Autism spectrum disorder (ASD) is also found to be an attribute of VPA teratogenicity (Ornoy, 2009). In 2005, Rasalam, found 8.9% risk of ASD in infants born to VPA monotherapy mothers compared with that of 11.7% with polytherapy, especially VPA with carbamazepine that gives 11.1%, while benzodiazepines generally are found to enhance the teratogenic effects of VPA. Due to increased teratogenic potentials of VPA it is placed in the FDA teratogenic category "D" (Rasalam *et al.*, 2005).

VPA like other AEDs possess antifolate activity and disrupts gene expression, enhances embryonic oxidative stress and alters protein synthesis. These disturbances may result in NTD, cardiac anomalies and neurodevelopment delay. In addition its intermediate reactive metabolites may increase oxidative stress and irreversibly damage the embryo or fetus (Na *et al.*, 2003; Ornoy, 2009).

VPA at therapeutic concentrations (0.3-0.7mM) inhibits histone deacetylase (HDACs) (Gurvich *et al.*, 2004; Murabe *et al.*, 2007a), which explains its anticancer properties (Chateauvieux *et al.*, 2010). VPA teratogenic effects are thought by some to be mediated through HDACs inhibition rather than antiepileptic activity (Phiel *et al.*, 2001). VPA inhibits HDACs, preferentially class I HDACs. It decreases histone acetylation, and thus changes in the chromatin structure and the activity of transcription factors of the genes may be induced. This action may interrupt the cell cycle, induce growth arrest and apoptosis, and in turn affect cell proliferation, which may explain its teratogenic properties (Ornoy, 2009). Earlier in 2003 Detich (Detich *et al.*, 2003) found that VPA triggers the active methylation of DNA along with acetylation of H3 Histone in human embryonic kidney cell culture. This is achieved by the direct action of VPA as a HDAC inhibitor, thus increasing demethylase accessibility to DNA, and demethylation of DNA induces changes at the gene level. Such changes may produce various epigenetic abnormalities (Blaheta & Cinatl, 2002; Ornoy, 2009). Beside this VPA affects a number of molecular pathways including PPAR δ, β-catenin, activator protein 1, PkC and Erk1/2 (Gotfryd *et al.*, 2011; Gurvich *et al.*, 2004).

Using embryoid bodies derived from murine ES cells, Lan Na found that VPA interrupted the cell cycle, but did not cause cytotoxic effects in the embryoid bodies in a dose dependent manner. The intracellular reactive oxygen species levels were found to be elevated and that may have inhibited cardiomyogenic differentiation (Na *et al.*, 2003). In the micromass system VPA affects neural differentiation and chondrogenesis (L'Huillier *et al.*, 2002).

4.2.2 Carbamazepine

One of the widely prescribed antiepileptic drug Carbamazepine (CBZ) acts by stabilizing voltage gated sodium channels or by modulating neurotransmitters release or uptake (Beutler *et al.*, 2005; Kwan *et al.*, 2001). CBZ was initially found to be devoid of teratogenic effects, but in 1989 it was observed that CBZ used alone or in combination may produce malformations similar to Phenytoin (Hansen et al., 1996). CBZ mostly causes cardiovascular anomalies (1.5-2.0%), NTDs (0.5-1%),

urinary tract malformations and cleft palate (Murabe et al., 2007b). CBZ increases risks of congenital malformation 2.89 fold, which increases to 3 fold during first trimester drug use and further elevates in AEDs polytherapy. Due to non-availability of adequate and well controlled studies on CBZ teratogenic effects in humans, it is placed in FDA pregnancy category "C" (Ornoy, 2006).

CBZ is metabolized in the body through arene oxide pathways; the chemically reactive epoxide produced may bind covalently with macromolecules like DNA and proteins and can produce teratogenic effects by disrupting normal process of fetal development (Kaneko et al., 1999; Lindhout et al., 1984). Phenobarbitone (PB) is also metabolized through the arene oxide pathways, by which xenobiotics can be hydroxylated through an epoxide intermediate. On the other hand VPA inhibits the production of epoxide hydrolase, which in VPA and CBZ polytherapy causes accumulation of CBZ epoxide. Therefore CBZ polytherapy with VPA or PB carries a greater risk of malformation (Matalon *et al.*, 2002). CBZ and its major metabolite CBZ-10,11-epoxide are histone deacetylase (HDAC) inhibitors, with an IC50 value of 2μM (Beutler et al., 2005) which is well within human therapeutic range (3-14μg/ml) (Murabe et al., 2007b). CBZ teratogenicity may be due to HDAC inhibition because HDAC inhibitors induce cell cycle arrest, apoptosis and differentiation (Beutler et al., 2005).

In an experiment to investigate the effects of CBZ on ES cell differentiation into cardiomyocytes using the R1 mouse embryonic cell line, Murabe found that expression of the primitive marker GATA6 increased in the differentiating endodermal lineage, but the expression of late differentiating markers TTR and HNF1 decreased with increasing concentration. While ALB was not detected, this shows CBZ promoted initial endodermal differentiation but inhibited differentiation into mature endodermal lineages (Murabe *et al.*, 2007b). In the mesodermal lineage the primitive marker BMP4 expression increased in a dose dependent manner. The early cardiac marker NKx2.5 also slightly increased, while late stage cardiac marker ANF was reduced. It was found that CBZ decreased the rate of undifferentiated ES cell differentiation into cardiomyocytes in a dose dependent manner. VPA inhibited the

expression of almost all the markers in all the stages of endodermal and mesodermal differentiation, whereas CBZ inhibits only later stages. This shows that VPA and CBZ exert embryotoxic effects using different mechanisms in endodermal and mesodermal developmental stages. In neural differentiation VPA induced differentiation in a lineage-specific manner, and the expression of late stage neuron specific markers like Nestin, Synaptophysin (Syn) and Neurofilament H (NFH) increased. The same increased expression is found with CBZ in a dose dependent manner. Expression of the glial markers GFAP, Oligo 2 and DM20 increased with CBZ but not with VPA (Murabe *et al.*, 2007b).

4.2.3 Phenytoin

Phenytoin (PHT) is an antiepileptic drug first used in 1937, since then its therapeutic spectrum covers a broad range of seizures (Temiz *et al.*, 2009). It acts primarily on the neuronal cell membrane sodium channel, limiting the spread of seizure activity and reducing seizure propagation (Kwan *et al.*, 2001; Tunnicliff, 1996). Like other AEDs PHT also affects the developing conceptus resulting in mental deficiencies, skeletal, CVS and nervous system malformations both in human and animal models (Okruhlicová *et al.*, 2003; Temiz *et al.*, 2009). Due to the well documented PHT teratogenic effects in humans and animal models it is placed in FDA pregnancy category "D" (Ozolins *et al.*, 1995).

In utero exposure of PHT has two to three fold increased risk of producing congenital malformations, with a characteristic constellation of anomalies termed as "Fetal Hydantoin Syndrome" (Ozolins *et al.*, 1995; Tiboni *et al.*, 2003). This syndrome exhibits facial and skull abnormalities, such as short nose, low nasal bridge, epicanthic folds, hypertelorism, abnormal ears, wide mouth, hypoplasia of distal phalanges and nails, finger like thumbs, short or webbed neck, low hairline, and abnormalities of growth and of mental and sometimes of motor development (Ornoy, 2006; Tiboni *et al.*, 2003). This specific syndrome is observed in 10% of children born to PHT treated mothers. PHT use during pregnancy may also cause increased neuroectodermal tumors in the offspring (Ornoy, 2006). At low doses (0.1mg closely resembles human therapeutic dose) in chick, PHT does not induce

NTDs, while at high doses (0.5mg) they are observed (Temiz *et al.*, 2009). Despite numerous reports the exact teratogenic mechanism and dose remains elusive. Many hypotheses have been put forward to explain the phenytoin mechanism of teratogenicity, including inhibition of detoxification enzymes, embryonic oxidative stress, glucocorticoid receptor interactions, inhibition of folate absorption and inhibition of prostaglandin production (Temiz *et al.*, 2009). Like other AEDs PHT also affects folate levels and produces NTD in the fetus, while folic acid supplements prevent these effects. (Ozolins *et al.*, 1995; Temiz *et al.*, 2009). Other notable mechanisms include production of the toxic arene oxide intermediates via enzymatic bioactivation by embryonic cytochrome P450. Arene oxides are highly reactive and can initiate the formation of reactive oxygen species, which at least in part is responsible for producing embryopathies (Ozolins *et al.*, 1995; Winn & Wells, 1996). PHT treatment affects membrane permeability and interferes with membrane transport of calcium ions, which changes membrane properties and could lead to alterations in cell-cell interactions. This explains why Phenytoin, more than Phenobarbitone and Valproate caused disaggregation of neuronal cell clusters resulting in cell death (Sedowofia & Clayton, 1985). On embryonic heart the adverse pharmacological actions on ion channels induces cardiac arrhythmias, cardiac arrests, and transient episodes of hypoxia (Azarbayjani *et al.*, 2006; Okruhlicová *et al.*, 2003). Beside these side effects it also alters vitamin D metabolism and inhibits calcium absorption resulting osteomalacia with hypocalcaemia (Ozolins *et al.*, 1995). PHT teratogenic effects are observed to different degrees in mice, rat, chicken and human. In addition to species variability there are remarkable strain differences in susceptibility to PHT teratogenesis (Ozolins *et al.*, 1995).

4.2.4 Primidone

The antiepileptic drug Primidone (PRM) belongs to the FDA pregnancy category "D". PRM was initially considered as a drug of choice in pregnant epileptic women, but later on this was refuted (McElhatton *et al.*, 1977). The exact anticonvulsant mechanism of action is not clearly understood, but is thought to inhibit high frequency repetitive firing action potential by interacting with sodium channels

(Macdonald & Kelly, 1995). It is often prescribed with Phenobarbital as an adjunct therapy. PRM is metabolized to two active compounds in the body, Phenobarbital and Phenylethylmalonamine. The latter has 20% of the antiepileptic potency of Phenobarbital and is less neurotoxic (Pizzi *et al.*, 1996). PRM is not a barbiturate but its anticonvulsant properties are believed to be the result of its metabolite phenobarbital, because of its similar behavioural effects to phenobarbital (Pizzi *et al.*, 1998). The PRM produced congenital malformations are dose independent, because even at non-toxic doses (25–150 mg/kg) it produces teratogenic effects (McElhatton *et al.*, 1977). The most commonly observed malformations with PRM are palatal defects, which include full length clefts (2.4%), submucosal clefts (9-17%) and abnormally shaped palatal bones, with ossification of bones and epithelial fusions. No skeletal defects are seen in PRM treated mice. On the other hand the PRM metabolite Phenobarbital when given alone produces skeletal defects (McElhatton *et al.*, 1977). PRM like other AEDs affects folic acid metabolism. The altered folic acid level may increases risk of DNA damage. PRM chronic exposure results in carcinoma (NTP, 2000; Ronemus *et al.*, 1996).

4.2.5 Folic acid

Folic Acid (FA), is a water soluble B-complex Vitamin (van Gelder *et al.*, 2010). Humans cannot synthesize FA, so they depend on dietary sources. The daily recommended dose for child bearing age women is 400µg per day, and up to 4mg per day for the mothers with a history of producing NTD children (Ornoy, 2009; Tamura & Picciano, 2006). In the body, folate is reduced by dihydrofolate reductase to the naturally bioactive form tetrahydrofolate, which is then converted into 5-methyltetrahydrofolate (5-MTHF). 5-MTHF enters the cells and acts as an essential co-enzyme being the donor or acceptor of one carbon unit, especially in nucleic acid metabolism and in DNA methylation (van Gelder *et al.*, 2010). Most AEDs impair folate absorption or metabolism thereby interfering with the supply of nucleic acid precursors in rapidly dividing cells and the control of gene expression by DNA methylation (van Gelder *et al.*, 2010). Decreased folic acid levels results in decreased conversion of homocysteine into methionine; elevated homocysteine

causes alteration in trans-sulphuration pathways, which results in increased ROS production. Increased homocysteine levels during pregnancy were found to produce malformation (Green, 2002; Rosenquist *et al.*, 1996). Folate deficiency causes various congenital malformations, growth retardation and intrauterine deaths (van Gelder *et al.*, 2010). It is widely accepted that the use of FA during pregnancy reduces the risk of malformations, especially NTDs and hearts defects (Green, 2002).

4.2.6 Reactive oxygen species and antioxidants

Reactive oxygen species (ROS) are highly reactive oxygen-containing molecules with unpaired valence shell electrons (Cengiz *et al.*, 2000). ROS are produced as a natural by-product of oxidative metabolism and have important roles in cell signalling and homeostasis (Devasagayam *et al.*, 2004). ROS is a second messenger in the signal transduction mechanism and plays an important role in cardiomyocyte differentiation (Sauer *et al.*, 2000; van Gelder *et al.*, 2010). However, increased ROS production may cause oxidative stress. These excessive ROS, if not detoxified, can interfere with the normal process of development and produce teratogenesis by binding to macromolecules (lipids, proteins, DNA and RNA), or by dysregulation of signal transduction (Cengiz *et al.*, 2000; van Gelder *et al.*, 2010; Wells *et al.*, 2009). In cells, antioxidants provide the defence mechanism to detoxify these species (Peng *et al.*, 2005), but the imbalance between ROS production and antioxidant defence mechanisms may cause embryopathy (Lee *et al.*, 2004). A number of xenobiotics within the embryo are enzymatically bioactivated to highly reactive electrophilic or free radical intermediates. If electrophilic reactive intermediates not detoxified they may covalently bind with embryonic macromolecules or free radical intermediates may reacts directly or indirectly with molecular oxygen to initiate the formation of reactive oxygen species which may induce oxidative stress. The increased ROS may interfere with embryonic signal transduction pathways and may adversely affect development (Wells *et al.*, 2005). It is hypothesized that during pregnancy the mother utilizes Vitamin C, E and antioxidant enzymes (like SOD, catalase and glutathione peroxidase) to provide a defence mechanism against ROS.

Ascorbic acid (AA) or vitamin C is a water soluble vitamin. It acts as an antioxidant by counteracting several hydroxy radicals in the cell cytosol and may contribute in protecting the fetus from free radical damage (Cem Ekmekcioglu *et al.*, 2010; Lee *et al.*, 2004). While on the other hand through the Fenton reaction it reduces Fe^{3+} to Fe^{2+}, which is involved in free radical formation (Cem Ekmekcioglu *et al.*, 2010). AA also promotes cardiomyocyte differentiation, unlike other antioxidants (Takahashi *et al.*, 2003). Superoxide dismutase (SOD) is the major superoxide anion antioxidant enzyme present in the cell. It catalyses superoxide anion conversion into hydrogen peroxide (Cengiz *et al.*, 2000), which in turn is reduced to water by enzymes catalase and glutathione peroxidase (Jauniaux *et al.*, 2004).

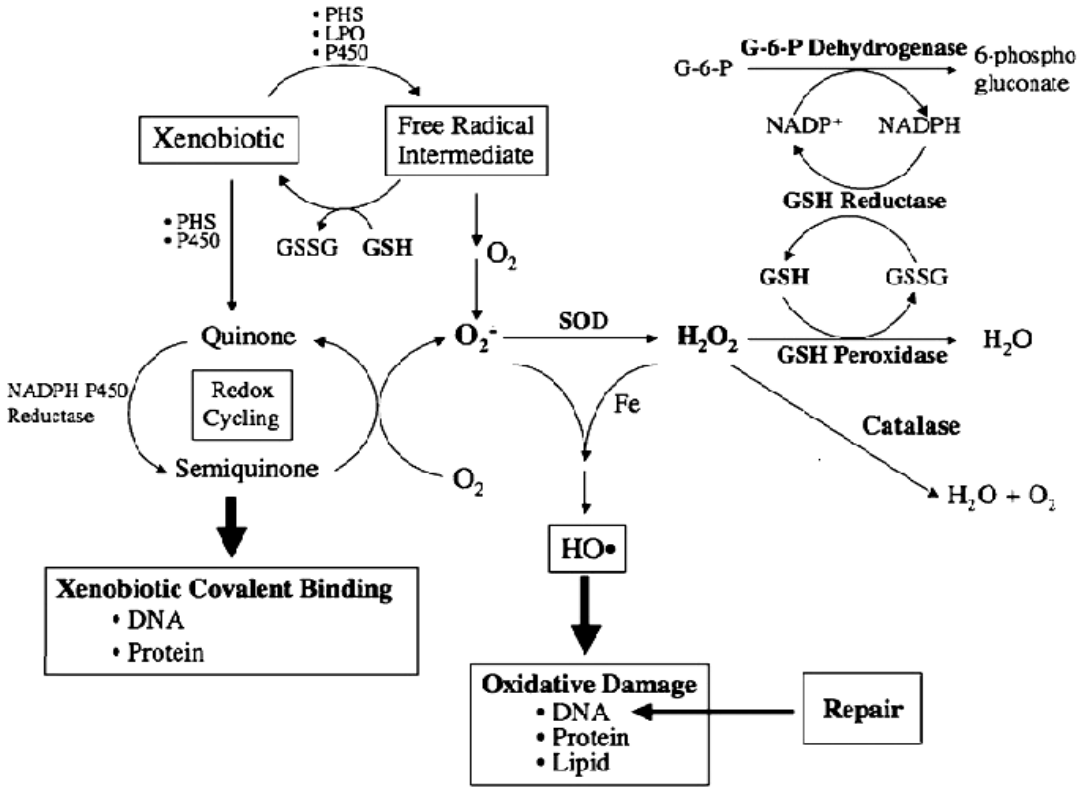


Fig 4.1 Biochemical pathways for xenobiotics metabolism to free radical intermediates and detoxification by antioxidants. PHS=prostaglandin H synthase, LPO=lipoxygenase, P450=cytochrome P450, SOD=superoxide dismutase, O^{-}_2=superoxide anion, H_2O_2=hydrogen peroxide, Fe=iron, HO^{-}=hydroxyl anion, GSH=Glutahione, GSSG=Glutathione disulphide, G-6-P=Glucose 6 phosphate (Wells *et al.,* 2009).

4.3 Methodology

4.3.1 Materials and Methods

Four antiepileptic drugs at 8 different concentrations were exposed to the chick embryonic cardiomyocyte cultured at a very high density using the micromass system (see section 2.4.1). While the same concentrations of drugs were exposed to ES cell derived cardiomyocyte for a period of 12 days (see section 2.4.2).

4.3.2 Test chemical dose preparation

The water soluble Sodium valproate stock solution was prepared by dissolving in complete culture medium, while for Carbamazepine and Primidone DMSO was used as a solvent to dissolve the drug first followed by preparation of stock solution in complete culture medium (see section 2.6). For Phenytoin the 45% w/v 2-hydroxypropyl-β-cyclodextrin was used as a solubilizing agent, which is found to be non-toxic (Pitha & Pitha, 1985). The final concentration of DMSO and cyclodextrin was kept constant for all doses including control. The FA, AA and SOD doses were prepared in complete culture medium either alone or in combination with CBZ.

4.3.3 End Points

In MM and ESDC, contractile activity was used as a marker for the estimation of the extent of the drug effects on cardiomyocytes, while cell viability and total protein content were also investigated at the end of experiments (see section 2.5).

4.3.4 Statistical analysis

The results three independent repeats were statistically analysed using Prism 5 software. All the drugs doses results were compared with control using one way ANOVA with Dunnet's post hoc test. $P<0.05$ was considered as a significant difference.

4.4 Results

4.4.1 Sodium valproate

4.4.1.1 Sodium valproate effects on cardiomyocytes in the Micromass system

The Valproic acid (VPA) salt sodium valproate at a concentration ranging from 5-800μM was tested in the MM system. The cardiomyocytes after drug exposure showed decreased ability to re-establish the contractile unit. A VPA dose of 100-800μM inhibited the contractile activity to statistically significant values ($P<0.05$) at 48 and 144h of cell culture (fig 4.2A). The Resazurin cell viability assay and Kenacid blue total protein content showed no signs of significant variations from the control (fig 4.2B and C). The drug treated cardiomyocytes at 144h of cell culture showed no large spaces between the foci, the proliferation was uniform and the numbers of floating cells were comparable to control.

4.4.1.2 Sodium valproate effects on ES derived cardiomyocytes

The same doses of VPA were used to treat the ESDC. The contractile activity of cardiomyocytes compared to control declined with increase in drug dose (fig 4.2D). A significant declining trend was observed when cardiomyocytes began to contract on day 10 of the experiment. The drug dose 100-800μM showed a statistically significant difference from control ($P<0.05$) on day 10, 11 and 12 of scoring (fig 4.2D). The other end point of cell viability (fig 4.2E) also followed the same pattern of decreased resorufin production with increased drug dose exposure. The drug dose 100-800μM showed some significantly decreased cell viability ($P<0.05$) compared to control. The total cellular protein content remained the same as the control (fig 4.2F) at all doses tested. At 800μM the day 3 and 5 EB's were slightly reduced in size, with some dead floating cells on day 12 of the experiment.

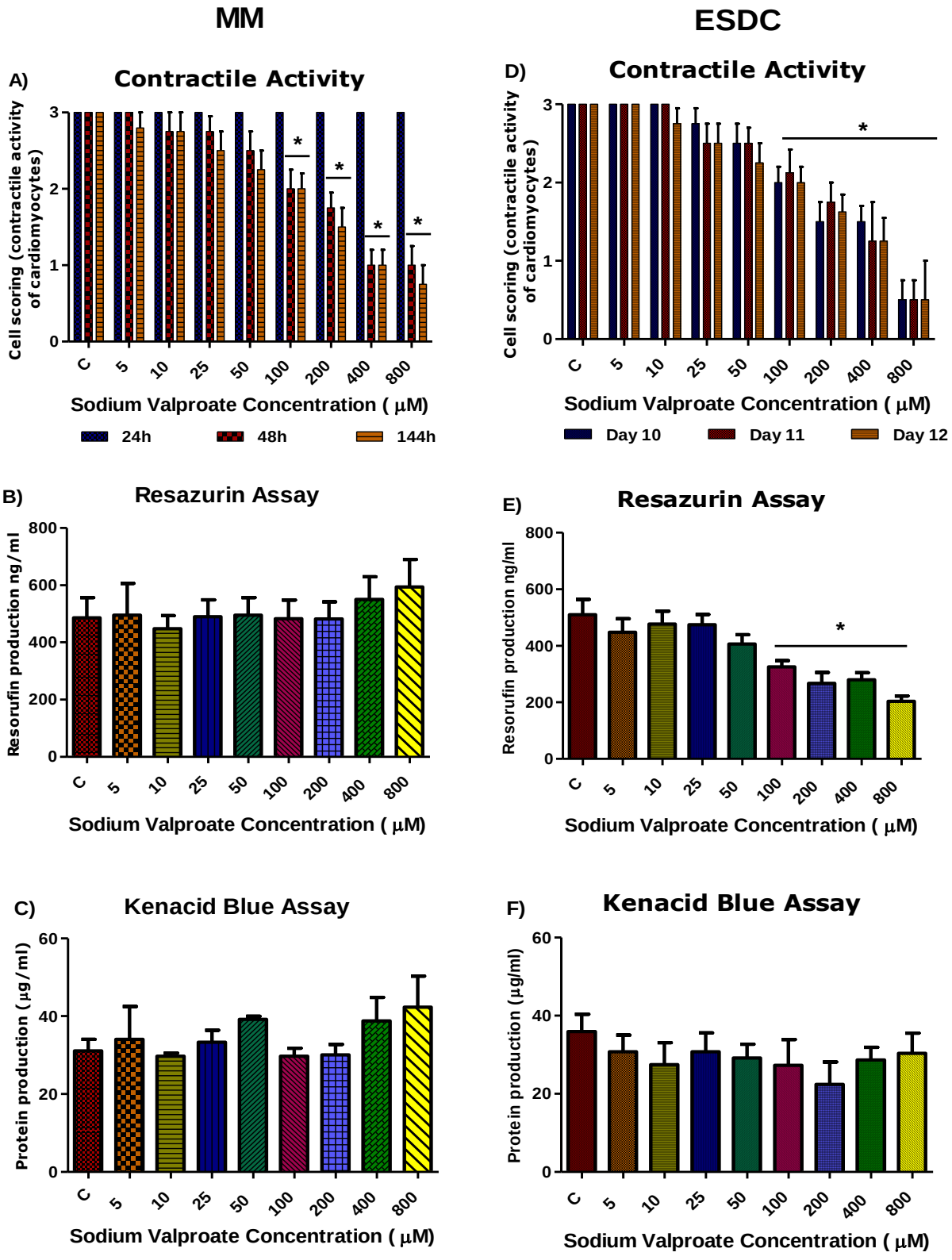


Fig 4.2 The effects of VPA on MM and ESDC systems. A) Effects on contractile activity in MM system, B) effects on cell viability (resazurin assay) in MM system, C) effects on total protein content (kenacid blue assay) in MM system, D) effects on contractile activity in ESDC, E) effects on cell viability (resazurin assay) in ESDC, F) effects on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control. (C=Control).

4.4.2 Carbamazepine

4.4.2.1 Carbamazepine effects on cardiomyocytes in micromass system

In the MM system, after 24h, cultured contracting chick cardiomyocytes were treated with 8 different concentrations of CBZ ranging from serum therapeutic concentration to 10-15 times higher. CBZ effects on differentiated cardiomyocyte contractile activity in the therapeutic range (50μM) was not significantly different from the control, but as the drug dose increased inhibitory effects on contractile activity were evident. 200-400μM CBZ showed a statistically significant decrease in contraction compared to control especially at 144h of incubation ($P<0.05$) (fig 4.3A). The chick cardiomyocyte viability remained unaffected at all doses tested, using the Resazurin cell viability assay (fig 4. 3B), while the total protein content (fig 4.3C) also did not showed any significant pattern of variation from the control ($P>0.05$).

4.4.2.2 Carbamazepine effects on ES derived Cardiomyocytes

With ES derived cardiomyocytes CBZ (1-400μM) decreased the cardiomyocyte differentiation with increased dose. Contractile activity was inhibited within the serum therapeutic range (50μM $P<0.05$), which further declined and completely ceased at 200μM CBZ (fig 4.3D). The cell viability assay (fig 4.3E) clearly showed a pattern of significantly decreased viability from the control with increased dose (50-400μM $P<0.05$). The effects on cellular Protein Content (Kenacid Blue Assay) were obvious as early as day 3 of the experiment, when the size of EBs decreased with increased in CBZ treatment dose (fig. 4.4A). On Day 12 of ES differentiation, cardiomyocytes also showed the same pattern of decreased Cellular Protein Content (50-400μM $P<0.05$) (fig 4.3F).

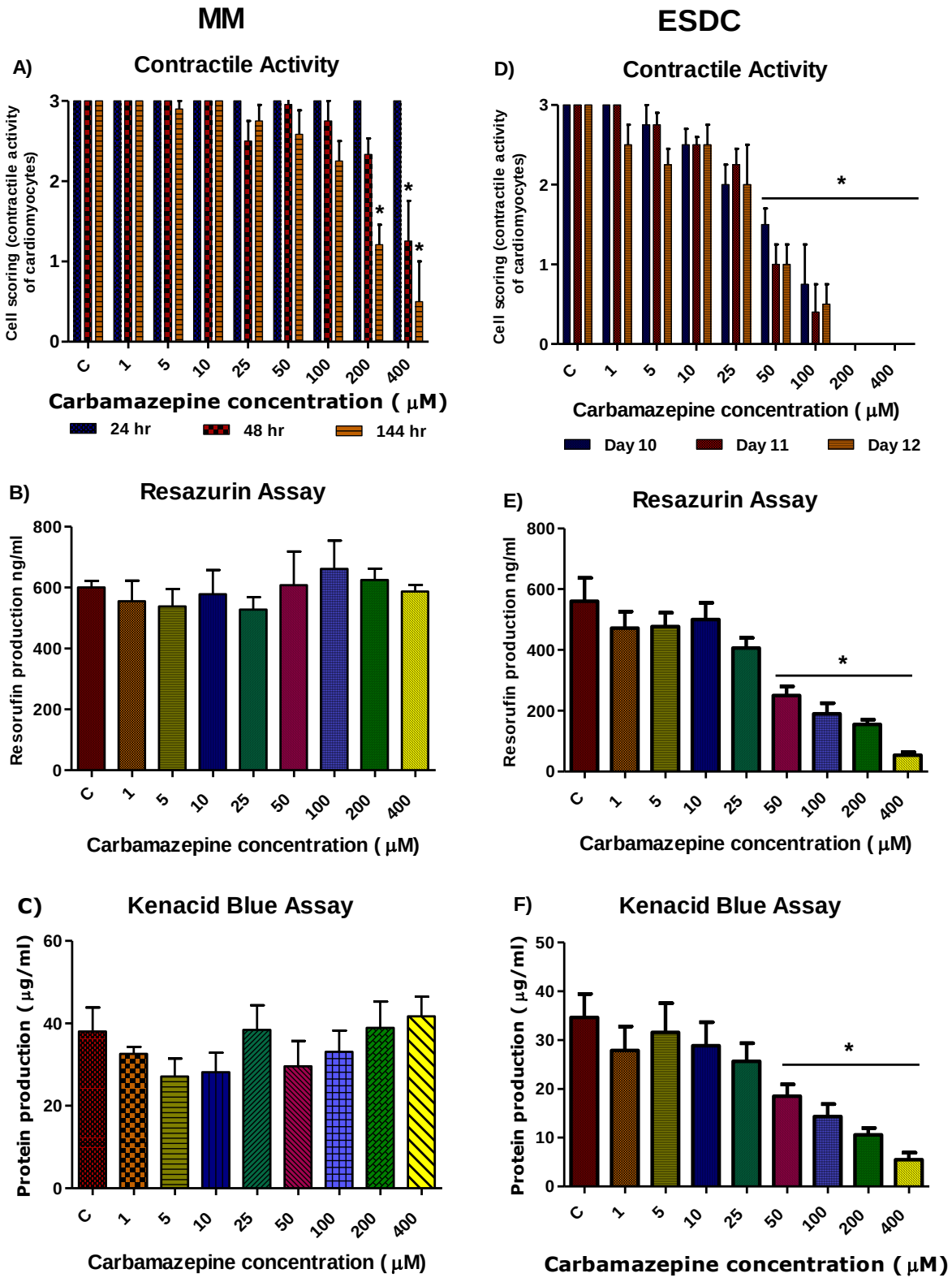


Fig 4.3 The effects of CBZ on MM and ESDC systems. A) Effects on contractile activity in MM system, B) effects on cell viability (resazurin assay) in MM system, C) effects on total protein content (kenacid blue assay) in MM system, D) effects on contractile activity in ESDC, E) effects on cell viability (resazurin assay) in ESDC, F) effects on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control. (C=Control).

4.4.2.3 Effects of CBZ on the size of EBs in ESDC

The treatment of CBZ 100-400μM reduced the size of EBs, after hanging drop on day 3 the size of EBs decreased as the drug dose increased. At 400μM EBs size reduced to less than half compared to Control, but still the EBs cells looked viable, containing less dark areas (necrotic cells) and few floating cells in the medium (fig 4.3A). On day five, there was not much effect on the size of EBs with 100 and 200μM CBZ, but EBs showed increased necrotic areas and floating cells in the medium. With 400μM CBZ, EB's on day five of differentiation were further reduced in size. EBs became irregular in shape, with the number of dark areas and floating cells in the medium also increased (fig 4.3B).

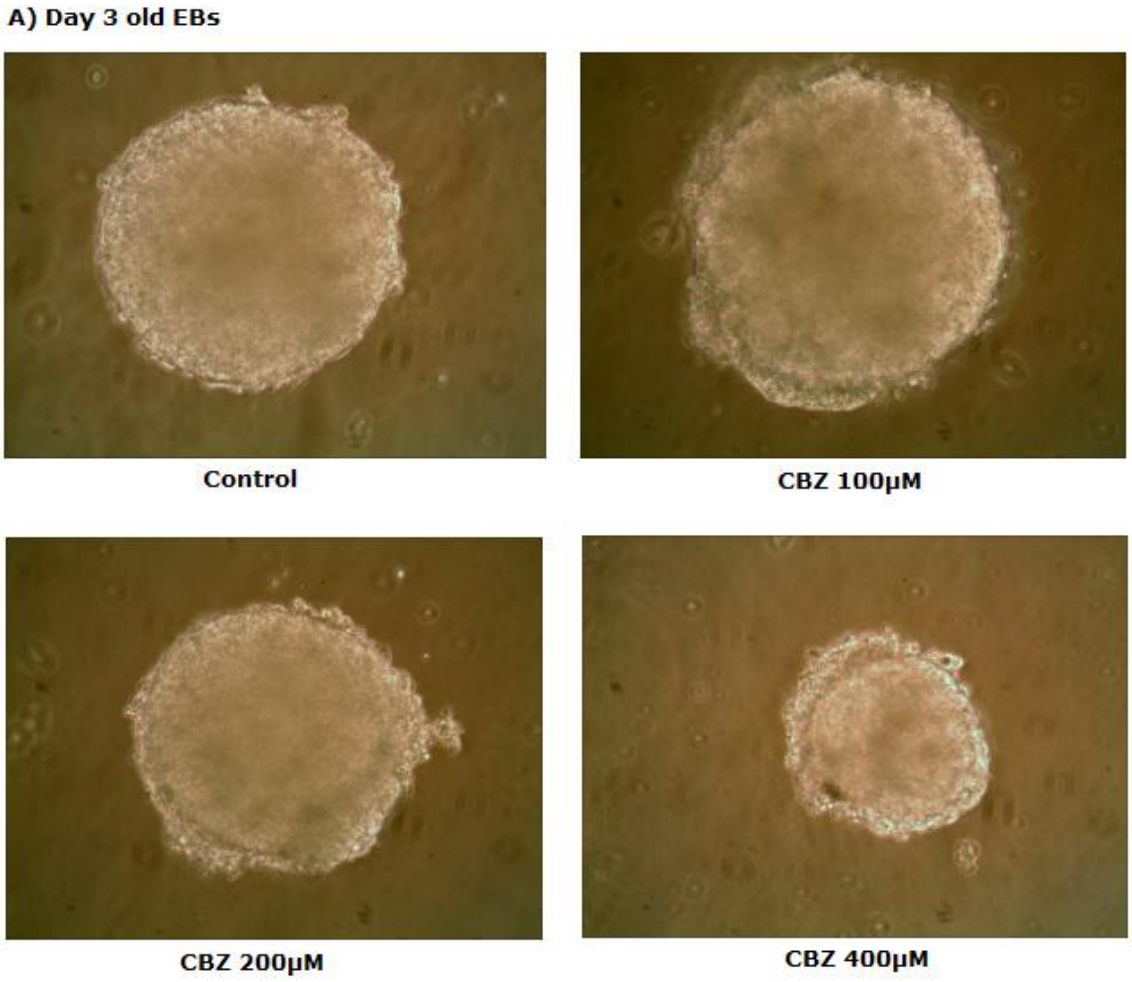


Fig 4.4A The effects of 100μM, 200μM, 400μM CBZ and untreated group (Control) on the size of EBs on Day 3 of ESDC system observed using the light microscopy (magnification ×25).

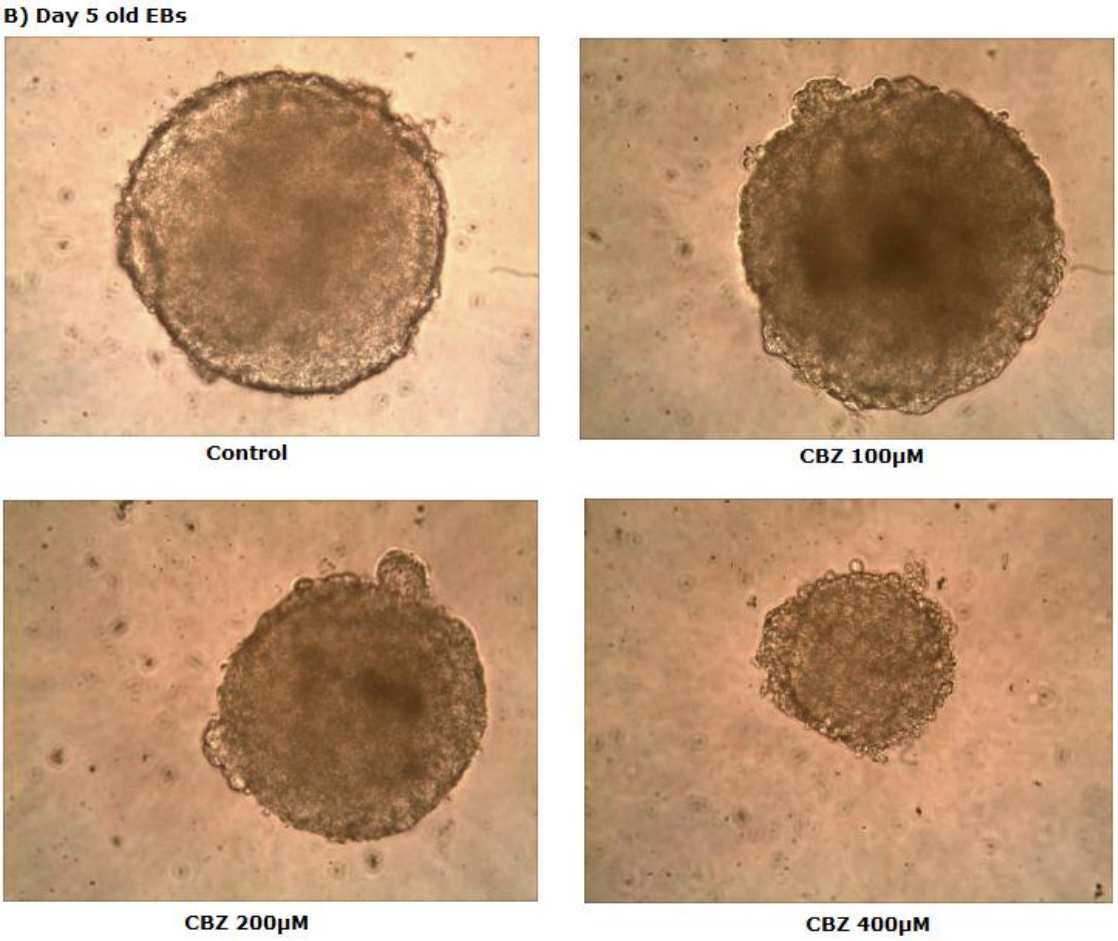


Fig 4.4B The effects of 100μM, 200μM, 400μM CBZ and untreated group (Control) on the size of EBs on Day 5 of ESDC system observed using the light microscopy (magnification ×25).

4.4.3 Phenytoin

4.4.3.1 Phenytoin effects on cardiomyocytes in the Micromass system

The third anticonvulsant molecule Phenytoin was used to treat the cultured chick embryonic cardiomyocytes in MM system at a dose ranging from 1-200μM. These doses did not show any specific trend of cytotoxicity. The conversion of resazurin to resorufin an indicator of cellular viability does not vary significantly in drug treated groups compared to control (fig 4.5B). The drug's effects on protein content which indicate cellular proliferation and total cell number also does not significantly alter after drug treatment compared to control (fig 4. 5C). PHT inhibitory effects on contractile activity at therapeutic concentrations were not significantly different from control compared to the higher doses at 200μM ($P<0.05$) (fig 4.5A).

4.4.3.2 Phenytoin effects on ES derived cardiomyocytes

When the drug Phenytoin concentration 1-200μM was applied to ESDC, the cardiomyocyte viability showed no specific declining trend with increased in drug dose. Only at 200μM Phenytoin dose some statistically significant decreased values from control was observed (fig 4.5E). The cellular protein showed no significant variation from control even at the highest dose tested (fig 4.5F). However the contractile activity of differentiating cardiomyocytes looked dose dependent. It decreased with increased in drug dose. The time of incubation does not show any significant drop in the values. 50-200μM of PHT showed statistically significant inhibitory effects on cardiomyocyte ability to contract at day 10, 11 and 12 of differentiation (fig 4.5D). The size of PHT treated EBs does not vary markedly compared to control. The cells looked healthy with less number of dark areas and floating cells.

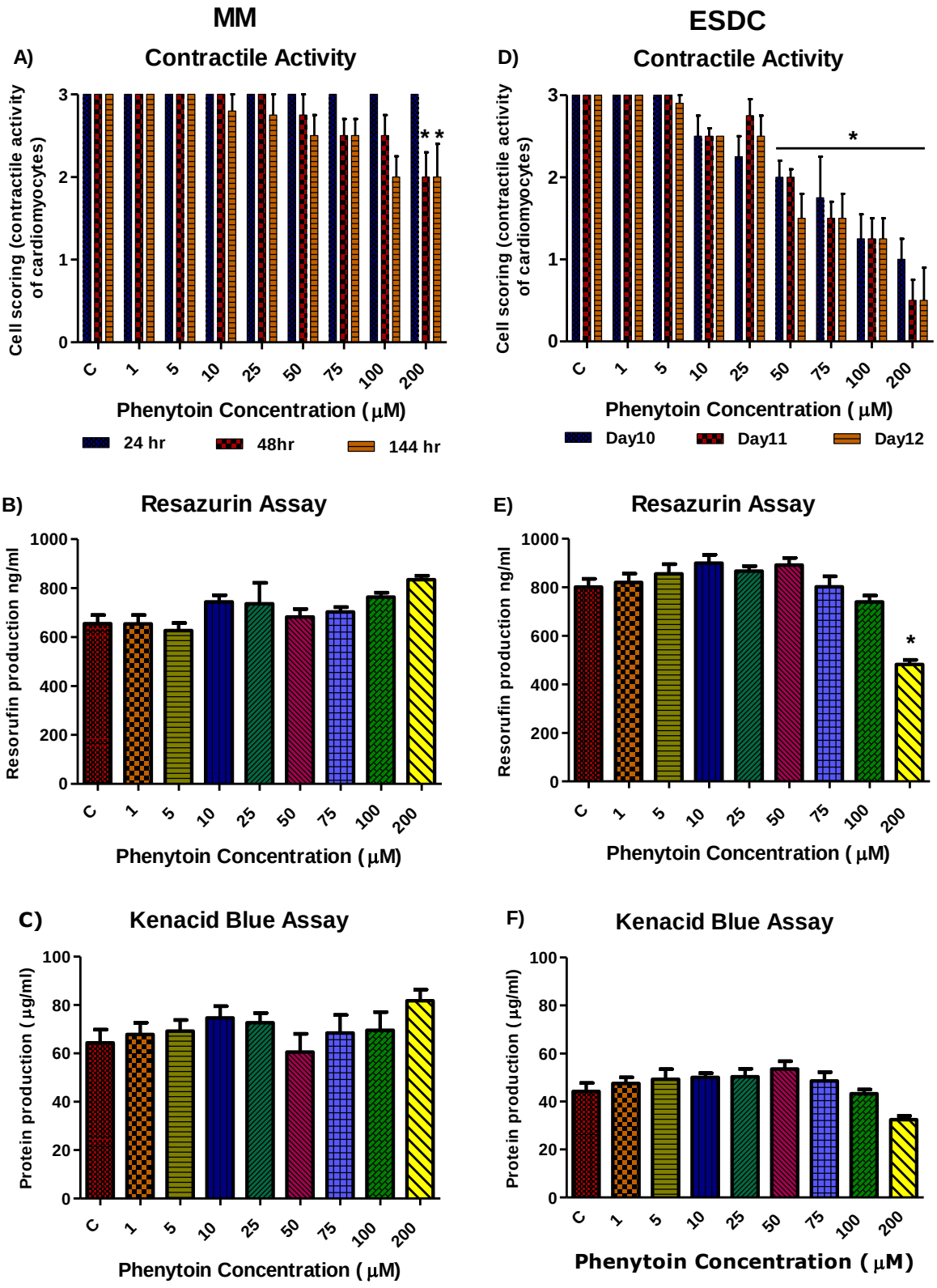


Fig 4.5 The effects of PHT on MM and ESDC systems. A) Effects on contractile activity in MM system, B) effects on cell viability (resazurin assay) in MM system, C) effects on total protein content (kenacid blue assay) in MM system, D) effects on contractile activity in ESDC, E) effects on cell viability (resazurin assay) in ESDC, F) effects on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control. (C=Control).

4.4.4 Primidone

4.4.4.1 Primidone effects on cardiomyocytes in the Micromass system

The fourth anticonvulsant drug Primidone effects on contractile activity were more pronounced in chronic exposure compared to acute at higher doses in the MM system (fig 4.6A). The drug dose 5-800μM inhibited cardiomyocyte contraction in a dose and time dependent manner. The drug dose at 48h (24h post drug incubation) does not inhibit the contractile activity significantly (except at 800μM). At 144h of cell culture, PRM 200-800μM reduces the cardiomyocyte contraction rate to less than half compared to control ($P<0.05$). The other two end points for PRM cytotoxicity estimation of cell viability and cellular protein do not exhibit any trend of significant variation from control (fig 4.6B and C).

4.4.4.2 Primidone effects on ES derived cardiomyocytes

The Primidone toxicity when tested using ES cell differentiation into cardiomyocytes showed no sign of cardiotoxicity on cardiomyocytes viability (fig 4.6E) or in cellular protein content (fig 4.6F). The drug treated group from 1-800μM showed no specific pattern of variation in resazurin assay or kenacid blue assay (fig 4.6E and F), but the cardiomyocyte contractile activity was inhibited with increase in drug dose. The decrease was not greatly different between the drug in dose in serum therapeutic levels and at 800μM. The drug dose 25-800μM showed some significantly decreased values when compared statistically with control (fig 4.6D).

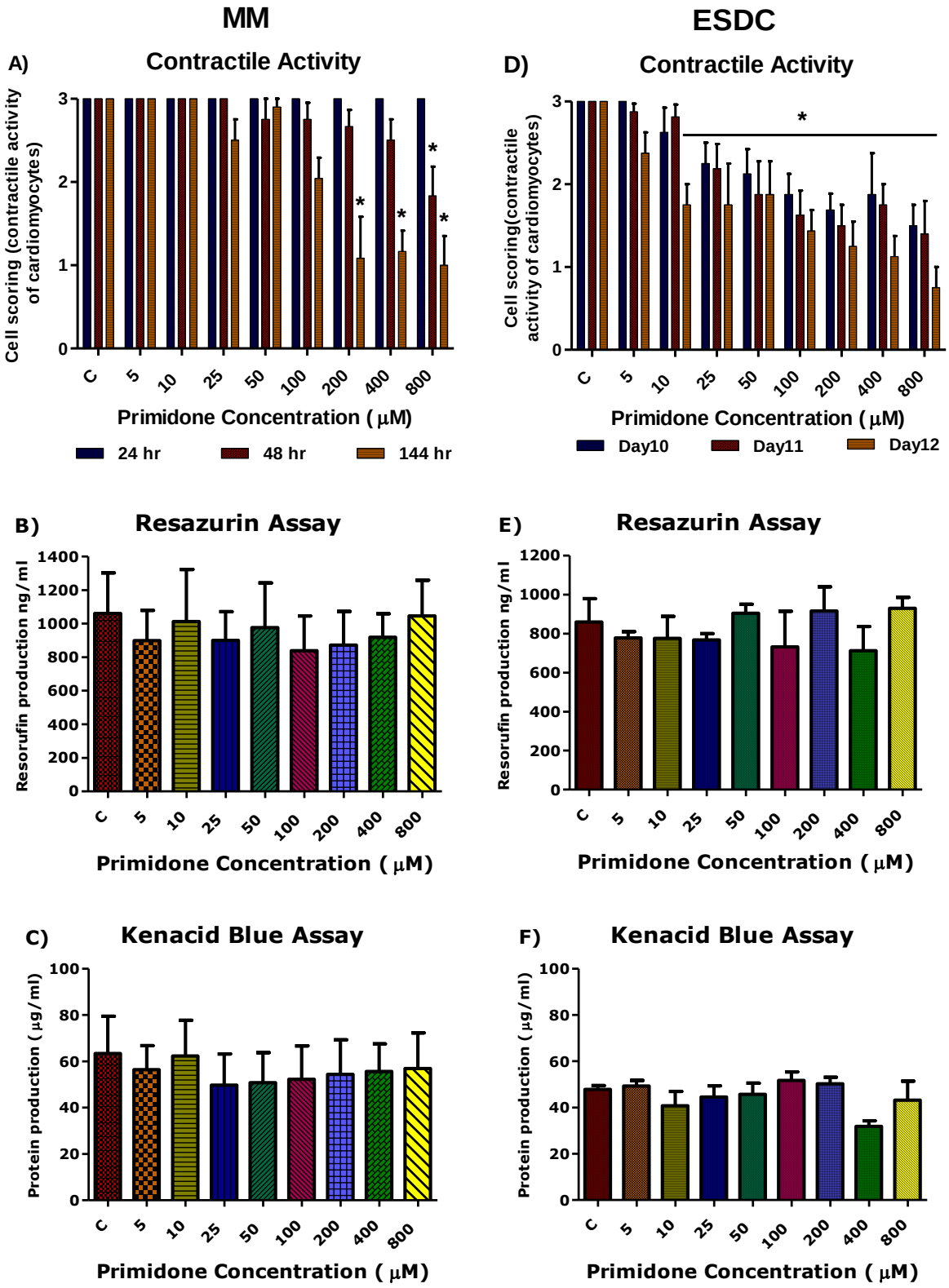


Fig 4.6 The effects of PRM on MM and ESDC systems. A) Effects on contractile activity in MM system, B) effects on cell viability (resazurin assay) in MM system, C) effects on total protein content (kenacid blue assay) in MM system, D) effects on contractile activity in ESDC, E) effects on cell viability (resazurin assay) in ESDC, F) effects on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control. (C=Control).

4.4.5 FA and AA supplements effects on Carbamazepine toxicity

4.4.5.1 Evaluation of non-toxic doses of FA and AA

The potential protective effects of FA and AA were evaluated on 200 and 400μM CBZ induced toxic effects in the MM and ESDC systems. Using ES cells different doses of FA and AA were applied to differentiating stem cells to find out the non-toxic dose. 50-500μM of both chemicals showed no signs of significant inhibition of cardiomyocyte viability or total protein content (fig 4.7B, C, E and F). Contractile activity in the 100μM AA treated group was found to be more vigorous compared to an increased dose, while the 100μM FA treated group showed no signs of inhibitory effects on contraction as compared to 250 and 500μM (fig 4.7A and D). For the MM system the same doses of FA (1mM) and AA (100μM) were applied for protective effects as were used by Memon *et al.,* 2009.

4.4.5.2 Effects of FA and AA supplements on Carbamazepine toxicity in MM system

In MM the chick cardiomyocytes were treated with 200 and 400μM of CBZ in the presence of non-toxic doses of either FA 1mM or AA 100μM. No significant improvement in contractile activity was recorded after FA and AA addition (Fig 4.8A and 4.9A). The effects of FA and AA with 200 and 400μM CBZ on cardiomyocytes viability (fig 4.8B and 4.9B) and total cellular protein contents (fig 4.8C and 4.9C) did not deviate from CBZ alone.

4.4.5.3 Effects of FA and AA supplements on Carbamazepine toxicity in ESDC

When the ES Cells were treated with 400μM CBZ in the presence of either 100μM FA or 100μM AA the differentiating stem cells showed no significant improvement in any end point (Fig 4.9A, B, C), some improvement in the size of EBs was observed with cells treated either with FA or AA (Fig 4.11). For the lower dose of CBZ 200μM, an improvement in AA treated group was observed; the cardiomyocytes were differentiated and formed beating foci compared to the drug treated group (Fig. 4.8D). The cell viability and protein content also improved with improvement in EBs size, while co administration of FA with 200 μM CBZ showed no signs of contraction or improvement in protein content, while cell viability improved (Fig.4.8E and F).

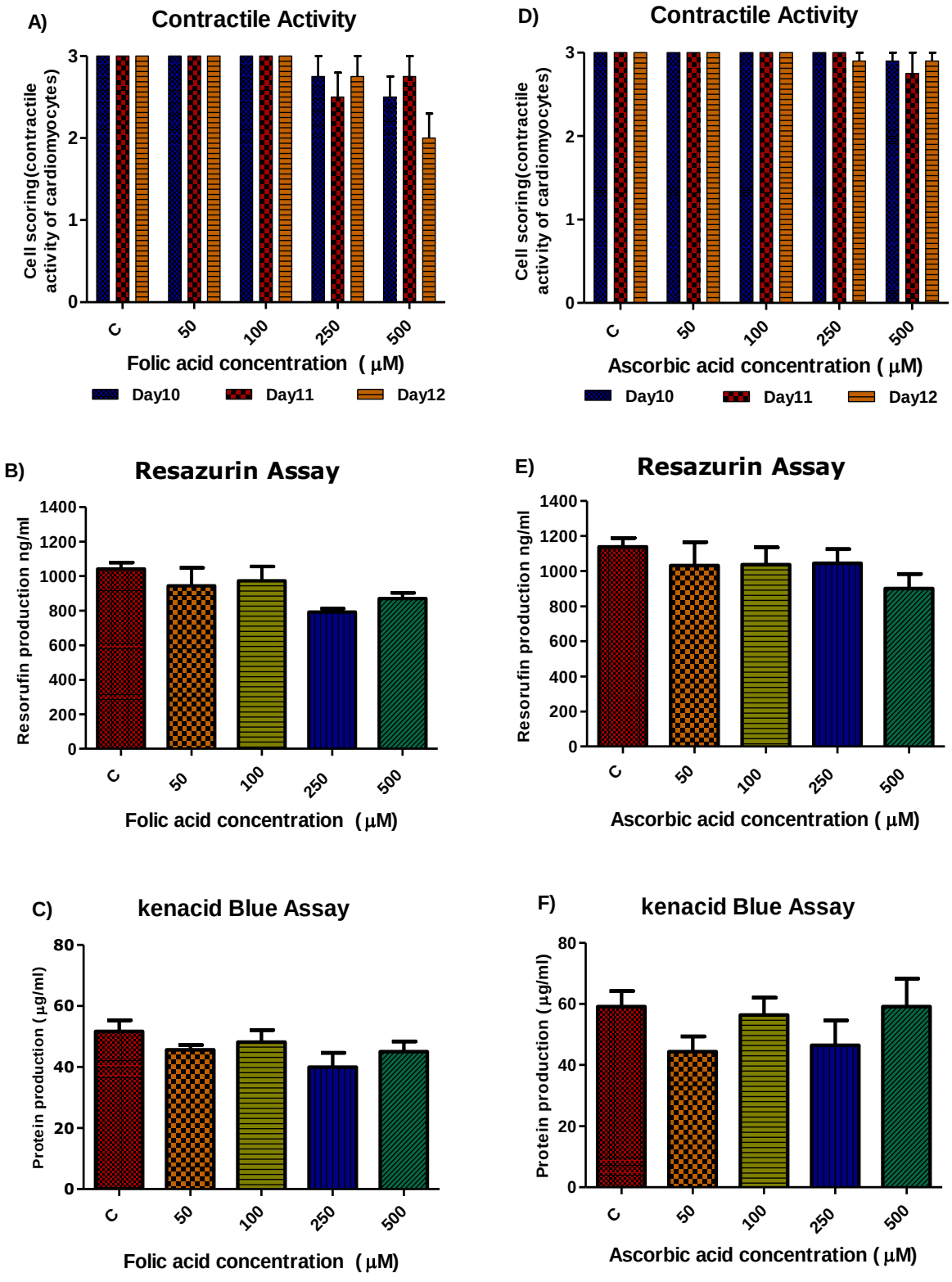


Fig 4.7 Effects of FA and AA on ESDC system. A) effects of FA on contractile activity in ESDC, B) effects of FA on cell viability (resazurin assay) in ESDC, C) effects of FA on total protein content (kenacid blue assay) in ESDC, D) effects of AA on contractile activity in ESDC, E) effects of AA on cell viability (resazurin assay) in ESDC, F) effects of AA on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control. (C=Control).

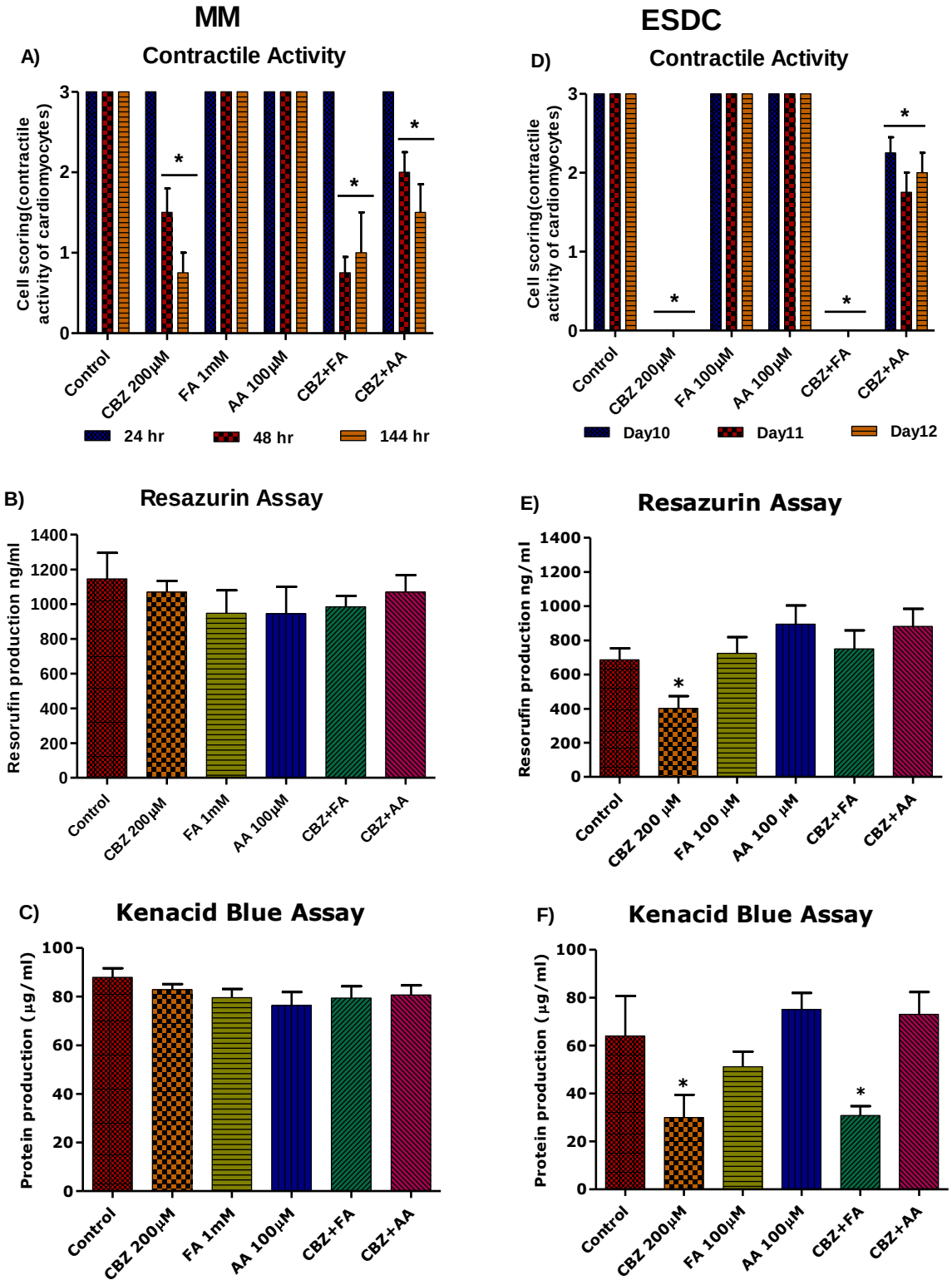


Fig 4.8 The protective effects of FA and AA on CBZ 200µM in MM and ESDC systems. A) protective effects on contractile activity in the MM system, B) protective effects on cells viability (resazurin assay) in MM system, C) protective effects on total protein content (kenacid blue assay) in MM system, D) protective effects on contractile activity in ESDC, E) protective effects on cells viability (resazurin assay) in ESDC, F) protective effects on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control (C=Control).

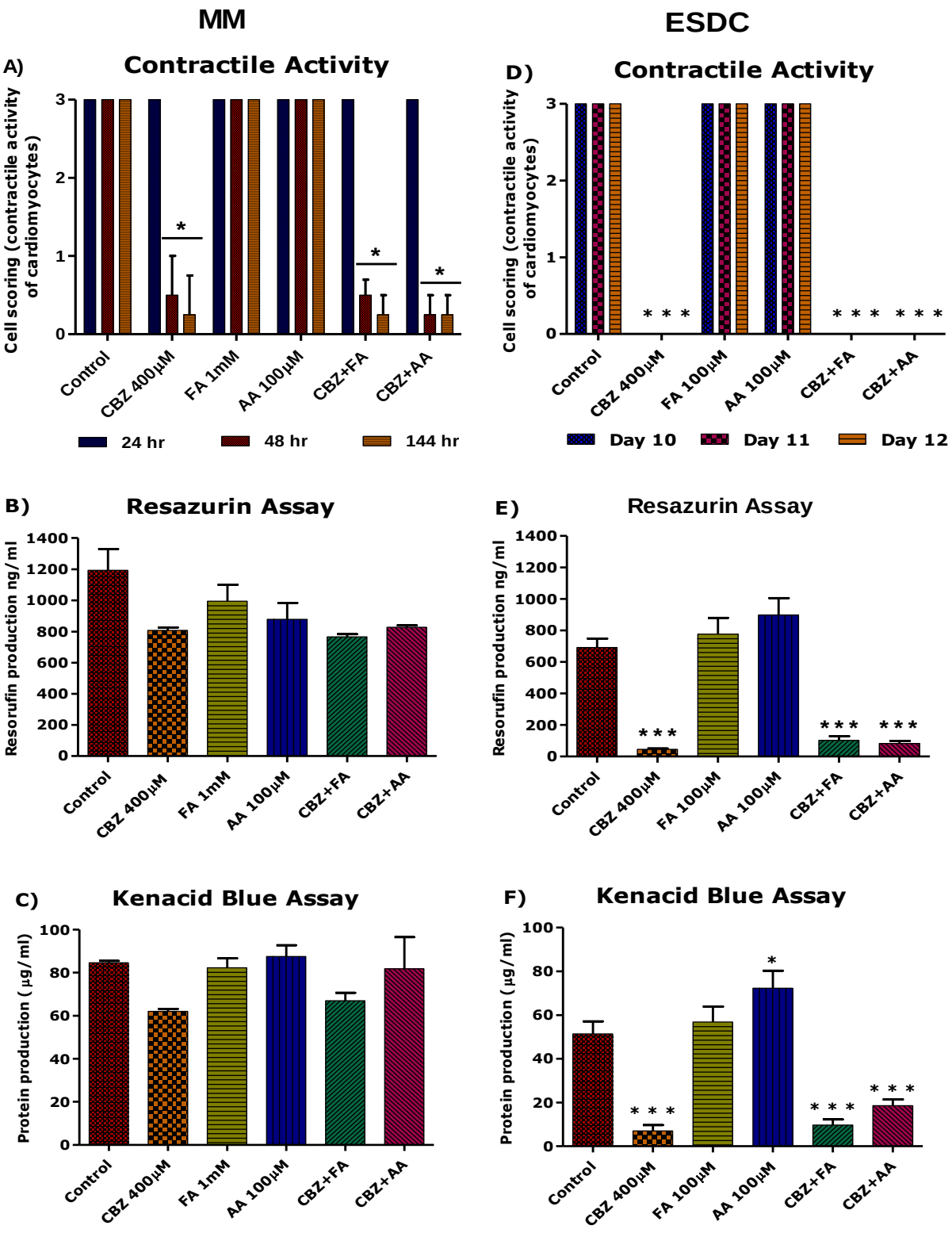


Fig 4.9 The protective effects of FA and AA on CBZ 400µM in MM and ESDC systems. A) Protective effects on contractile activity in the MM system, B) protective effects on cells viability (resazurin assay) in MM system, C) protective effects on total protein contents (kenacid blue assay) in MM system, D) protective effects on contractile activity in ESDC, E) protective effects on cell viability (resazurin assay) in ESDC, F) protective effects on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control. (C=Control).

4.4.6 Effects of Superoxide dismutase (SOD) supplements on Carbamazepine toxicity in MM and ESDC systems

In order to confirm the protective effects of AA on CBZ cytotoxicity was due to its action as an antioxidant, the antioxidant enzyme superoxide dismutase (2μM) (Memon & Pratten, 2009) was added to CBZ treated groups in both systems. In MM the contractile activity (fig. 4.10A) improved at all doses of CBZ and reached near to control, even the 400μM CBZ showed a significant improvement, while cell viability and protein content remained unaffected (fig 4.10B, C). In ESDC there was not much change in the contractile activity of cardiomyocytes, the cell viability improved compared to drug treated groups (fig 4.10D and E). The protein content remained significantly low (fig 4.10F), but the size of EBs improved with SOD treatment (fig 4.11).

4.4.7 Effects of FA, AA and SOD supplements on the size of Carbamazepin treated EBs in ESDC systems

When the FA or AA was added with CBZ the size of EBs improved. Day 3 EBs showed improved EB size especially with AA treated groups. The 400μM CBZ treated group showed the improved EB size with AA treatment, as with the other antioxidant (SOD) treated groups, while with FA the improvement was marginal (fig 4.11A). When these EBs were further cultured for two days in the presence of drugs with or without FA and AA, the 200μM group continued to grow in size and the number of dead and floating cells decreased. But at 400μM the size decreased and the floating and dead cells could be easily seen. With SOD the size of EBs improved compared to the drug treated groups (fig 4.11B).

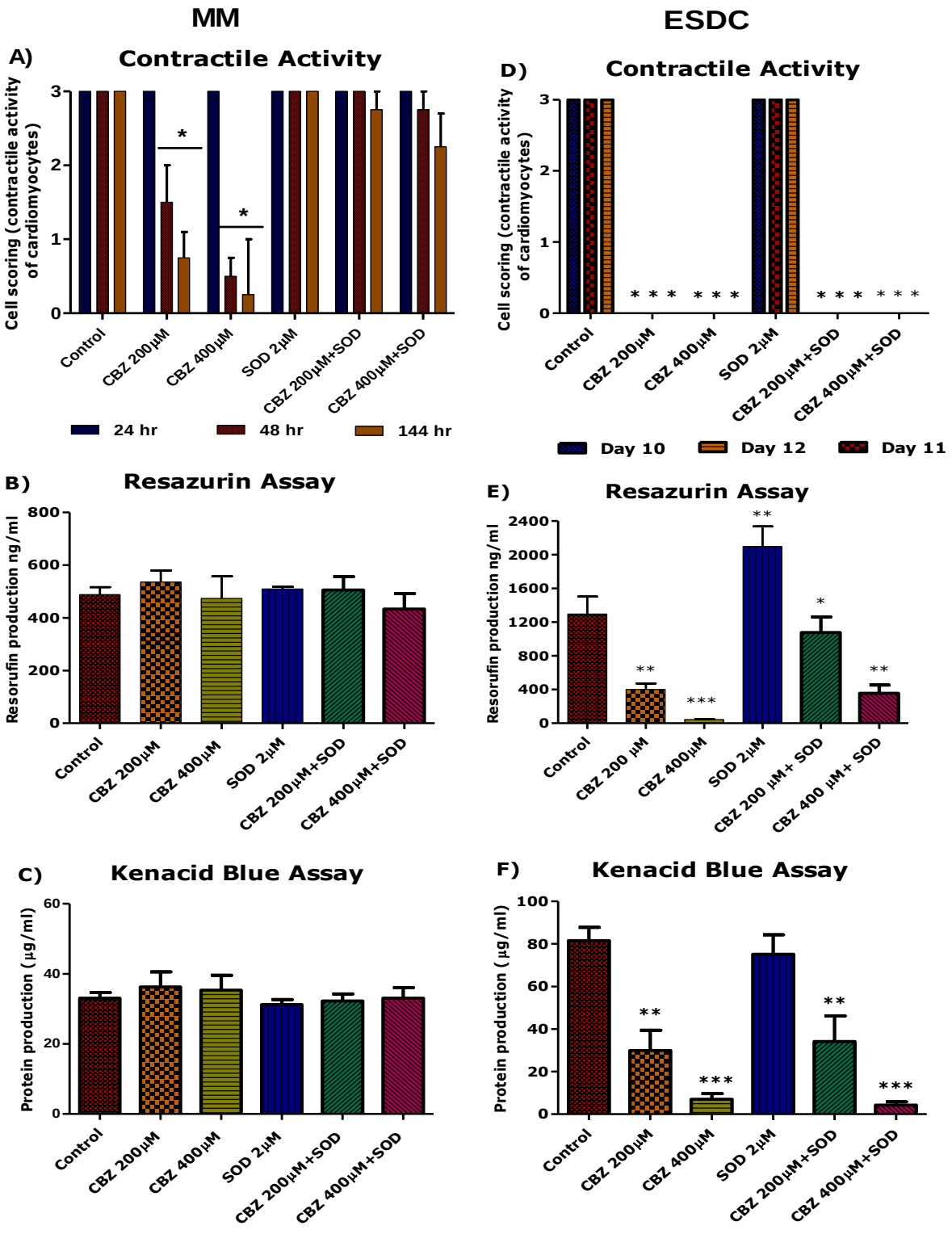


Fig 4.10 Protective effects of SOD on CBZ toxicity in MM and ESDC systems. A) Protective effects on contractile activity in the MM system, B) protective effects on cells viability (resazurin assay) in MM system, C) protective effects on total protein content (kenacid blue assay) in MM system, D) protective effects on contractile activity in ESDC, E) protective effects on cells viability (resazurin assay) in ESDC, F) protective effects on total protein content (kenacid blue assay) in ESDC. * represents the significant difference from control. (C=Control).

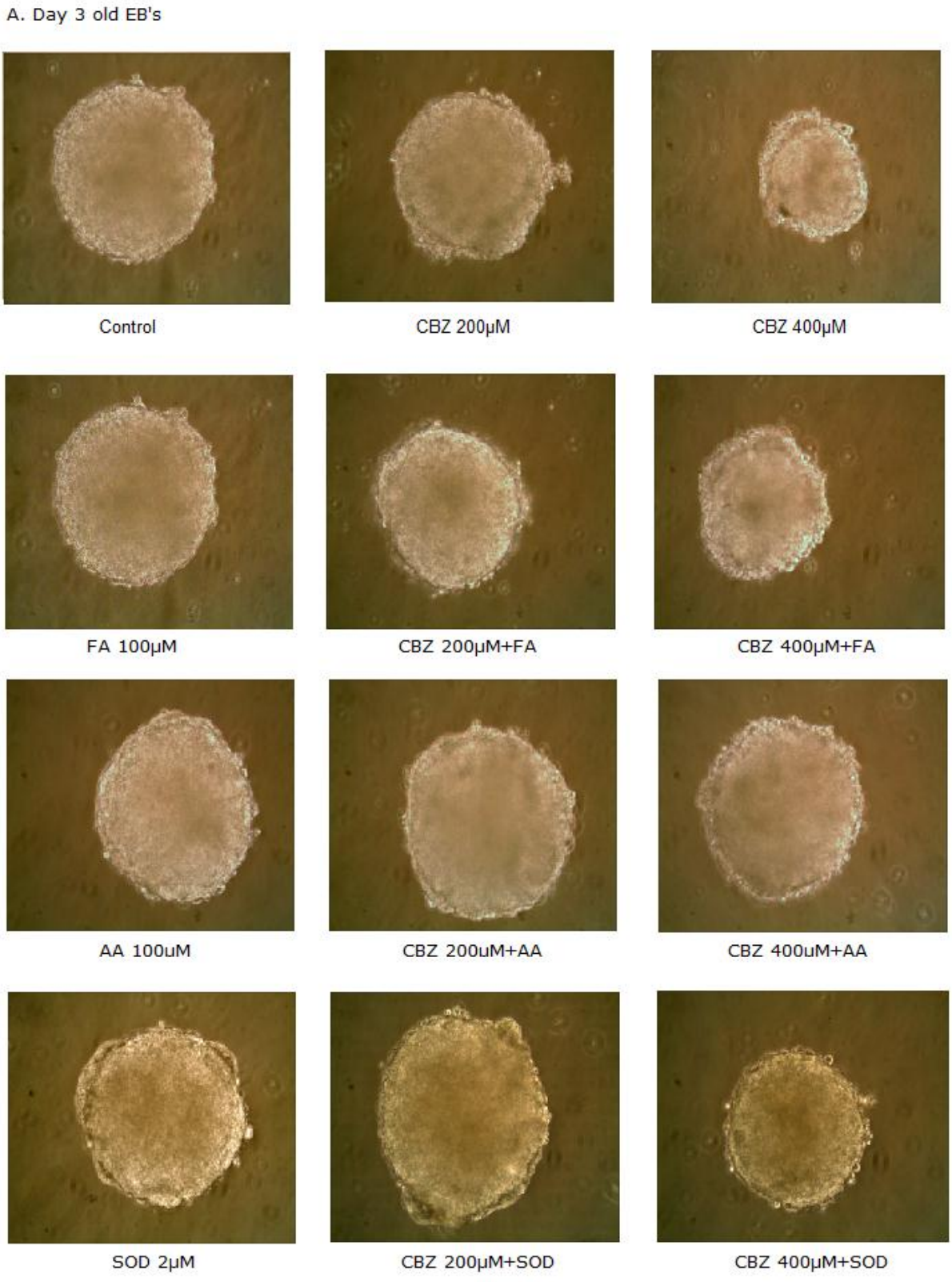


Fig 4.11A The protective effects of FA, AA and SOD on 200 µM, 400 µM CBZ and untreated group (Control) on the size of EBs at Day 3 of ESDC observed using the light microscopy (magnification ×25).

B. Day 5 old EB's

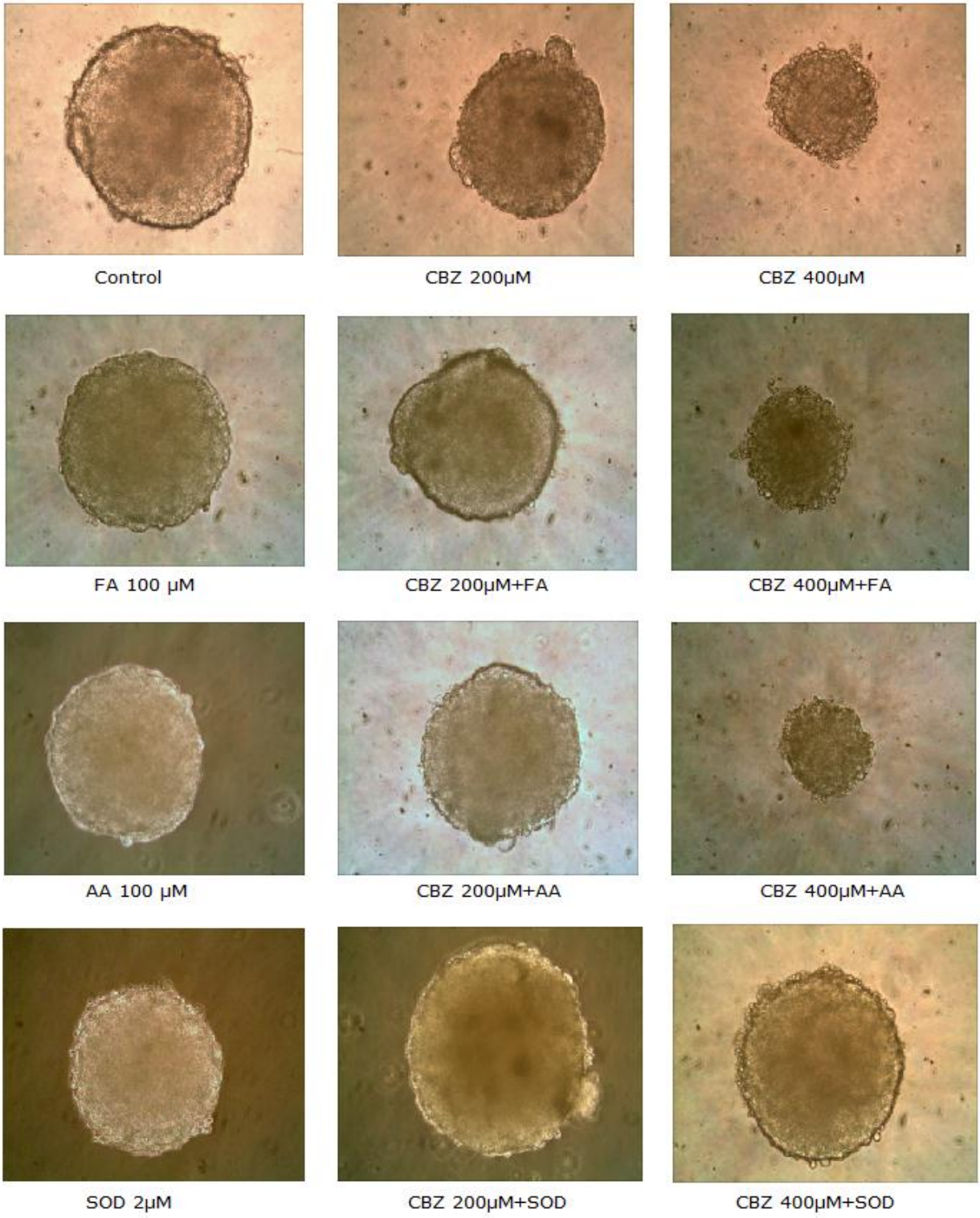


Fig 4.11B The protective effects of FA, AA and SOD on 200 μM, 400 μM CBZ and untreated group (Control) on the size of EBs at Day 5 of ESDC observed using the light microscopy (magnification ×25).

4.4.8 Effects on ROS production with drug treatment in MM and ESDC system

4.4.8.1 Effects of VPA on ROS production in MM and ESDC system

When Valproic acid was used to treat the 24h cultured chick cardiomyocytes in MM system, some increase in ROS production was observed. The increase in dose from 400 and 800μM VPA did not produced a big difference in ROS production (fig 4.12A). When the results were compared with control the increase was insignificant, while the positive control hydrogen peroxide (10μM) increased the ROS production to significant levels ($P<0.05$). In ESDC the H_2O_2 proved to be a positive control again with increased ROS values ($P<0.05$). Compared to MM, VPA in ESDC showed increased ROS production. The VPA dose 800μM showed a statistically significant increase compared to the control at 2 and 4 h of treatment (fig 4.12B).

4.4.8.2 Effects of CBZ on ROS production in MM and ESDC system

The CBZ effects in MM system were not different from VPA at the doses tested. The micromass system failed to show an increase in ROS production after 200 and 400μM treatment compared to control (fig 4.13A). The ES derived cardiomyocytes showed some significant increase with both doses. The 200 and 400μM drug showed significant ROS production compared to control, which became more evident with the time of incubation (fig 4.13B).

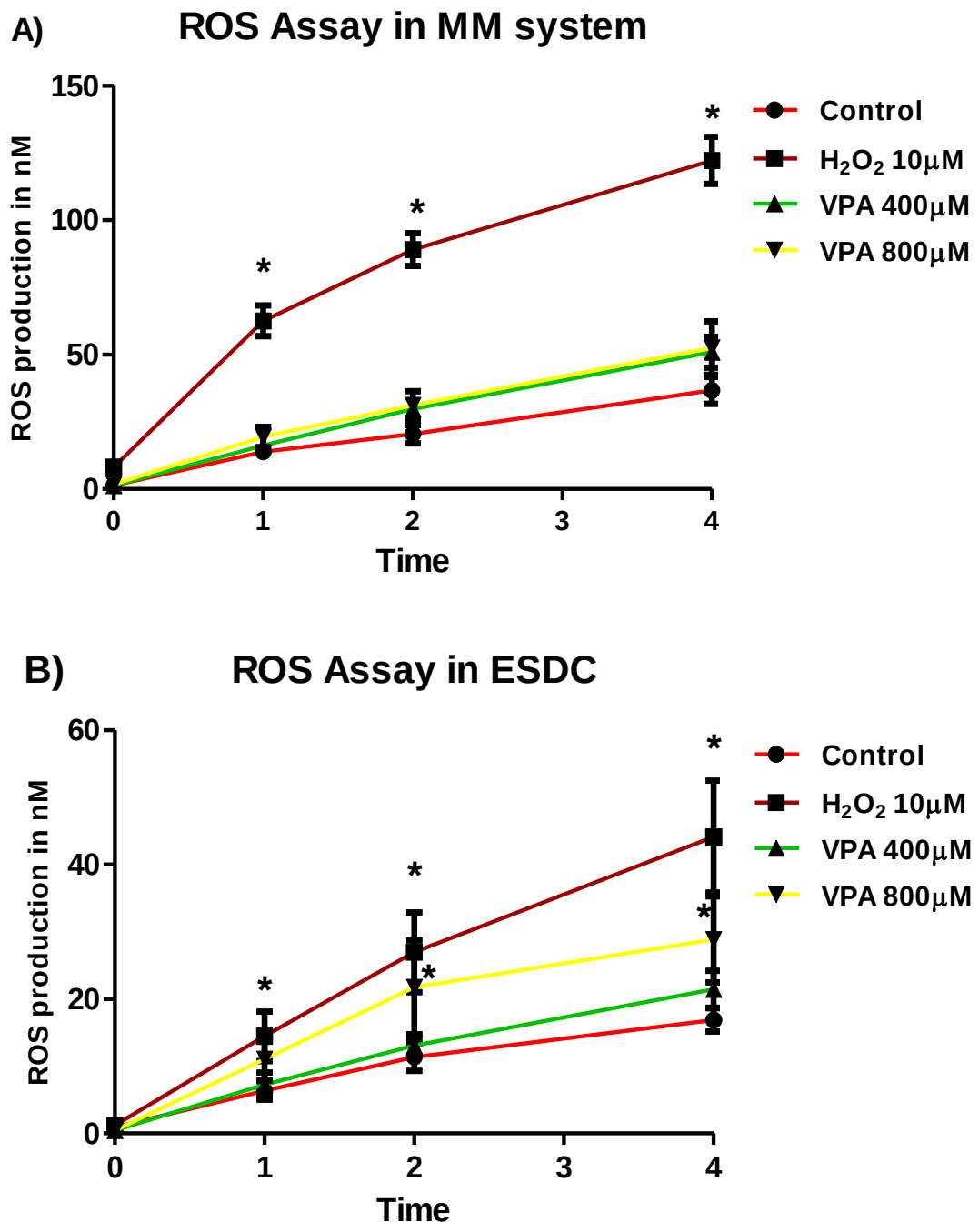


Fig 4.12 Effects of VPA ROS production in MM and ESDC system. A) Effects of VPA on ROS production in MM system and B) effects of VPA on ROS production in ESDC system. * represents the significant difference from control.

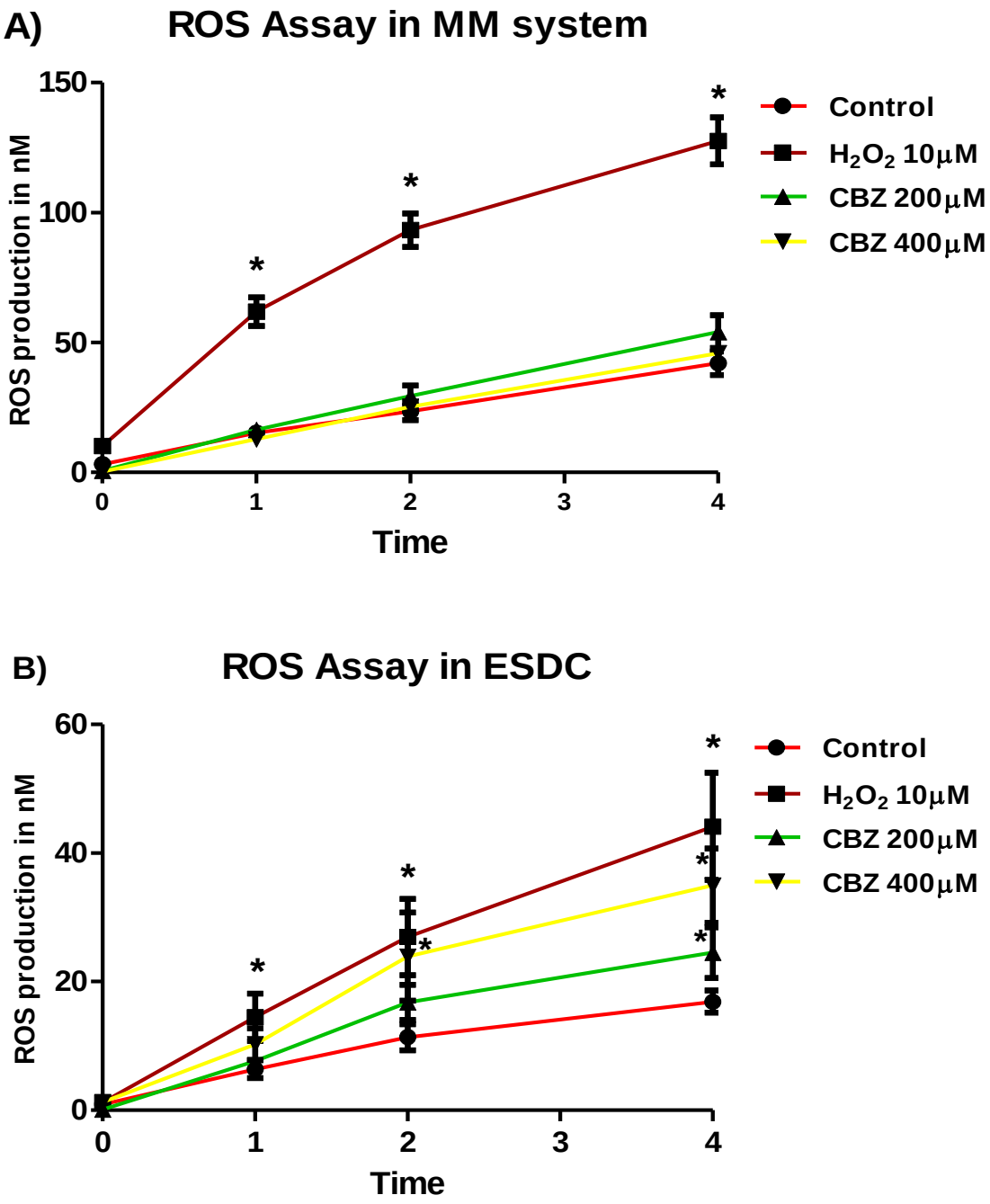
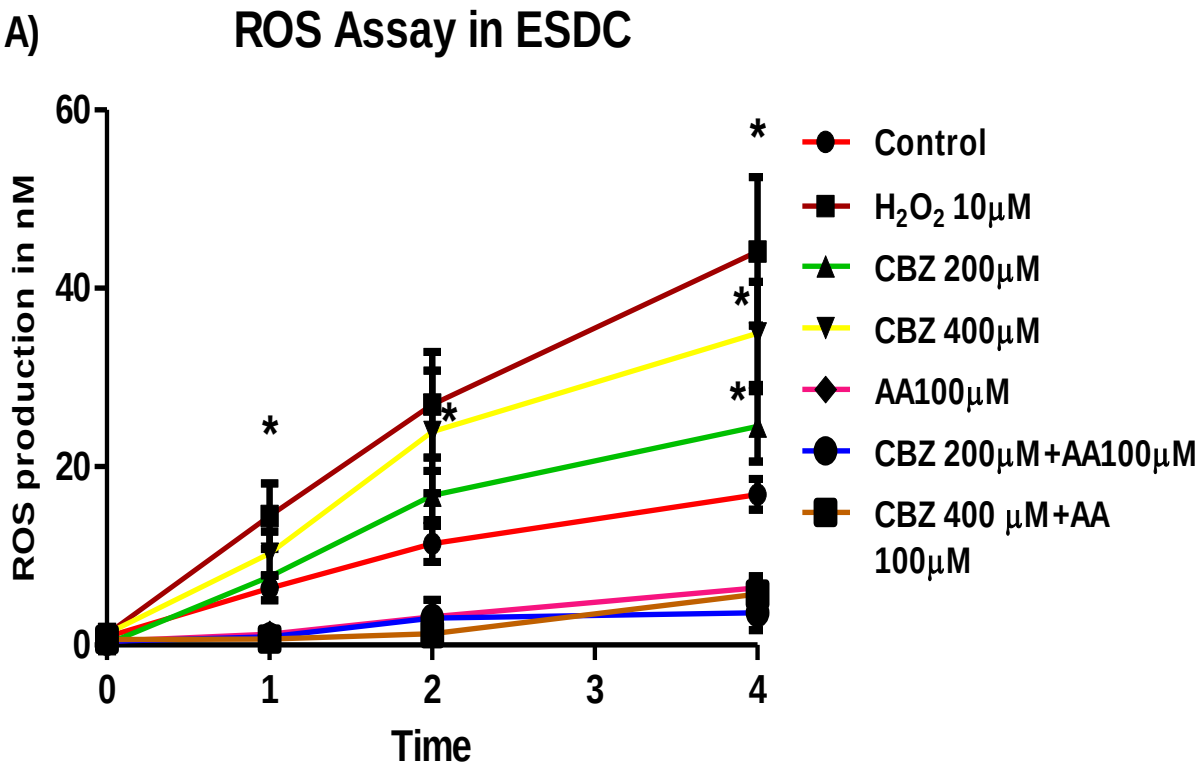
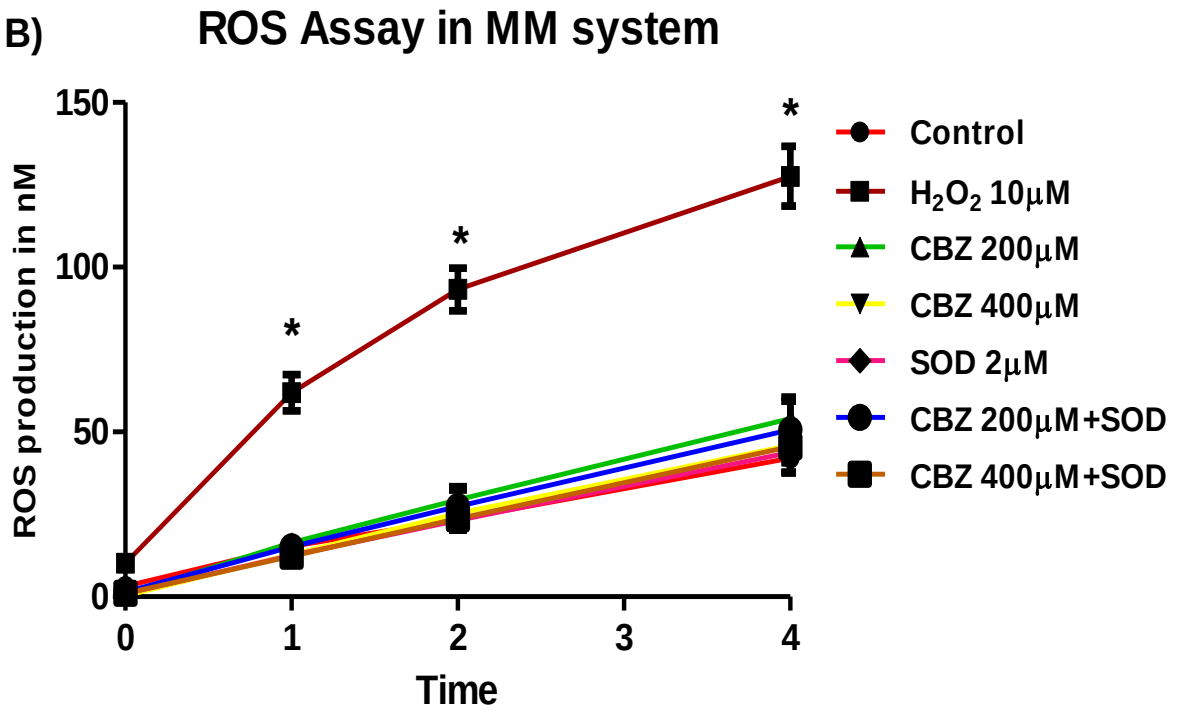


Fig 4.13 Effects of CBZ on ROS production in MM and ESDC system. A) Effects of CBZ on ROS production in MM system and B) effects of VPA on ROS production in ESDC system. * represents the significant difference from control.

4.4.8.3 Effects of AA and SOD supplements on CBZ ROS production

In the micromass system the addition of AA showed no improved contractile activity for this reason the SOD protective effects on CBZ was only evaluated. As there was no significant ROS increase with CBZ addition in MM system, the addition of SOD with CBZ in MM system did not alter the ROS levels (fig 4.14B). In ESDC AA was found to be associated with decreased ROS production, the values were lower than control. When the AA was used with CBZ 200 and 400µM in ESDC, it showed protective effects on ROS production compared to drug alone (fig 4.14A). With the other antioxidant SOD in ESDC systems a decrease in ROS production was observed and protective effects were also evident on CBZ doses (fig 4.14C).





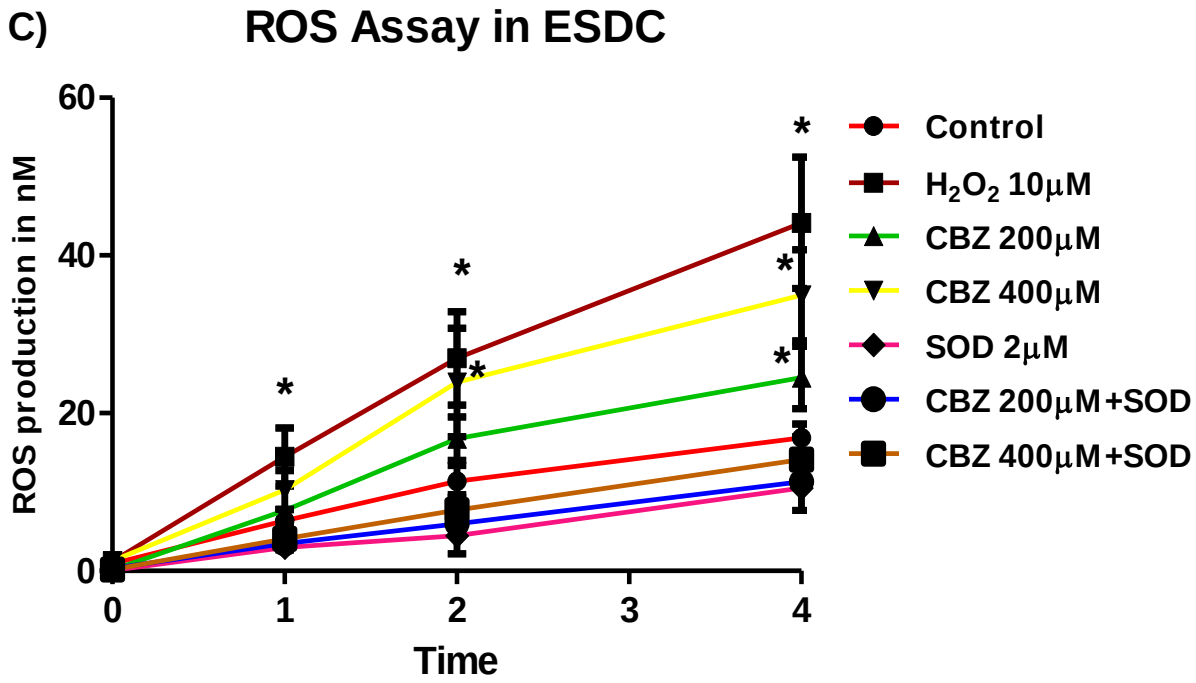


Fig 4.14 Protective effects of AA and SOD on CBZ ROS production in MM and ESDC system. A) Protective effects of AA on CBZ ROS production in ESDC system, B) protective effects of SOD on CBZ ROS production in MM system, C) protective effects of SOD on CBZ ROS production in ESDC system. * represents the significant difference from control.

4.5 Discussion

Neurological disorders like epilepsy may have profound effects on fetal development. The use of AEDs during pregnancy reduces the seizure frequency, while untreated seizures may directly or indirectly put the life of the mother and fetus at risk (Fried *et al.*, 2004). Most AEDs are teratogenic, their use during pregnancy elevates the occurrence and severity of congenital defects (Ornoy, 2009). It is now widely accepted that the combination of seizure disorders and AED use during pregnancy increases the incidence of dysmorphological characteristics in the new born (Adams *et al.*, 1990). AED exposed children exhibit some common malformations like facial dysmorphism, NTD, cognitive and behavioural defects (Ornoy, 2009). There seems to be some common mechanism between most of the AEDs. Investigation over the years have found that the AED teratogenic mechanism shows some similarities. For example most of the AEDs interrupt folic acid absorption or inhibit its metabolism. Folic acid deficiency and its relationship to congenital defects is well known (van Gelder *et al.*, 2010). AED induced NTD are prevented by folic acid supplements although the exact pathogenesis is still unclear (Dawson *et al.*, 2006; Green, 2002). Another proposed mechanism is that of highly reactive intermediate metabolites produced with most of the AEDs. These intermediates if not detoxified may lead to the formation of reactive oxygen species and in turn may cause oxidative damage (Cengiz et al., 2000; van Gelder et al., 2010; Wells et al., 2009). Also, for VPA and CBZ, inhibition of HDAC cannot be ruled out (Beutler *et al.*, 2005). Histone acetyl transferases (HATs) loosen and HDAC compacts chromatin structure and regulates gene expression. HDACs repress transcription and are involved in cell cycle regulation, proliferation and differentiation (Karamboulas *et al.*, 2006). The HDAC inhibitors interrupt the cell cycle and induce growth arrest and apoptosis (Ornoy, 2009).

In this study sodium valproate was used as a reference drug because of its known effects in both the systems (Ahir & Pratten, 2011). VPA has been used for many years for its anticonvulsant and mood stabilizing properties (Gurvich & Klein, 2002). In the past few decades its therapeutic profile has enormously extended with a new

addition of anticancer effects due to its HDAC inhibition properties (Gotfryd *et al.*, 2011). In utero exposure to VPA in humans results in neural, craniofacial, cardiovascular and skeletal malformation (Bennett *et al.*, 2000). These malformation significantly increase during first trimester administration of the drug (Gurvich & Klein, 2002).

VPA effects on the contractile activity of chick cardiomyocytes *in vitro* and cardiomyocytes derived from stem cells do not show a big variation when the doses were compared. The effects observed in both the systems were comparable with previous studies (Ahir & Pratten, 2011; Murabe *et al.*, 2007a). VPA at 800μM is a high dose compared to the drug's serum therapeutic levels, but due to the presence of high levels of VPA in umbilical cord this dose may correspond to the embryo/fetal *in vivo* effects (Ornoy, 2009). The mechanism of teratogenicity by HDAC inhibition (Blaheta & Cinatl, 2002) does not seem to be the case in the MM system, because the cardiomyocytes viability and proliferation remains unaffected, while the inhibition of HDAC results in altered cell proliferation and induction of apoptosis (Chateauvieux *et al.*, 2010; Gurvich *et al.*, 2004). VPA through HDAC inhibition also triggers DNA demethylation which inhibits maintenance of cell cycle (Chateauvieux *et al.*, 2010). Cell cycle arrest in G1 phase has already been observed with VPA derivatives in other cell lines (Gotfryd *et al.*, 2011). This kind of inhibitory effect was observed with ESDC, where decreased differentiation into contracting cardiomyocytes and cell viability was observed. Murabe in 2007b using R1 ES cell line has already proved that VPA inhibits mesodermal differentiation of embryonic stem cells. In another study it was observed that VPA at high doses inhibits ES cells differentiation into cardiomyocytes as a result of oxidative damage (Na *et al.*, 2003). The highly reactive epoxide intermediate produced during bioactivation may trigger the formation of free radical species, which affects normal development by binding to various macromolecules (Defoort *et al.*, 2006). When the reactive oxygen species production is compared in the two systems the MM cardiomyocytes do not seem to increase ROS production to significantly high levels after VPA treatment, while with EBs the ROS levels were found to be significantly increased compared to control.

This may because the cardiomyocytes in MM system were differentiated and have acquired the detoxifying mechanism against oxidative stress, while the EBs cells, which correspond to very early embryonic developmental stages have low levels of cellular antioxidants that neutralize ROS produced (Defoort *et al.*, 2006). The MM system may behave differently with ROS production if the drug dose was increased to high levels which would not be therapeutically relevant. The effects on contractile activity may be the result of disturbances in establishing the contractile system either by gap junctions or some other mechanism. Beside these, VPA also disturbs the FA cycle (Chateauvieux *et al.*, 2010) along with a number of signalling molecules that may affect cell differentiation (Gurvich *et al.*, 2004). Folic acid supplements, free radical scavengers and antioxidant enzymes were found to counteract VPA-induced teratogenic effects (Defoort *et al.*, 2006; Na *et al.*, 2003; Ornoy, 2009; Zhang *et al.*, 2010).

Pregnant epileptic women on CBZ therapy may have a normal outcome, because they may be less susceptible to CBZ teratogenicity either by altered epoxide hydrolase enzyme levels or polymorphisms in folate metabolism associated genes (Nicolai *et al.*, 2008; van Gelder *et al.*, 2010), but generally a higher rate of cardiovascular malformations and NTDs are experienced with CBZ treated mothers compared to control groups (Hansen *et al.*, 1996; Murabe *et al.*, 2007b). The teratogenic mechanisms of CBZ can be either by inhibition of FA absorption (van Gelder *et al.*, 2010), epoxide intermediates (Lindhout *et al.*, 1984), HDAC inhibition (Beutler *et al.*, 2005) or increased free radical production. As it is believed that the *in vitro* system lacks drug metabolising systems, the extent of epoxide intermediate production is unknown. HDAC inhibitory effects are already established, as with valproic acid (Beutler *et al.*, 2005; Murabe *et al.*, 2007b). The remaining two mechanism of teratogenicity were counteracted in this study by direct addition of FA and AA or the antioxidant enzyme superoxide dismutase to cardiomyocytes in culture.

CBZ at 200μM-400μM caused statistically significant inhibitory effects on cardiomyocyte contractile activity in primary culture. The results clearly show that

the drug does not inhibit cardiomyocyte proliferation nor cause growth arrest at higher doses, but only interferes with the process that involves the cardiomyocytes re-establishing the beating process. In ESDC, at higher doses, CBZ inhibitory effects were observed for all end points. Beating was completely stopped at 200μM, which indicates the drug's teratogenic effects on cardiomyocyte differentiation at higher doses. The CBZ IC50 value for cell viability (0.31mM) observed by Murabe in ESDC using the R1 cell line is well below that observed here (Murabe *et al.*, 2007b). The antiproliferative activity of CBZ increased with increased dose. According to Peres Martin, at higher doses (400μM), CBZ arrests cell division from metaphase to anaphase, by forming monopolar spindles and impaired centrosome separation (Perez Martin *et al.*, 2008). These results were consistent with the findings of decreased cell number by total protein content estimation and reduced EBs size, which indicated decreased cell division. The other mechanism of toxicity could be oxidation of DNA by hydroxyl free radicals to form 8-oxoguanine, which is involved in producing mutation and teratogenesis (Wells *et al.*, 2009). It may be that CBZ use before the start of organogenesis is more toxic to cardiogenesis than its use at the later stages of gestation. The HDAC inhibitory effects of CBZ cannot be ruled out in case of ESDC due to decreased cell proliferation (Karamboulas *et al.*, 2006).

FA and AA were used in an attempt to counteract the embryotoxic effects of CBZ at 200-400μM. In MM, the addition of FA and AA showed no signs of improvement in the contractile ability of the cardiomyocytes. This could be because homocysteine is not involved in the inhibitory mechanism or these molecules were not sufficient to prevent damage to the contractile activity mechanism. Alternatively, it may involve some other pathways or some disturbance in gap junction communications. However, the addition of SOD in MM improved the contractile ability of the cardiomyocytes even in the CBZ 400μM treated group. This may suggest that superoxide free radicals produced by oxidative stress were involved in the inhibitory mechanism of contraction and these were detoxified by SOD, or the increased production of peroxide by SOD promotes cardiogenesis (Crespo *et al.*, 2010) or

increases Ca^{2+} release, which ultimately led to increased contractile activity (Xi *et al.*, 1999).

FA (100μM) and AA (100μM) addition to the CBZ treated groups in ESDC showed a different result compared to MM system. The stem cells, especially when treated with AA, showed very fast beating foci compared to control, which totally supports the idea that AA addition could increase cardiogenesis *in vitro*. This, on the other hand, deviates from the previously reported antioxidant inhibitory effects on cardiogenesis (Takahashi *et al.*, 2003). AA acts as pro-oxidant in the presence of transition metals, AA increases the levels of peroxides which is found to be cardiogenic in stem cells differentiation (Crespo *et al.*, 2010). The addition of the antioxidant AA to the 200μM CBZ treated group's induced contractile activity, and improved cell viability, protein content and size of EBs. In the 400μM CBZ treated groups there was not much improvement compared to control and it may be that the AA antioxidant effect, or induction of cardiogenesis, was not sufficient to reverse the growth arrest of cardiomyocytes. The addition of SOD to CBZ groups showed no improvement signs in contractile activity but the cell viability and the size of EBs improved on day 3 and 5 of treatment. In both the case of MM and ESDC the production of peroxide was critical for the initiation of cardiogenesis signalling or induction of contractile activity either by direct conversion of superoxide anions by SOD or the increased production of peroxide by some extrinsic factors like Ascorbic acid (fig 4.14). FA in ESDC showed some protective effects on cell viability and protein content, especially with CBZ 200μM treated group. The CBZ impairment of FA transfer inside the rapidly dividing cells might have affected their growth.

When the reactive oxygen species production was measured in both the systems, the CBZ treated chick cardiomyocytes showed no significant increase. However, the increase ROS observed in ESDC with CBZ treatment indicates that ROS production is the mechanism that inhibits cardiogenesis and induces teratogenesis. As stated earlier the early stage cells exhibit low levels of antioxidant enzymes and a weak defence system (Defoort *et al.*, 2006; van Gelder *et al.*, 2010), which was not sufficient in the case of ESDC to neutralize the excessive ROS produced. In the case

of ESDC the ROS production in the presence of AA was below the control level, but the AA treated ESDC contracts with full pace at all days of scoring (fig 4.8D and 4.9D). With ESDC AA showed protective effects on CBZ produced ROS which was evident with the results observed after ES differentiation. The CBZ 200μM treated ESDC were rescued by AA antioxidant effects, while the ROS level after AA treatment did not look different in either dose. It may be that CBZ 400μM along with ROS production also induces growth arrest from metaphase to anaphase, by forming monopolar spindles and impaired centrosome separation (Perez Martin *et al.*, 2008). The difference in effects of AA in the MM system may be due to different stages of development or the low level of H_2O_2 produced has a role in cardiogenesis induction. As it is observed that undifferentiated ES cell do not sense ROS production mechanism even with H_2O_2 (data not shown here). In MM system the SOD protected the drug's effects on contractile activity that might be its effects on detoxification and conversion of superoxide's into peroxides, which indirectly protects the other mechanism interfering contractile activity. But in ESDC SOD failed to recover lost contractile compare to AA which has cardiogenic induction properties.

Putting it together it is concluded that CBZ teratogenic effects are dose and developmental stage dependent. The CBZ teratogenic mechanism is mainly due to induction of oxidative stress through increased ROS production in both the systems. The ESDC were affected directly by high levels of ROS production while low levels of ROS increase might have affected gap junctional communication or some other mechanism indirectly in MM system. This indicates that inhibition of cardiogenesis at low doses involves a mechanism that can be improved by production of peroxide by the addition of AA, but for the induction of contractile activity in MM the detoxification of the superoxide anion is important. The antioxidant enzyme SOD can rescue the later stage oxidative stress, which might be because of the differentiated cells' ability to respond SOD effects. The AA induced cardiomyocyte differentiation does not depend on its antioxidant properties solely but its ability to promote cardiac differentiation through peroxide mediated pathways which cannot protect growth arrest at higher doses.

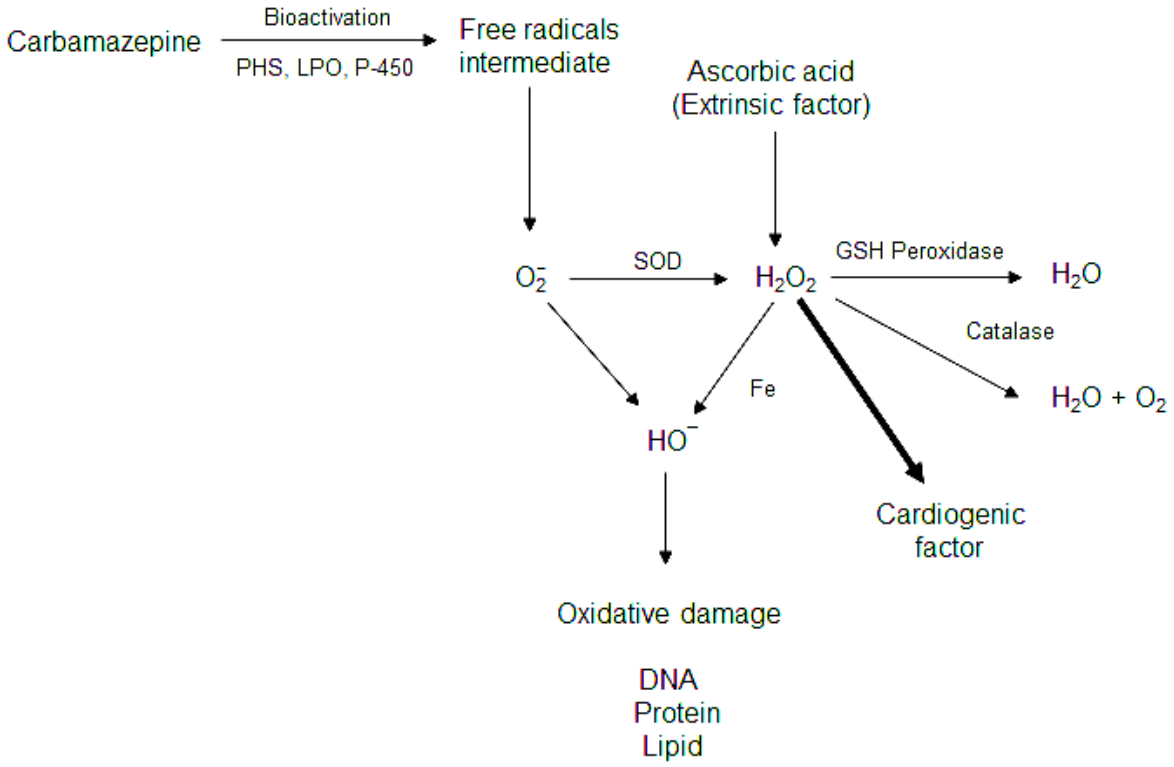


Fig 4.14 Biochemical pathways for carbamazepine metabolism to free radical intermediates and detoxification by antioxidants. PHS=prostaglandin H synthase, LPO=lipoxygenase, P450=cytochrome P450, SOD=superoxide dismutase, O^{-}_2=superoxide anion, H_2O_2=hydrogen peroxide, Fe=iron, HO^-=hydroxyl anion, (modified from wells et al (Wells *et al.*, 2009)).

The third anticonvulsant molecule tested in this study was a hydantoin derivative (Tiboni *et al.*, 2003), the diphenylhydantoin or commonly known as phenytoin. PHT has been used as an antiepileptic drug nearly for half a century (Temiz *et al.*, 2009). The drug mainly acts by affecting sodium depolarization (Tunnicliff, 1996). Previous studies confirmed the structural and behavioural teratogenic properties of PHT in both human and animals. Like other AEDs, PHT also crosses the placenta and produces the specific fetal hydantoin syndrome (Tiboni *et al.*, 2003). Phenytoin affects the developing heart at a sensitive stage by inducing hypoxia/ischemia, which relates to PHT induced teratogenic effects (Okruhlicová *et al.*, 2003).

PHT in both the systems inhibited the cardiomyocytes contractile activity. In the MM system the same effects were observed at 4 times higher dose compared to the ESDC system. Earlier studies confirm PHT dose-dependent induced bradycardia and

arrhythmias in the mouse (Danielsson *et al.*, 1997). Danielson in 2007 also reported that PHT caused irregular embryonic heart rhythm by blocking special K^+ channels at 100μM and above. Therefore, it may be hypothesized that PHT inhibits the contractile activity by blocking the potassium channel (IKr) in the ESDC and chick heart MM culture system (Danielsson *et al.*, 2007).

The PHT toxic mechanism is mainly attributed to its reactive intermediate metabolite production and folic acid inhibition (Ozolins *et al.*, 1995; Temiz *et al.*, 2009; Winn & Wells, 1996). If the electrophilic arene oxide intermediates are not detoxified by epoxide hydrolase or by glutathione S-transferases they may lead to the formation of ROS thereby initiating teratogenesis (Ozolins *et al.*, 1995). The epoxide can be bioactive-reactive chemically with nucleophilic centres in nucleic acid (DNA, RNA), lipids, or proteins-resulting in a variety of toxic effects including necrosis, mutagenesis, carcinogenesis, or teratogenesis. Formation of a trans-dihydrodiol by microsomal epoxide hydrolase catalysed hydrolysis of an electrophilic epoxide may deactivate the metabolite and decrease the risk of cellular damage (Hartsfield *et al.*, 1995). When the highly reactive arene oxide intermediates are detoxified the oxidative balance is not disturbed and cells function normally. But there are some reports of receptor-mediated effects of unmetabolized phenytoin (Ozolins *et al.*, 1995). Several studies confirm increased ROS production by PHT through the production of intermediate metabolites which are responsible for initiating teratogenesis (Parman *et al.*, 1998). The *in vitro* system lacks the drug bioactivation system which results in no production of arene oxide intermediates but studies confirm the increased ROS production with PHT treatment in the *in vitro* system (Abramov & Wells, 2011; Gallagher & Sheehy, 2001; Parman *et al.*, 1998). The effects on cardiac development may be the result of increased ROS production, which impairs macromolecules like proteins and DNA by irreversible binding (Parman *et al.*, 1998). In this study the PHT effects on cell viability and protein content observed in the MM and ESDC system were not very different from control. The ESDC with 200μM dose showed some reduced cell viability and some insignificant trend of reduced cell proliferation. These altered effects were expected at high doses

(Okruhlicová *et al.*, 2003) but in differentiated cells of MM system the PHT at these high doses failed to produce toxic effects on cell viability and proliferation. Some *in vitro* studies confirm these results of reduced ES differentiation into cardiomyocytes without affecting cell activity (zur Nieden *et al.*, 2001). Using the CHO cell line Winn observed no significant effects on cell viability at 240μM but the effects became significant at 800μM and inversely increased oxidation of DNA (Winn *et al.*, 2003). The other proposed mechanism of folic acid inhibition of intestinal absorption by PHT (Ariel *et al.*, 1982) would not be relevant in the *in vitro* system. This is confirmed by the use of FA with PHT resulting in no change to the protective effects or enhancement in teratogenicity (Azarbayjani *et al.*, 2006).

The species and strain differences with PHT induced teratogenesis is mainly the results of different enzyme levels which detoxifies the toxic intermediates (Danielsson *et al.*, 2007). The possibly of multiple teratogenic mechanisms in case of PHT, which depends on gestational age, tissue type and drug dose (Ozolins *et al.*, 1995), seems likely. The extent of the embryotoxic effect of PHT at similar doses *in vivo* and *in vitro* was not comparable, which may be explained by differences in exposure characteristics (Beekhuijzen *et al.*, 2000)

The fourth anticonvulsant molecule in this study was primidone. In the body it is metabolized to PEMA and phenobarbital. The phenobarbital is thought to be responsible for its activity (Pizzi *et al.*, 1998). In both systems PRM inhibited contractile activity. The inhibition in contractile activity observed in MM system was very much dependent on the time of exposure with the drug dose. The contractile activity at 200-800μM PRM, which is a very high dose, showed the same values of contractile activity at 144h of cell culture. This means the dose increase after 200μM showed the same toxic effects on chronic exposures. But *in vivo* studies showed toxic effects even at non-toxic doses (McElhattn et al., 1977) which is not observed in case of MM system. When the effect on contractile activity was recorded in ESDC the results were dose dependent the contractile activity drops down with increased dose. But the inhibitory effects were observed at very low doses compares to MM system. The contractile activity inhibition results in ESDC resemble *in vivo* toxic

doses (McElhattn et al., 1977). The viability and cell proliferation remain unaltered in both systems. The common mechanism for most AEDs of folic acid interference and ROS production (van Gelder *et al.*, 2010) might be not be responsible for the contractile activity inhibition. Since folic acid inhibition affects cell proliferation, which does not show any trend in either the system. Nor does oxidative stress look to be a likely candidate for cell viability and proliferation effects. Maybe the low levels of ROS produced disturb the oxidative balance after prolonged exposure and affects cell contractile activity by some molecular mechanism or disturbance in gap junction communication. As stated earlier in the *in vivo* system PRM effects are mainly due to its metabolite phenobarbital (Pizzi *et al.*, 1998) but here in the *in vitro* system there is no PRM bioactivation, so the results are effects of PRM itself. The PRM compound itself showed anticonvulsant effects, while its metabolite phenobarbital along with anticonvulsant effects showed decreased cardiomyocytes contractile activity in MM and ESDC systems (Ahir, 2009).

The complexity of epilepsy and AEDs induced teratogenic effects remains unresolved. From the results observed in study it can be concluded that antiepileptic drugs affect the cardiomyocyte differentiation at an early stage more pronouncedly compared to late developmental stage cells. The potential teratogenic mechanism differs between drugs but mostly involves disturbance in cellular oxidative state or FA acid pathway. These toxic mechanisms can be counteracted by adding protective molecules to the culture system.

Chapter 5

Evaluation and comparison of antidepressant teratogenic effects in chick cardiomyocyte micromass system and embryonic stem cell derived cardiomyocyte

5.1 Depression and Antidepressants in Gestation

Statistically, women have a higher life time risk of developing major depression (10-25%), with a peak prevalence during childbearing age (Alwan *et al.*, 2007; Bonari *et al.*, 2004a). 1 in 5 pregnant women experience depression but fewer are treated (Bonari *et al.*, 2004b). Major depression significantly increases the morbidity rate in mothers and shows adverse effects on the developing fetus (Bellantuono *et al.*, 2007). The fetuses and neonates of depressed mothers differ biochemically, physiologically and behaviourally (Field *et al.*, 2004). These psychopathologic symptoms during pregnancy may have physiological effects on the developing fetus indirectly by the mother's abnormally depressed behaviour causing an increased consumption of cigarettes and alcohol during pregnancy or even a suicide attempt (Bonari *et al.*, 2004b). These may confound the pregnancy outcome. Untreated maternal depression may lead to spontaneous abortions, low birth weight, small head circumference, increased uterine resistance, low Apgar score, preterm delivery, neonatal retardation and high cholesterol levels in the new born, as well as hypertension and preeclampsia in the pregnant woman (Bonari *et al.*, 2004a; Bonari *et al.*, 2004b; Field *et al.*, 2004). The use of antidepressants (ADP) is necessary to avoid these maternal and fetal life complications due to depression. Investigations over the last several decades on the use of ADPs during the first trimester of pregnancy suggest the absence of major congenital malformation risk, but the Swedish medical registry data concluded that Paroxetine (a SSRI) use increases major congenital malformation incidence 1.5 to 2 fold (Cohen *et al.*, 2006). ADP use during pregnancy may cause pre-term delivery. The use of tricyclic ADPs may cause a reduction in limb size (Ericson *et al.*, 1999). Increased cardiovascular defects were found in new born whose mothers were treated with ADPs during pregnancy, the malformation differing with the type of ADP used. Strongest effects on the cardiovascular system were on ventricular and atrial septa. Other random effects on the cardiovascular system include hypoplastic left heart or aortic atresia, transposition of large vessels, tetralogy of Fallot and abnormal pulmonary vein return (Kallen *et al.*, 2006).

5.1.1 Bupropion

Bupropion is an aminoketone class drug, chemically and structurally unrelated to classical ADPs. It was first marketed as an oral antidepressant agent in the mid 1980's, then removed from the market due to seizure side effects. In 1989 it was reintroduced at a low dose with necessary contraindications and precautions (Alwan *et al.*, 2010; Shepherd *et al.*, 2004). Bupropion shows antidepressant properties and is used as non-nicotinic aid in smoking cessation (Alwan *et al.*, 2010; Chun-Fai-Chan *et al.*, 2005; Cole *et al.*, 2007). The drug's pharmacological activity is primarily by strong inhibition of dopamine reuptake and nicotinic receptor antagonist activity, as well as a weak blockage of norepinephrine and serotonin reuptake (Shepherd *et al.*, 2004; Slemmer *et al.*, 2000). The mechanism of the ability of bupropion to help the patient abstain from smoking remains unknown, but appears to be pharmacologically mediated by its effects on the noradrenergic and dopaminergic systems (Chun-Fai-Chan *et al.*, 2005). These indirect sympathomimetic effects might be explained by the close resemblance of its molecular structure with phenylethylamine (Cremers *et al.*, 2003).

Bupropion is extensively metabolized in the body, mainly into 4-hydroxybupropion. 4-hydroxybupropion blood levels are ten times higher than peak levels of the parent drug, and this is thought to be involved in the drug's toxic effects (Cremers *et al.*, 2003; Shepherd *et al.*, 2004). The therapeutic blood plasma level for bupropion ranges from 10-100ng/ml, while for seizures, a plasma concentration above 170ng/ml was found (Hill *et al.*, 2007). The major adverse effects of bupropion resemble those of amphetamines, with some conduction disturbances (Cremers *et al.*, 2003). Like cocaine it exerts biphasic effects on myocardial contractile activity; the positive inotropic effect is mediated by catecholamine release due to its indirect sympathomimetic properties, while these effects are reversed (negative inotropic) when cardiac β-adrenoceptors are blocked. The latter may be the result of the drug's toxic effects, or like cocaine it may block calcium release and influx (Cremers *et al.*, 2003).

Presently there are no control studies concerning bupropion safety in pregnancy (Chun-Fai-Chan *et al.*, 2005). The results do not provide a clear picture of its teratogenic profile. Studies in animals show no signs of teratogenicity with bupropion use, nor is it found to be involved in increasing congenital malformations above the normal. Only increased abortion rates were found in bupropion treated mothers (Chun-Fai-Chan *et al.*, 2005), but Cole et al, 2007 have observed that in rabbits it slightly increased malformations and skeletal abnormalities, with decreased fetal weight. The GSk pregnancy registry data shows 2.5% major malformations, most with cardiovascular defects, from bupropion use in humans (Cole *et al.*, 2007). A statistically significant number of congenital heart defects were observed in infants with bupropion maternal exposure, specially increased cases of left outflow tract defects (Alwan *et al.*, 2010). For these uncertain effects of bupropion and the lack of control studies during pregnancy, the United States Food and Drug Administration (FDA) placed bupropion in the pregnancy category "C" (Cole *et al.*, 2007).

5.1.2 Lithium carbonate

Lithium carbonate (Li_2CO_3) is widely prescribed for the treatment of acute manic depression (Gralla & McIlhenny, 1972). During the last few decades, the clinical profile for Li_2CO_3 has extended beyond manic depression, to also include management of alcoholic patients (Messiha, 1986). The serum therapeutic concentration of Lithium in humans ranges from 0.6-1.2mM (Ikonomov *et al.*, 2000), with a small therapeutic to toxic ratio (0.7), which on chronic exposure, even at therapeutic doses, produces malformations (Giles & Bannigan, 1997). In humans Li_2CO_3 crosses the placenta (Nokhbatolfoghahai & Parivar, 2008) and perturbs normal development (Klein & Melton, 1996). Earlier investigations show lithium use during pregnancy reduces litter size, and causes infertility in males by inhibition of testicular glycolysis and reduced spermatozoa motility. Since the discovery that lithium salts produce embryos with profound exgastrulation in sea urchins, it became evident that lithium may induce morphological deviations in primitive development (Gralla & McIlhenny, 1972). At serum therapeutic levels lithium is shown to induce cardiovascular malformations specially the Ebstein's anomaly, disturbance in

embryonic vascular development, yolk sac vessels vascular stasis and vascular dilation in the cranial region of the embryo. These effects were reversed by treatment with myo-inositol (Giles & Bannigan, 1999). Treatment of mouse embryos with lithium at the neurulation stage causes discrete ruptures in certain cranial blood vessels (Giles & Bannigan, 1999). In Xenopus embryos, lithium treatment induces the formation of a second dorsal axis, but these teratogenic effects were reported to be preventable by a co-injection of myo-inositol (Hedgepeth *et al.*, 1997). Lithium affects DNA by competing with Mg^{2+} in binding and may impair DNA synthesis and repair (Léonard & Gerber, 1997). Lithium also enhances granulopoiesis and thereby induces neutrophilia (Barr & Galbraith, 1983; Nokhbatolfoghahai & Parivar, 2008).

Therapeutic and teratogenic mechanisms of Li_2CO_3 remain to be fully elucidated. However, its interference with the phosphatidylinositol (PI) cycle is thought to be the major factor in mood stabilizing and in teratogenic properties (Giles & Bannigan, 1999; Klug *et al.*, 1992; Segal, 2004). More recently the toxic mechanism for lithium has been associated with inactivation of glycogen synthase kinase-3β (GSK3-β), which mimics the canonical Wnt signalling pathway (Ikonomov *et al.*, 2000; Manisastry *et al.*, 2006; Martin *et al.*, 2011).

In the phosphatidylinositol cycle, phospholipase C (activated by G protein receptors) metabolizes the plasma membrane phosphatidylinositol into two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 regulates the release of Ca^{2+} from the non-mitochondrial pool (mostly ER). The Ca^{2+} activates the calcium and calmodulin dependent kinases and promotes extracellular Ca^{2+} influx, While DAG activates PKC (De Jonge *et al.*, 1995; Papaleo *et al.*, 2009; Segal, 2004). The IP_3 is then dephosphorylated by a number of steps; finally inositol monophosphate is hydrolysed into free inositol by the enzyme inositol monophosphatase (IMPase). The free inositol is recycled back for the regeneration of phosphatidylinositol (Klug *et al.*, 1992). In the cardiovascular system IP_3 plays a key role in vascular smooth muscle mechanical coupling, thereby regulating peripheral resistance and blood pressure. In the developing heart IP_3 initiates the pacemaker activity, promotes cardiogenesis and regulates cardiomyocyte contraction by promoting calcium release and influx

(Kockskämper *et al.*, 2008). Inhibition of the PI cycle affects early developmental events, especially the development of the heart and vessel formation (Giles & Bannigan, 1999; Kockskämper *et al.*, 2008; Méry *et al.*, 2005).

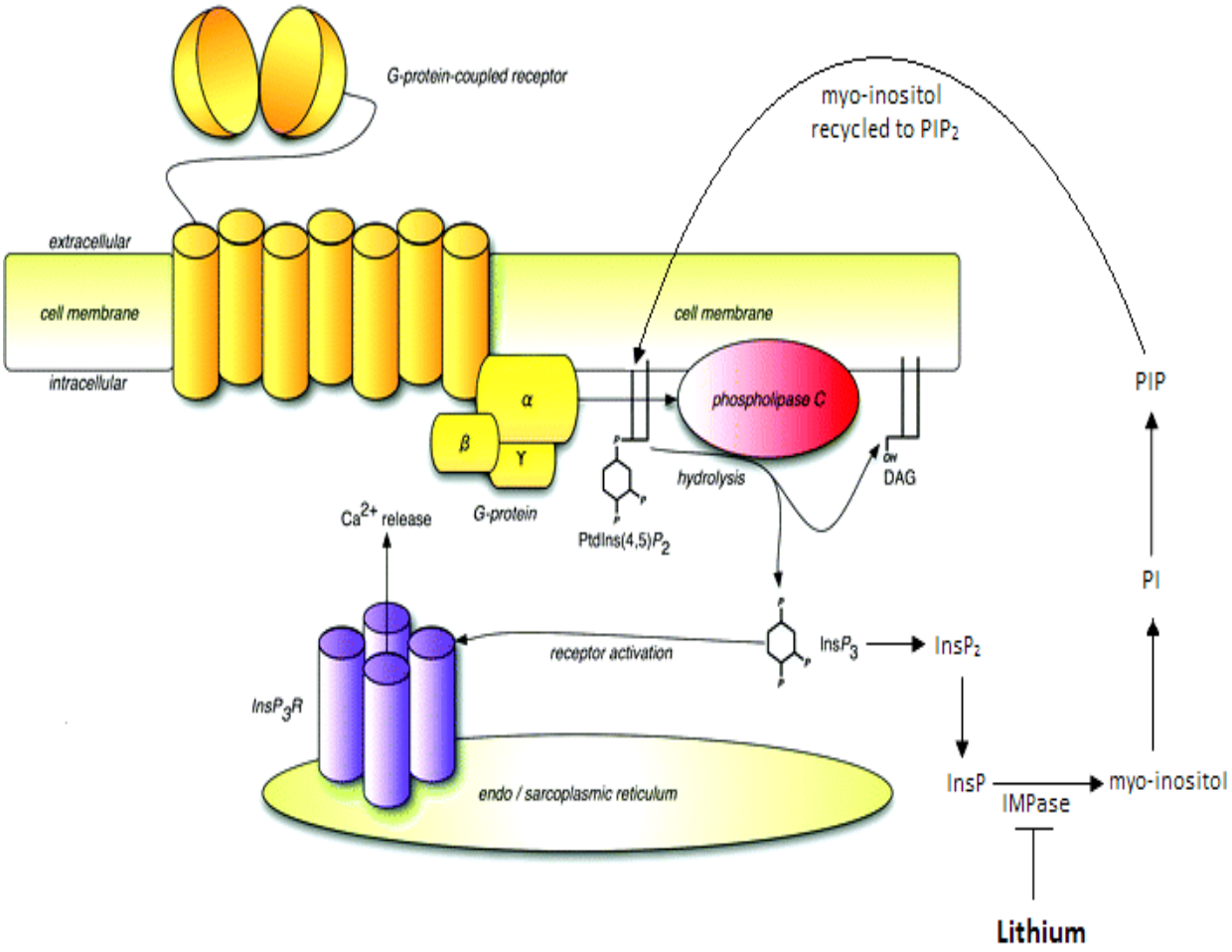


Fig 5.1 PI cycle and effects of lithium on IMPase inhibition. Phospholipase C (PLC), phosphosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), inositol 1,4,5 triphospahte ($InsP_3$), diacyl glycerol (DAG), inositol monophosphatase (IMPase), phosphatidylinositol (PI). Modified from Conway (Conway & Miller, 2007).

Lithium targets the enzymes IMPase in the PI cycle (King *et al.*, 2010; Segal, 2004), thereby inhibiting the formation of free inositol. Inositol depletion consequently results in failure of PI to reconstitute at the cell membrane (Giles & Bannigan, 1999). When exogenous inositol is not readily available, the cells do not respond to PI dependent extracellular signal transduction. IP_3 production is reduced and the regulation of Ca^{2+} is affected (Giles & Bannigan, 1999; King *et al.*, 2010). These effects can be negated by the addition of myo-inositol (Klug *et al.*, 1992).

In another mechanism of teratogenicity, lithium mimics Wnt signalling and produces a hyperdorsalized embryo (Klein & Melton, 1996). Wnt signalling has an important role in development, axis formation, cancer, stem cell biology and neural function. Wnt inactivates GSK3-β, which results in β-catenin cellular accumulation. In turn, β-catenin forms a transcriptional enhancer complex and upregulates expression of selected target genes (Eisenberg & Eisenberg, 2007; Martin *et al.*, 2011). In the absence of Wnt signals, GSK3-β phosphorylates β-catenin leading to its ubiquitination and proteosomal degradation (Eisenberg & Eisenberg, 2007). Lithium mimics the Wnt pathway by inhibiting phosphorylation of GSK-3β resulting in β-catenin accumulation in the cell (Manisastry *et al.*, 2006). Wnt signalling inhibits early cardiac specification and the proper orchestration of cardiac development by acting via its major transducer β-catenin (Eldad, 2007; Manisastry *et al.*, 2006).

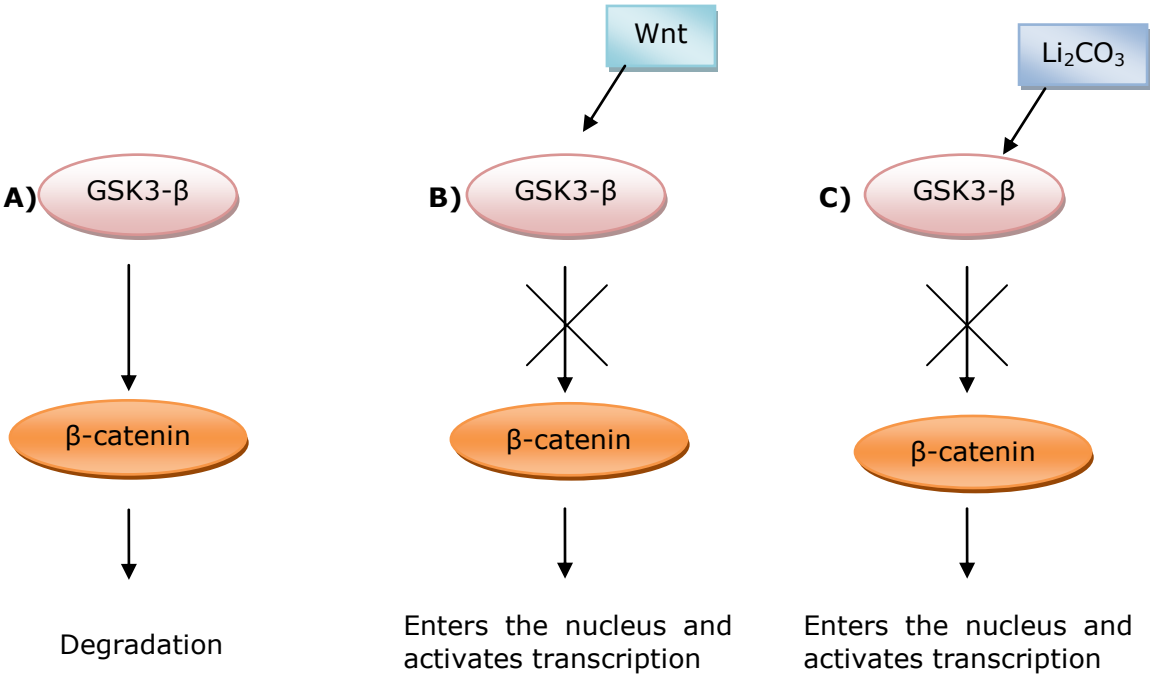


Fig 5.2 Wnt/β-Catenin Pathway. A) The phosphorylation of β-catenin by GSK3-β leads to β-catenin degradation. B) and C) the inactivation of GSK3-β by Wnt and Li_2CO_3, which allows β-catenin accumulation in the cells and entry into the nucleus.

5.1.2.1 Myo-inositol

Myo-inositol belongs to the vitamin B complex group. It is one of the nine possible stereoisomers of C6 sugar alcohol (Papaleo *et al.*, 2009). Humans consume 1g of inositol per day (Papaleo *et al.*, 2009). Myo-inositol is fairly ubiquitous, and is extensively studied, particularly myo-inositol containing phospholipids in biological membranes. It has an essential role in cell growth, cell morphogenesis, lipid synthesis, cell membrane structure and especially phosphatidylinositol (PtdIns) signal transduction which controls diverse cell processes including proliferation (Holub, 1986; Papaleo *et al.*, 2009). Providing the requisite amount of inositol to continue PtdIns signalling in the cells can reverse the lithium effects (Giles & Bannigan, 1999; Klug *et al.*, 1992).

5.2 Methodology

5.2.1 Materials and Methods

The materials utilized are discussed in detail in appendix 1. The antidepressant drugs bupropion hydrochloride and lithium carbonate at various doses were investigated for toxic effects in the chick embryonic cardiomyocyte micromass (see section 2.4.1) and ESDC systems (see section 2.4.2).

5.2.2 Preparation of test chemicals

Both bupropion HCl and lithium carbonate are water soluble, thus their stock solutions were prepared using the culture medium. The lithium carbonate doses with myo-inositol (water soluble) were prepared in complete culture medium using the stock solutions.

5.2.3 End points

The cardiomyocyte contractile activity at pre and post drug exposure were recorded in the MM system, while in ESDC it was recorded on day10, 11 and 12 of differentiation (see section 2.5.1). The cell viability (resazurin assay) (see section 2.5.2) and total protein content (kenacid blue assay) (see section 2.5.3) were measured at the end of the culture period.

5.2.4 Statistical analysis

The results of three independent repeats were statistically analysed using Prism 5 software. All the drugs doses results were compared with control using one way ANOVA with Dunnet's post hoc test. For non-parametric scoring the Kruskal wallis test was used. $P<0.05$ was considered as a significant difference.

5.3 Results

5.3.1 Bupropion hydrochloride

5.3.1.1 Bupropion effects on cardiomyocytes in the Micromass system

The first antidepressant molecule bupropion when tested in the MM system, showed more pronounced cytotoxic effects on cardiomyocytes, compared to the known anticonvulsant teratogens tested. Cardiomyocyte contractile activity declined with increase in drug dose. The length of exposure did not seem to significantly vary the results (fig 5.3A), except for BPN 75μM. This showed some chronic exposure inhibitory effects compared to 100 and 200μM. BPN 200μM dose at 144h of MM, significantly reduced the contractile activity compared to control ($P<0.05$). The cell viability estimation using resazurin assay followed the same inhibitory trend with increased dose. The BPN dose 50-200μM significantly ($P<0.05$) decreased cardiomyocyte viability compared to control (fig 5.3B). The total protein content also decreased with increase in drug dose (fig 5.3C), 25-200μM drug dose showed significant inhibitory effects ($P<0.05$). The cultured cardiomyocytes do not resemble the control at high doses tested. Increased floating cells and decreased proliferation were observed compared to the control. The foci size decreases and the spaces between the contracting foci increases with increased dose, especially observed at BPN 200μM (fig 5.4)**.** To find out if the effects on cell viability were either a direct effect of the drug or due to decreased cellular number, the ratio of cell viability to total protein was calculated, which suggests the effects on cell viability might be the result of decreased proliferation rather than a direct effect of BPN on cell viability (fig 5.4F).

5.3.1.2 Bupropion effects on ES derived cardiomyocytes

When the ESDC were treated with bupropion, the drug showed inhibitory effects on differentiated cardiomyocyte contractile activity. The declining trend started at 5μM and continued up to the highest dose used (200μM). The scoring recorded on day 10, 11 and 12 did not show a big variation except 75μM, which became significant on day11 and 12 ($P<0.05$). The BPN dose 100 and 200μM continued to significantly differ from the control on all days ($P<0.05$) (fig 5.3D). The other end point of cell viability showed some variation but not a particular trend. Even the BPN 200μM failed to differ significantly from control ($P>0.05$) (fig 5.3E). The cellular protein content was reduced with increased BPN dose. The drug dose from 50μM showed decreased protein, with significant values at 75-200μM ($P<0.05$) (fig 5.3F). The EBs resemble the control when observed under the microscope. Unlike MM, cellular proliferation was even with no void spaces between contracting foci, but EB proliferation to spread on the surface after attachment looked slightly reduced in size compared to control.

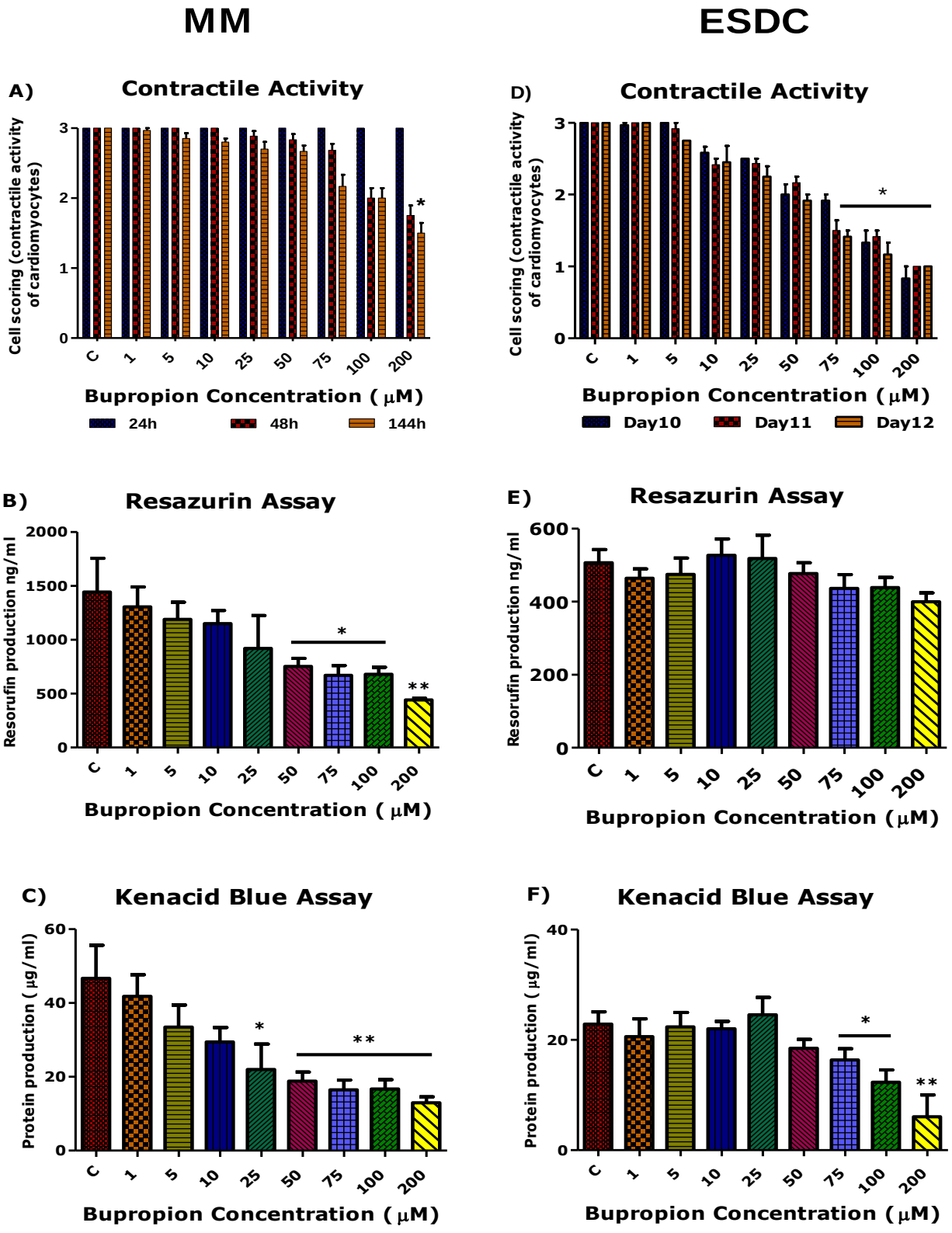


Fig 5.3 The effects of BPN on MM and ESDC systems. A) Effects of BPN on contractile activity in MM system, B) effects of BPN on cell viability (resazurin assay) in MM system, C) effects of BPN on total protein content (kenacid blue assay) in MM system, D) effects of BPN on contractile activity in ESDC, E) effects of BPN on cell viability (resazurin assay) in ESDC, F) effects of BPN on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control (C=Control).

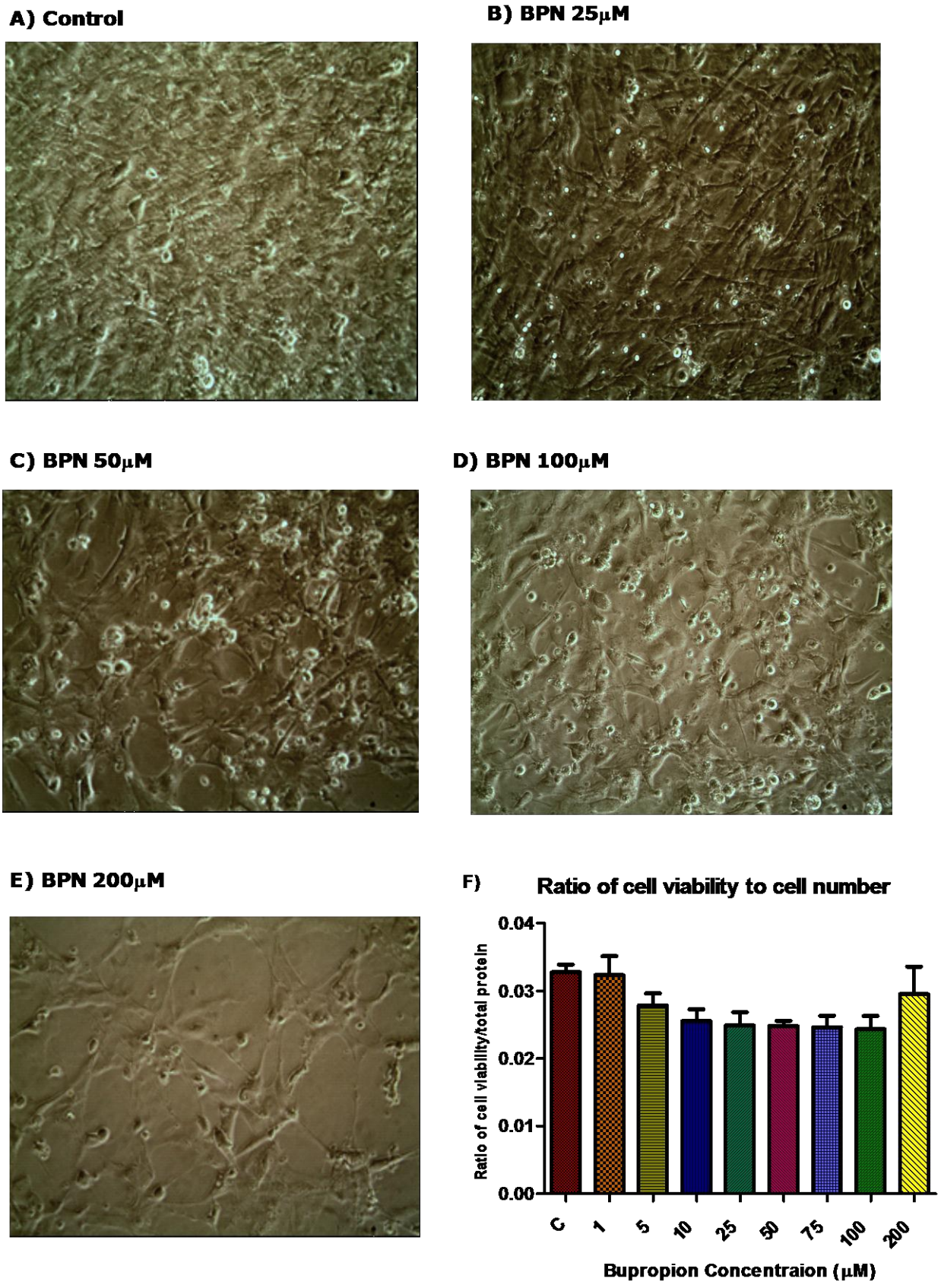


Fig 5.4 Effects of control and BPN doses on cardiomyocytes proliferation in MM system. **Fig 5.4F** Ratio of cell viability to cell numbers (total protein) in BPN treated cardiomyocytes using MM system.

5.3.2 Lithium carbonate

5.3.2.1 Lithium carbonate effects on cardiomyocytes in the Micromass system

Lithium carbonate, when at a concentration ranging from 50-2000μM in the MM system, showed no significant inhibitory signs on cell viability and total cellular protein content (fig 5.5B and C). The cardiomyocyte contractile activity remained unaffected at low doses tested. At higher doses 1200-2000μM some decrease in contractile activity was observed which was not statistically significant (fig 5.5A). The drug exposure for a short period of time and chronic exposure did not affect the values to a great extent. The cardiomyocytes looked healthy, well attached and formed large beating foci comparable to the control.

5.3.2.2 Lithium carbonate effects on ES derived cardiomyocytes

The results with lithium carbonate in ES derived cardiomyocytes were completely different from those with MM system. The cardiomyocyte contractile activity started to decreases below the therapeutic serum levels (>0.6mM) of drug and became significant at around 800μM on all scoring days recorded. The drug dose 1500μM showed no cell contraction at all, only some dull beating was observed at day 10 in very few repeats. The increased dose of 2000μM caused the contractile activity in ESDC to cease completely (fig 5.5D). The drug's effects on cell viability were not different to those observed in the MM system (fig 5.5E). The drug showed some increased levels of resorufin production but failed to attain significant difference from control. But interestingly the total protein contents of ESDC increased with increased drug dose (fig 5.5F). The 1500 and 2000μM Li_2CO_3 increased the cellular protein contents to significant levels ($P<0.05$). At 2000μM the EBs after attachment expanded and covered a large area compared to the control.

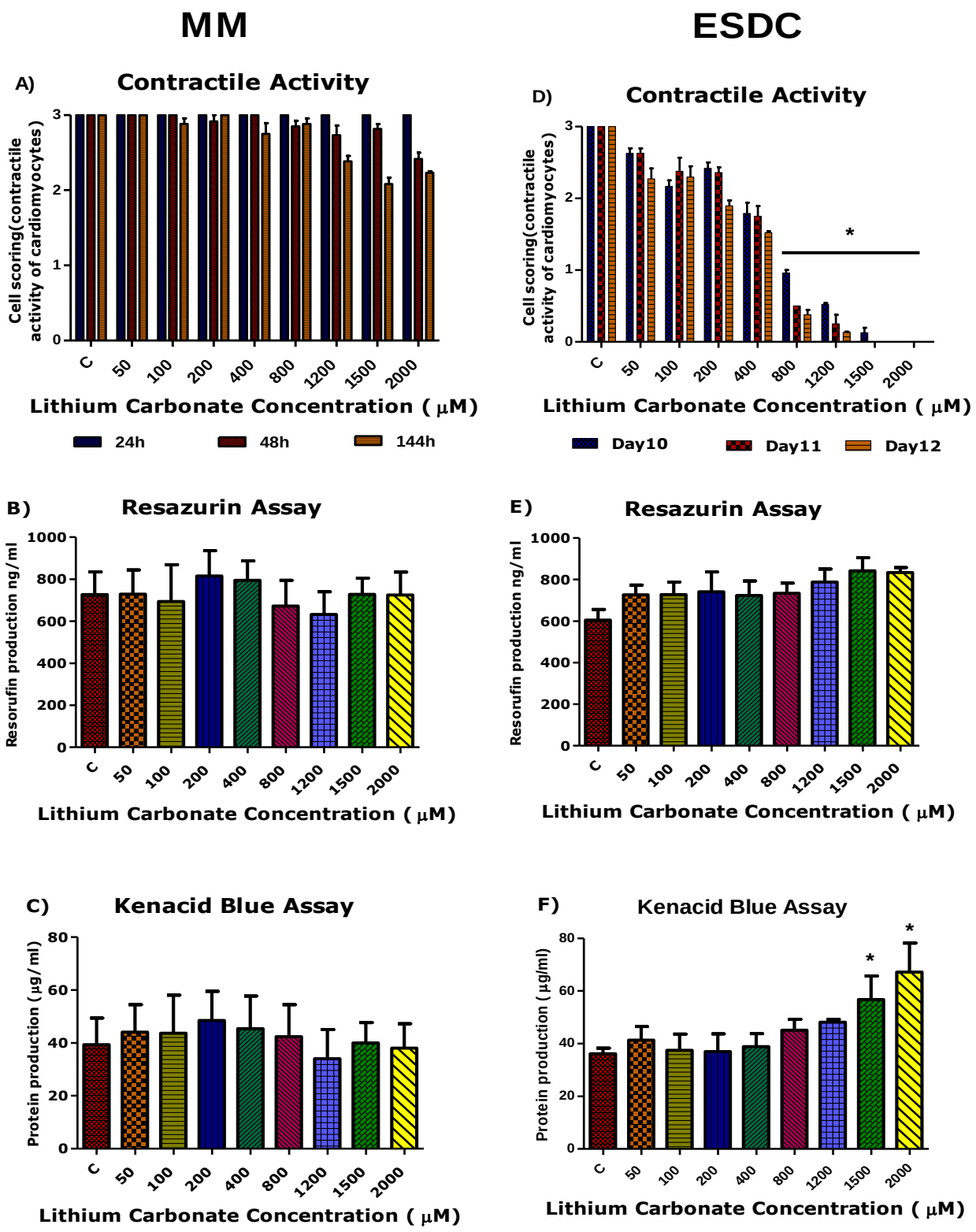


Fig 5.5 The effects of Li_2CO_3 on MM and ESDC systems. A) Effects of Li_2CO_3 on contractile activity in the MM system, B) effects of Li_2CO_3 on cell viability (resazurin assay) in the MM system, C) effects of Li_2CO_3 on total protein content (kenacid blue assay) in the MM system, D) effects of Li_2CO_3 on contractile activity in ESDC, E) effects of Li_2CO_3 on cell viability (resazurin assay) in ESDC, F) effects of Li_2CO_3 on total protein content (kenacid blue assay) in ESDC. * represents a significant difference from control (C=Control).

5.3.3 Evaluation of myo-inositol non-toxic dose in ESDC

Myo-inositol doses ranging from 100-2000μM were tested on ESDC for evaluation and identification of the non-toxic dose. Differentiating cells exposed to this dose range did not show any strong cytotoxic effects. The cardiomyocytes continued contraction at all doses tested, but the 2000 μM showed some inhibition compared to low doses (fig 5.6A). Besides this, for 2000 μM treated cardiomyocytes (fig 5.6B), compared to other doses and the control, the viability was relatively low, but failed to attain a significant difference ($P>0.05$). Cellular protein content remained unaffected at all doses tested (fig 5.6C). 1000 μM was found to be the highest non-toxic dose in ESDC.

5.3.4 Protective effects of myo-inositol on Lithium carbonate treated ESDC

Attempts were made to counteract the inhibitory effects of lithium carbonate, as observed earlier, using myo-inositol 1000μM. The cells were exposed to Li_2CO_3 doses of 800, 1500 and 2000 μM along with myo-inositol. The contractile activity at Li_2CO_3 800μM, which previously was reduced to significantly lower score compared to the control in ESDC, showed improvement. The contraction was still not identical to the control but rose to a level not significantly different. At Li_2CO_3 1500μM the ESDC were not contracting but with the addition of myo-inositol the cells started to contract. The contraction was very slow but easily recognisable. However, the 1500μM Li_2CO_3 treated cells still showed a significant difference to control ($P<0.05$). With 2000μM Li_2CO_3 dose no contraction was observed after supplementing the cells with myo-inositol (fig 5.6D). The cell viability remained unaffected after myo-inositol addition (fig 5.6E). The total protein content dropped down at all doses especially with Li_2CO_3 2000μM, which had shown a significant increase in protein content. After myo-inositol addition it reduces and was not significant compared to the control (fig 5.6F).

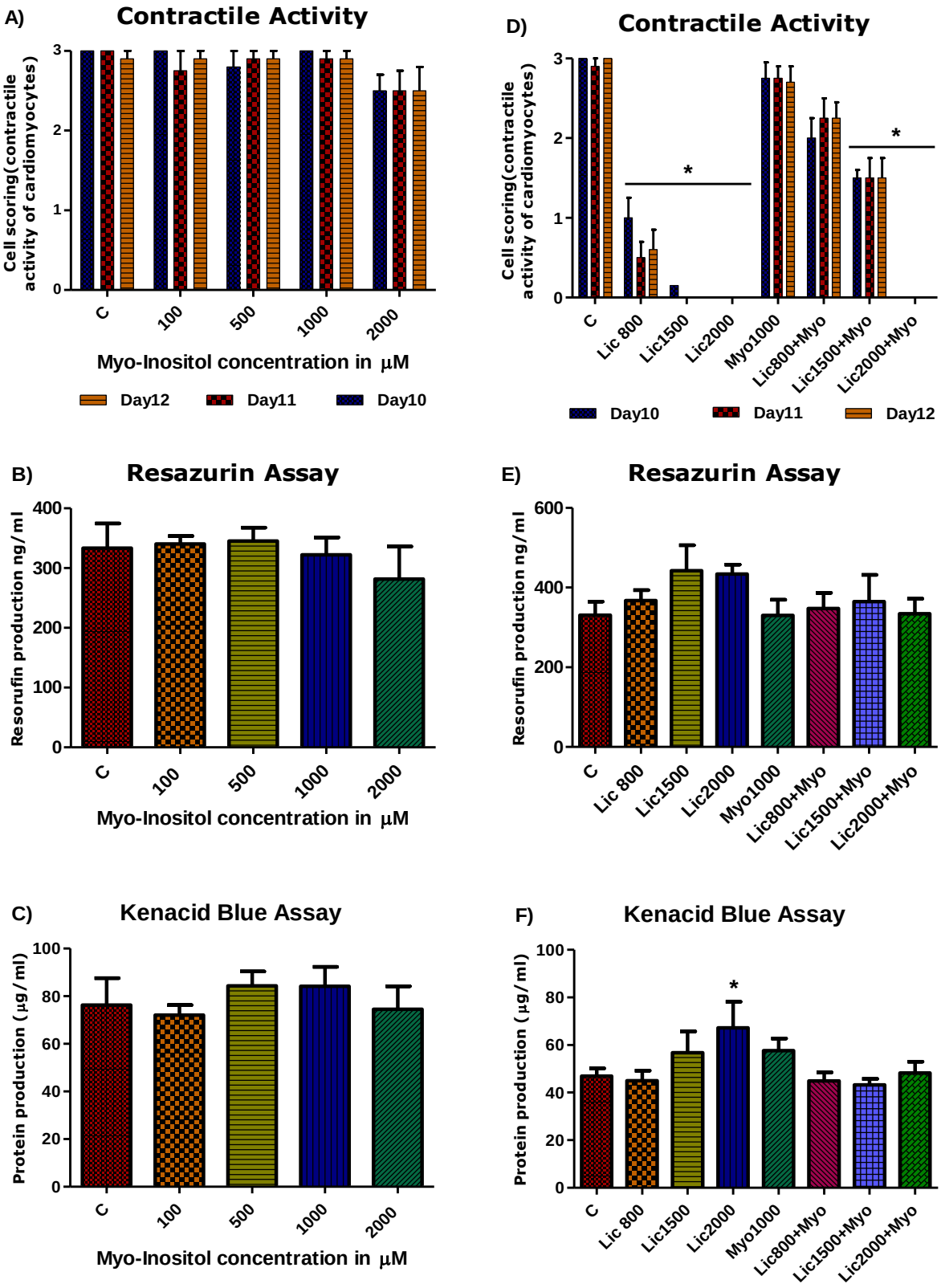


Fig 5.6 The effects of myo-inositol and Li_2CO_3 on ESDC. A) Effects of myo-inositol on contractile activity, B) effects of myo-inositol on cell viability (resazurin assay), C) effects of myo-inositol on total protein contents (kenacid blue assay), D) protective effects of myo-inositol on contractile activity, E) protective effects of myo-inositol on cell viability (resazurin assay), F) protective effects of myo-inositol on protein contents (kenacid blue assay). * represents a significant difference from control (C=Control).

5.3.5 Antidepressant effects on Reactive oxygen species production

When the antidepressant drugs bupropion hydrochloride and lithium carbonate were evaluated for the production of ROS, neither showed any significant effect on ROS production in the MM or the ESDC system (fig 5.7 and 5.8). The positive control H_2O_2 10µM in both of the systems produced significant ROS levels compared to control.

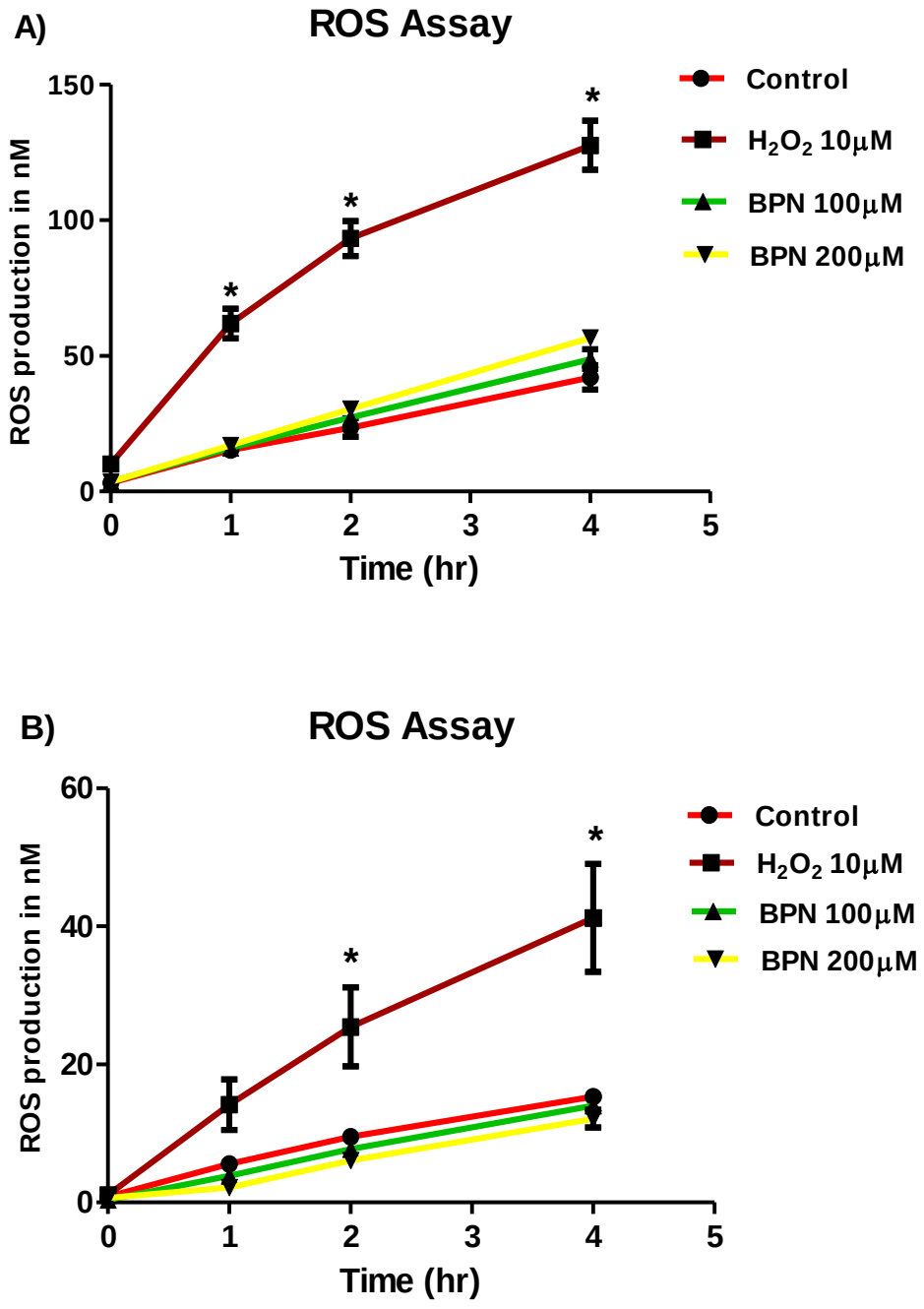


Fig 5.7 Effects of BPN on ROS production. A) BPN effects in MM system, B) BPN effects in ESDC. * represents a significant difference from control.

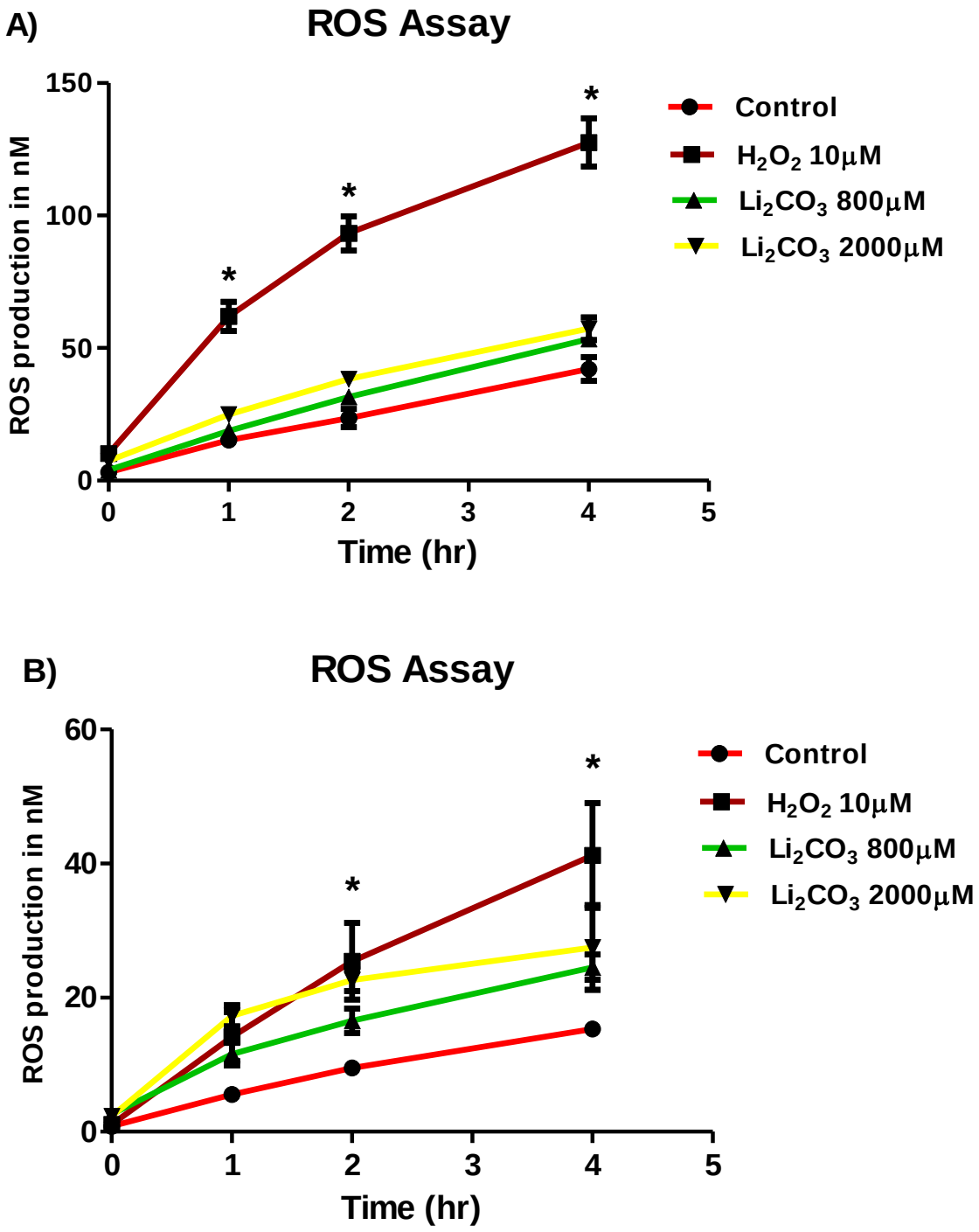


Fig 5.8 Effects of Li_2CO_3 on ROS production. A) Li_2CO_3 effects in MM system B) Li_2CO_3 effects in ESDC. * represents a significant difference from control.

5.3.6 Blind trials

To make the experimental outcome more reliable and to avoid any biased scoring with contractile activity a blind study was performed in both the systems. Toxic, moderately toxic and non-toxic drugs doses were selected, primarily on basis of the results of contractile activity three repeats as observed earlier. The drugs doses were prepared and with the help of colleague the falcon flasks were coded. The MM and ESDC experiments were performed as described in chapter 2, using coded drug solutions and endpoints were recorded. After the three repeats the doses were decoded, analysed and results of this blind study was compared with the results observed earlier. For MM system the codes were A=PRM 800µM, B=VPA 400µM, C=Li_2CO_3 2000µM, D=Control and E=BPN 25µM. While for ESDC A=VPA 400µM, B=Li_2CO_3 2000µM, C=PRM 800µM, D=Control and E=BPN 25µM. When the results of the MM system blind trials were compared with the previously obtained results, the results were comparable. The controls with a beating score of 3, with no significant effects with BPN and Li_2CO_3 treated cells. The VPA and PRM continued to reduce the contractile activity in more or less the same fashion. In ESDC Li_2CO_3 2000µM stopped the cells beating, with VPA and PRM showing some significantly decreased effects, as observed earlier. The results for cell viability and protein content were also comparable. The effects observed on contractile activity in both the systems were comparable with no huge difference. The results of this study eliminate the fragility of the contractile activity recording procedure due to manual scoring. This further supports the study and the validity of system adopted.

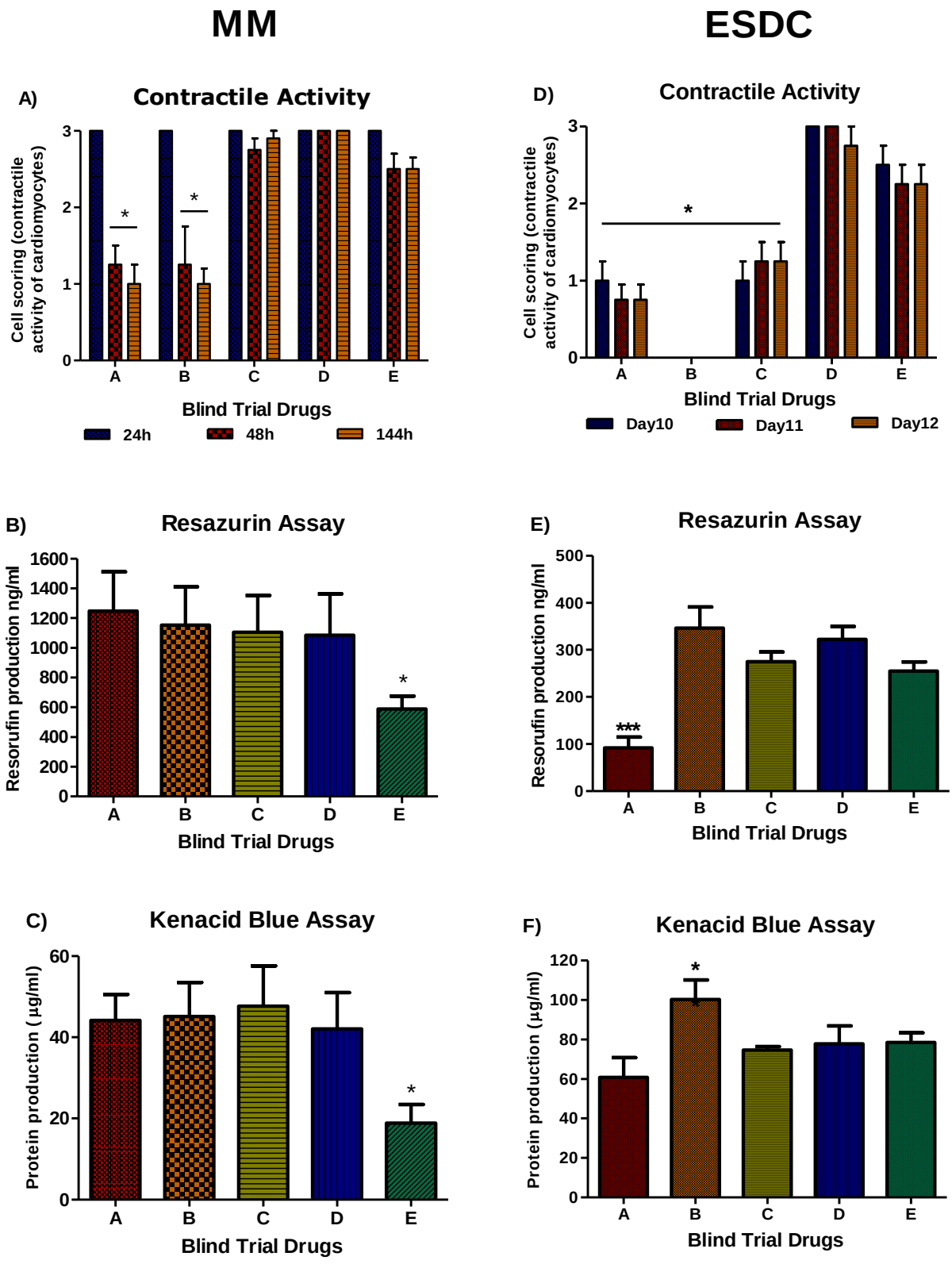


Fig 5.9 Blind trial study of different drug doses in MM and ESDC systems. A) Effects on contractile activity in the MM system, B) effects on cell viability (resazurin assay) in MM system, C) effects on total protein content (kenacid blue assay) in MM system, D) effects on contractile activity in ESDC, E) effects on cell viability (resazurin assay) in ESDC, F) effects on total protein contents (kenacid blue assay) in ESDC. * represents a significant difference from control (C=Control).

5.4 Discussion

Depression affects 10-25% of women (Field *et al.*, 2004). Depression in pregnancy complicates normal embryonic development, including that of the placenta, as well as causing preeclampsia, spontaneous abortions or premature deliveries (Field *et al.*, 2004). The affected neonates experience elevated fetal activity, growth retardation, and altered behavioural, biochemical and physiological states (Field *et al.*, 2004). Women being most hit by depression, especially during the child bearing years and disturbed hormones level during pregnancy further added a natural elevating factor. Prenatal depression increases in the second trimester compared to the first and negatively regulates fetal development and pregnancy outcome (Field *et al.*, 2004). It may affect pregnancy by influencing the maternal mental state and the consumption of substances of abuse during pregnancy and due to depressed behaviour may weave a web of complications for prenatal and postnatal environments (Bonari *et al.*, 2004b). The use of antidepressants during pregnancy confounds the pregnancy outcome. But untreated depression leads to severe complications (Bonari *et al.*, 2004a). In this study using *in vitro* systems ADP effects were evaluated with particular reference to cardiac development.

The first antidepressant molecule bupropion HCl was tested in both the systems. This molecule has been used for depression, and most recently as a non-nicotinic aid in smoking cessation (Alwan *et al.*, 2010). The antidepressant mode of action is by indirectly acting as a sympathomimetic amine, but it can also act with nicotinic receptor antagonist activity (Cremers *et al.*, 2003; Shepherd *et al.*, 2004; Slemmer *et al.*, 2000). In the body bupropion is principally metabolized by the CYP2B6 isoenzyme to hydroxybupropion, threohydrobupropion and erythrohydrobupropion. Hydroxybupropion is the pharmacologically active metabolite, its plasma levels are higher than the parent drug and it is thought to be involved in the drug's toxic effects (Cremers *et al.*, 2003; Hesse *et al.*, 2000; Shepherd *et al.*, 2004). A number of congenital heart defects were associated with bupropion use during pregnancy, especially anatomically malformed hearts, which is not observed with other antidepressants like SSRIs (Alwan *et al.*, 2010).

The use of the MM and ESDC systems may elucidate the mechanism of suspected bupropion toxic effects, for which it comes under FDA pregnancy category C (Cole *et al.*, 2007). In the MM system bupropion affects the cardiomyocyte ability to re-establish the beating system with increasing dose; cardiomyocyte proliferation in particular is mostly affected by the drug. The cells divide but their ability to form flat sheets with beating foci is suppressed. The foci connect with each other with thin longs strands. Contractile activity was present, even at 200μM BPN and the synchronised contraction of foci was not decreased drastically by the decreased size and connections between the foci. From the results it is observed that bupropion with increase in dose affects cells proliferation either by arresting cell division by senescence or by inducing apoptosis. Jang et al, 2011 in a study using the human neuroblastoma SH-SY5Y cell line confirms the activation of the caspase 3, 8 and 9 pathways after BPN treatment, with caspase 3 having particularly noticeable increased levels compared to the others. The induction of apoptotic pathways might have caused reduced cell proliferation. The same cardiomyocytes, when subjected to viability testing, showed decreased oxidoreductase levels as the drug dose increased. This might be explained as the consequence of decreased proliferation, which results in a decreased number of cells. The effects on cell viability were purely the result of decreased cell numbers.

When it comes to bupropion effects on differentiating cardiomyocytes using embryonic stem cells, the effects were more pronounced on contraction compared to the MM system. The higher doses lower the contractile activity more compared to the MM system. The effects may be the result of decreased cell proliferation making it impossible to form beating foci, but in ESDC the EBs are plated in 24 well plates, not as a single cell suspension like the MM system. The space between foci is therefore not so apparent. The EBs have already formed a compact system which show toxic effects on cell proliferation or maybe the ES cells do not sense the mechanism of bupropion toxicity compared to differentiated MM cardiomyocytes. Interestingly in ESDC any effects on cell viability do not decline to significant difference levels. The EBs size on day three and five was not very different compared

to the control, but when the EBs were plated a slight decrease in expansion was observed at the higher BPN doses tested.

Conduction disturbances, delay and worsening of angina and myocardial infarction have already been reported with bupropion use (Cremers *et al.*, 2003). Bupropion exerts biphasic effects in human myocardium in a similar way to cocaine. Increased contractile activity is not observed with BPN use. The negative inotropic effect at higher doses could be by the inhibition of calcium release from sarcoplasmic reticulum or calcium influx across the sarcolemma, as with cocaine (Cremers *et al.*, 2003). This may be the cause of reduced contractile activity in both the systems but it requires proper elucidation before making any conclusion. BPN may have effects on cellular oxidative state, but when analysed, ROS levels were not altered after treating the cardiomyocytes in either system. This is in accordance with the results of Jang et al, 2011 using a neuroblastoma cell line (Jang *et al.*, 2011). When bupropion treated cells were supplemented with ascorbic acid and superoxide dismutase to see the protective effects, no improvement in any end point was found in either of the systems (data not shown). There may be some other unidentified toxic mechanism or cardiac induction by ascorbic acid may occur (Takahashi *et al.*, 2003).

The second antidepressant molecule, lithium carbonate, was tested for teratogenic effects, in particular with cardiogenesis. The therapeutic prolife of the lithium ion has enormously expanded during the last few decades (Messiha, 1986). The therapeutic and toxic effects are mediated either by interference with the PI cycle or GSK3-β inactivation (Giles & Bannigan, 1999; Ikonomov *et al.*, 2000). Teratogenic effects of lithium are well established. It perturbs normal embryonic development in diverse organisms, producing a secondary dorsal axis (Hedgepeth *et al.*, 1997) and CVS defects (Giles & Bannigan, 1999).

In this study micromass and ESCD showed stage dependent effects of lithium on cardiogenesis. In the MM system, cardiomyocytes are differentiated and have already acquired contractile ability before isolation, but anatomically they are still under development to orchestrate the heart (Bellairs & Osmond, 1998). However,

contractile activity is established before drug addition (Memon & Pratten, 2009). In comparison, the ES cells were undifferentiated and have not even acquired or expressed lineage commitment (Davila *et al.*, 2004; Friel *et al.*, 2005). The lithium dose range used was well below the therapeutic level up to serum toxic levels (Ikonomov *et al.*, 2000). In the micromass system any toxic effects were not statistically significant. The cardiomyocytes contractile activity does not seem to be much affected by a therapeutic dose. This may be because the cells are well differentiated and they can withstand lithium carbonate inhibition of IMPase in the PI cycle, or the cells have become tolerant to these conditions. The other mechanism of toxicity associated with lithium is that of mimicking Wnt pathways (Klein & Melton, 1996). Wnt controls heart development but is also modulated during adult heart remodelling, but Wnt has a limited role in the developed heart compared to during organogenesis (Bergmann, 2010). That is perhaps why it did not affect differentiated cardiomyocytes in the MM system to any extent. Cell viability and cell proliferation was not affected at all, even at toxic doses. It may be that these pathways do not trigger a toxic mechanism that inhibits cells division or formation of the contractile system in differentiated cardiomyocytes.

When ES were differentiated to cardiomyocytes in the presence of lithium the results were completely different in comparison to the MM system. The ESDC showed a decreased contractile activity with increased dose. The effects were observed at therapeutically relevant doses. ESDC treated with the serum toxic concentration of 2000μM had no beating areas. The lithium inhibition of IMPase in the PI cycle reduces the production of IP_3, which eventually results in decreased Ca^{2+} release (Hedgepeth *et al.*, 1997) and this might have shown the inhibitory effects on contractile activity. Beside calcium being a major ion in cardiomyocyte contractile activity, it also has an important role in development and signalling (Whitaker & Smith, 2008). IP_3 initiates pace maker activity and promotes cardiogenesis (Kockskämper *et al.*, 2008), and lithium reduces the endogenous production of IP_3 (King *et al.*, 2010) which affects the cells' ability to initiate the beating mechanism.

Lithium treatment in Xenopus decreases cardiac gene and protein expression (Martin *et al.*, 2011).

In ESDC the lithium effects on cell viability remained insignificant compared to control, rather like in the MM system, but the total protein content was increased, especially with toxic dose treatment. This may be the result of IMPase inhibition by lithium, but IMPase inhibitors do not produce embryo dorsalization. Thus the inhibition of IMPase was not sufficient for dorsalization of Xenopus embryo (Hedgepeth *et al.*, 1997) but could affect the contractile mechanism. Lithium activation of Wnt signalling might initiate the mechanism of increased cell proliferation, because Wnt is found to be associated with producing hyperdorsalized embryos. Myo-inositol could block dorsal axis induction by lithium through a more indirect mechanism that is independent of IMPase inhibition. Inhibition of GSK3-β is a general mechanism for lithium action. It is difficult to see why co injection of myo-inositol reverses the effects when this does not affect the Wnt pathway and IP cycle and does not produce embryo dorsalization. Depletion of inositol is necessary, but not sufficient for the teratogenic effects of lithium**.** The mechanism of myo-inositol rescue is uncertain. There may be some relation between inositol depletion and GSK3-β (Hedgepeth *et al.*, 1997). The increased ROS production observed with lithium is in accordance with the results observed by Shu-Huei Kao et al at high concentration (20mM) using a different cell type (Shu-Huei Kao *et al.*, 2008). Addition of myo-inositol reversed the lithium inhibition of contractile activity. There is some evidence of myo-inositol and derivatives having antioxidant effects (Jiang *et al.*, 2011; Rao *et al.*, 1991), but ROS production in this study was not significantly different to the control. This might be different at high doses of lithium carbonate.

It can be concluded that bupropion effects may be mediated through activation of apoptotic pathways, while effects on contractile activity could be the results of the drug's negative inotropic effects at toxic doses. The formation of EBs due to gravitational force using the hanging drop method might make the cells behave differently compared to some other methods of EB formation. It would be interesting to see how these cells come together to form EBs in the presence of BPN using some

other method for differentiation into cardiomyocytes. While the lithium effects were the results of PI cycle inhibition and GSK3-β inactivation as expected. Addition of myo-inositol to the cells compensates for the depleted inositol. If the PI cycle continues, IP_3 is formed, which promotes cardiogenesis and pace maker initiation of contractile activity. This rescue was observed in ESDC. From the results it can be concluded that lithium carbonate effects on ESDC contractile activity are due to IMPase inhibition of the PI cycle, which plays a pivotal role in ES derived cardiogenesis and pacemaker induction unlike that which is observed in the differentiated cardiomyocytes of the MM system. While the increased protein content might be attributed to lithium, Wnt pathway activation shows cardiac stage dependent effects (Bergmann, 2010).

Chapter 06

Effects of the active constituents of the herbal antidepressant St. John's Wort on chick cardiomyocyte in the micromass system

6.1 Depression, herbal medicines and pregnancy

Depression is a commonly prevailing mental disorder in our society (Morelli, 2003). Many women of child bearing age, especially between 25 and 44 years of age suffer from major depression. The estimated life time risk during pregnancy of these psychopathological symptoms may vary between 10-25%. Untreated depression during pregnancy may have direct physiological effects on the developing fetus, or indirectly by the abnormally depressed behaviour of the mother (Bonari *et al.*, 2004a). The possible side effects of synthetic antidepressant drugs directed the need for alternative medicines. The use of herbal medicines may provide the solution for this problem (Rodríguez-Landa & Contreras, 2003). Herbal medicines are officially classified as dietary supplements and are readily available (Klepser *et al.*, 2000). It is a general belief that herbal medicines are harmless, although statistical data do not confirm this (Kober *et al.*, 2008). The use of herbal medicines in expectant mothers varies from 7-55%. The pregnant woman often fails to appreciate the chemicals in herbal medicines may have the power to trigger abnormal embryonic/fetal development through toxic mechanisms. The lack of adequate safety data for the use of natural medicines during pregnancy, mostly because of the number of pharmacologically active compounds present, further affects safety precautions (Denise, 2003).

6.2 Hypericum Perforatum (St. John's Wort)

Hypericum perforatum, commonly known as St. John's Wort (SJW) is a top selling over the counter herbal antidepressant drug (Chan *et al.*, 2001; Wurglics & Schubert-Zsilavecz, 2006). SJW has been used for nearly 2000 years to treat diarrhoea, wound healing, urine and bladder problems and mostly frequently for mild to moderate depression (da Conceição *et al.*, 2010; Walker *et al.*, 2002; Wurglics & Schubert-Zsilavecz, 2006). It was the Swiss physician Paracelsus (ca. 1493-1541) who first used it to treat depression (Dugoua *et al.*, 2006). The SJW extract contains a number of pharmacologically active constituents including flavonoids, napthodianthrones, phloroglucinol, proanthocyanidins, xanthones, and essential oils (Hostanska *et al.*, 2003; Wurglics & Schubert-Zsilavecz, 2006). However, with the

presence of a number of pharmacologically active compounds, the idea of one plant, one active compound and one mechanism seems incorrect. It is more like that multiple active compounds contribute in a more complex antidepressant mechanism (Butterweck & Schmidt, 2007). SJW is found to be a more effective antidepressant than placebo, while its effects are comparable to synthetic tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRIs) (Hammerness *et al.*, 2003; Kober *et al.*, 2008). The antidepressant effects of SJW were previously attributed to phloroglucinol, napthodianthrones and several flavonoids by MAO inhibition, but *in vitro* studies suggested these effects are achieved only at very high concentrations, which cannot be achieved by the recommended 300mg dose of SJW (Butterweck & Schmidt, 2007). The antidepressant effects are more likely the result of the inhibition of a number of neurotransmitters, especially involving serotonin reuptake (Denise, 2003; Moretti *et al.*, 2009).

The SJW extract, beside its therapeutic effects, also is known to interfere with calcium transport, G protein coupled receptors, ion channels, phosphodiesterase and DNA (da Conceição *et al.*, 2010; Khan *et al.*, 2011). SJW use is not generally associated with serious side effects, with some reports of mild GIT disturbances, anxiety, fatigue, palpitation, restlessness, dry mouth and photosensitivity (an average of 2.4% of patients) (Lawvere & Mahoney, 2005). Beside these effects SJW induces the cytochrome P-450 system, which affects a number of drug metabolisms and may results in altered effectiveness (Chan *et al.*, 2001; Lawvere & Mahoney, 2005). SJW's consumption during pregnancy may result in decreased offspring weight, while its emmenagogue (stimulation of pelvic blood flow), uterine stimulant and abortifacient properties were confirmed in animal studies and some cases of spontaneous abortions in humans (Chan *et al.*, 2001; Dugoua *et al.*, 2006; Moretti *et al.*, 2009).

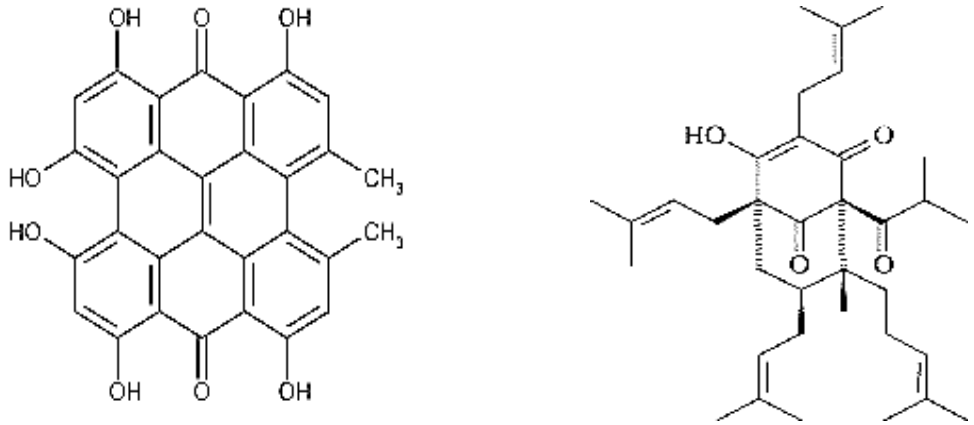
Two pharmacologically active compounds, Hypericin (0.3%) a napthodianthrone, and Hyperforin (3%) a phloroglucinol (Mennini & Gobbi, 2004; Vitiello, 1999), are used to standardize SJW extracts and are the most widely studied compounds in its

extract because of their possible antidepressant activity (da Conceição *et al.*, 2010; Lawvere & Mahoney, 2005; Moretti *et al.*, 2009).

6.2.1 Hypericin usually constitutes 0.3% of the total SJW extract, and produces antidepressant effects by inhibiting MAO-A (IC50 68µmol/L), MAO-B (IC50 420µmol/L) (Eckert *et al.*, 2004; Kubin *et al.*, 2005; Wurglics & Schubert-Zsilavecz, 2006) and serotonin reuptake (Gregoretti *et al.*, 2004). It also has affinity for dopamine D3 receptors (Mennini & Gobbi, 2004). The 50% MAO inhibition activity is 100-1000 fold higher than normal C_{max} values after oral administration of SJW extract, which cannot be achieved without the severe phototoxic effects of hypericin (Kubin *et al.*, 2005; Wurglics & Schubert-Zsilavecz, 2006). In humans its therapeutic plasma concentration ranges from 1.3-91ng/ml with poor absorption and bioavailability and a half-life of 6-48.2h (da Conceição *et al.*, 2010). Hypericin may cause infertility by denaturing the DNA of sperm and affecting its penetration into the ovum. Hypericin treatment decreases morphological score, yolk sac diameter and somite number in whole embryo culture (Chan *et al.*, 2001). Hypericin enters the cell by diffusion and a membrane associated translocation process. It interacts with human serum albumin (HSA), low density lipoprotein (LDL) and high density lipoprotein (HDL), which facilitates its solubility and dissolution in aqueous solution. Due to its lipophilic properties it incorporates into phospholipid bilayers, enters the cell and is localized in the Golgi apparatus and endoplasmic reticulum. Only at increased exposure time is it found in the nuclear membrane or inside the nucleus (Kober *et al.*, 2008; Sauviat *et al.*, 2007). Hypericin interacts with purine in DNA by forming a hydrogen bond with the OH group at N7 (Kober *et al.*, 2008). Hypericin at higher doses is phototoxic. It may produce skin irritation on exposure to UV light by generating singlet oxygen and other ROS, which may lead to cell death and necrosis. This photodynamic property is used for cancer treatment (photodynamic therapy) (Karioti & Bilia, 2010; Theodossiou *et al.*, 2004; Wurglics & Schubert-Zsilavecz, 2006), but these effects are associated with interference in calcium mobilization which limits its use (da Conceição *et al.*, 2010). These light dependant toxic properties were confirmed by Stupakova *et al*, since when HUVEC, U-87 MG and U-

373 MG cells were exposed to nM and μM concentrations of hypericin they showed no effect, but on photo activation the cell viability was significantly affected (Stupáková *et al.*, 2009). An increased hypericin concentration 150-250μgml^{-1} decreases the cell viability with cell vacuolization, tumefaction and cell death (da Conceição *et al.*, 2010). Beside this hypericin antiviral activity has already been established (Lopez-Bazzocchi *et al.*, 1991).

Hypericin interacts with a number of enzymes like PKA (IC50 >80μmol/L), PKC (IC50 3.37μmol/L), tyrosine kinase (epidermal growth factor kinase or insulin receptor tyrosine kinase), glutathione reductase (IC50 2nmol/L) and phosphatidylinositol-3-kinase or dopamine-beta-hydrolase that plays a critical role in cell signalling. But these interactions are mostly light dependent (Kober *et al.*, 2008; Kubin *et al.*, 2005; Sauviat *et al.*, 2007).



Hypericin **Hyperforin**

Fig 6.1 Hypericin and Hyperforin chemical structures (Hostanska *et al.*, 2003).

6.2.2 Hyperforin is considered mainly responsible for the antidepressant effects in SJW because of its comparable effects with commercially available synthetic antidepressant drugs (Wurglics & Schubert-Zsilavecz, 2006). It acts by affecting synaptosomal uptake of serotonin, dopamine, glutamine, GABA and noradrenaline neurotransmitters (Medina *et al.*, 2006). These effects are probably due to an alteration in sodium conductive pathways or modified neurotransmitter storage (Wurglics & Schubert-Zsilavecz, 2006). The mean plasma concentration of hyperforin after 3h of oral administration of 300-600mg dose reaches 150-370ng/ml (Wurglics & Schubert-Zsilavecz, 2006). Which is in the range of its IC50 value (120-3.300nM)

for synaptosomal uptake inhibition of serotonin (Butterweck & Schmidt, 2007). The pure compound is poorly susceptible to light and oxidation (Beerhues & Beerhues, 2006). Hyperforin inhibits leukotriene biosynthesis by inhibiting the enzyme 5-lipoxygenase (Feißt *et al.*, 2009). Hyperforin triggers apoptosis by initiating caspase dependent pathways. Hyperforin and hypericin synergistically can produce light independent antiproliferative effects (Hostanska *et al.*, 2003). Hyperforin is secreted at quantifiable levels in breast milk, which is reported to result in a higher incidence of lethargy and drowsiness in breast feeding infants (Chan *et al.*, 2001; Hammerness *et al.*, 2003).

6.3 Materials and Methods

The micromass culture system materials (Appendix 1) and methods are discussed in detail in chapter 2. In brief 5 day's old chick cardiomyocytes were seeded in a 20µl droplet in 24 wells plate at a cellular density 3×10^6 cellsml^{-1}. After 2h of cell attachment the wells were flooded with 500µl of complete culture medium.

6.3.1 Preparation of test chemicals

The four concentrations doses of hypericin (50, 100, 200 and 400ngml^{-1}) and hyperforin (100, 200, 400 and 800ngml^{-1}) were prepared by diluting a stock solution (see section 2.6) and this was protected from light. Both the drugs, either alone or in combination, were used with increasing concentrations. 500µl of medium with double the drug concentration required was added 24h after cell seeding.

6.3.2 End Points

For cardiomyocytes contractile activity the culture was morphologically inspected using the microscope and the score was recorded at 24h, 48h and 144h of cell culture (see section 2.5.1). For estimation of drug cytotoxic effects on cell viability, the resazurin reduction assay was performed at 144h of cell culture (see section 2.5.2), followed by kenacid blue total protein content estimation (see section 2.5.3).

6.3.3 Statistical analysis

The results of three independent repeats were statistically analysed using Prism 5 software. All the drugs doses results were compared with the control using one way

ANOVA with Dunnet's post hoc test. For non-parametric data the Kruskal Wallis test with Dunn's post hoc test was used. The $P<0.05$ was considered as a significant difference.

6.4 Results

The active constituents of St. John's Wort, hypericin and hyperforin, were tested at and above blood serum therapeutic concentrations. Both the drugs appeared to inhibit contractile activity at a dose above serum levels, but hypericin, even at the higher doses tested (400ng/ml) failed to achieve statistical significance from the control ($P>0.05$), while hyperforin showed some significant difference at 144h of cell culture with 800ng/ml dose ($P<0.05$). The drug combination follows more or less the same pattern of contractile activity inhibition as hyperforin alone. The combination of 50 and 100ng/ml hypericin with 100 and 200ng/ml hyperforin receptively showed no significant inhibitory effects on contractile activity, while higher dose combinations above serum levels 200ng/ml HP+400ng/ml HF at 48h and 400ng/ml HP+800ng/ml HF at 48 and 144h showed a statistically significant difference from control ($P<0.05$). Cardiomyocyte cell viability, using the resazurin assay, showed no significant pattern of variation from control with 50-400ng/ml hypericin or, hyperforin. When both the drugs were combined, up to 200ng/ml HP with 400ng/ml HF, the drugs also showed no significant effect on cell viability, while the drug combination above the serum level (400ng/ml HP and 800ng/ml HF) showed a statistically significant decreased conversion of resazurin to resorufin compared to control. The third end point of effect on total cellular protein content more or less showed the same pattern of results as the resazurin assay. HP at all doses does not significantly alter the cellular protein content compared to control. HF appeared to inhibit cell proliferation at 400 and 800ng/ml compared to control but it failed to achieve statistical significance, while the drug combination of 400ng/ml HP and 800ng/ml HF showed significantly decreased protein level compared to control ($P<0.05$).

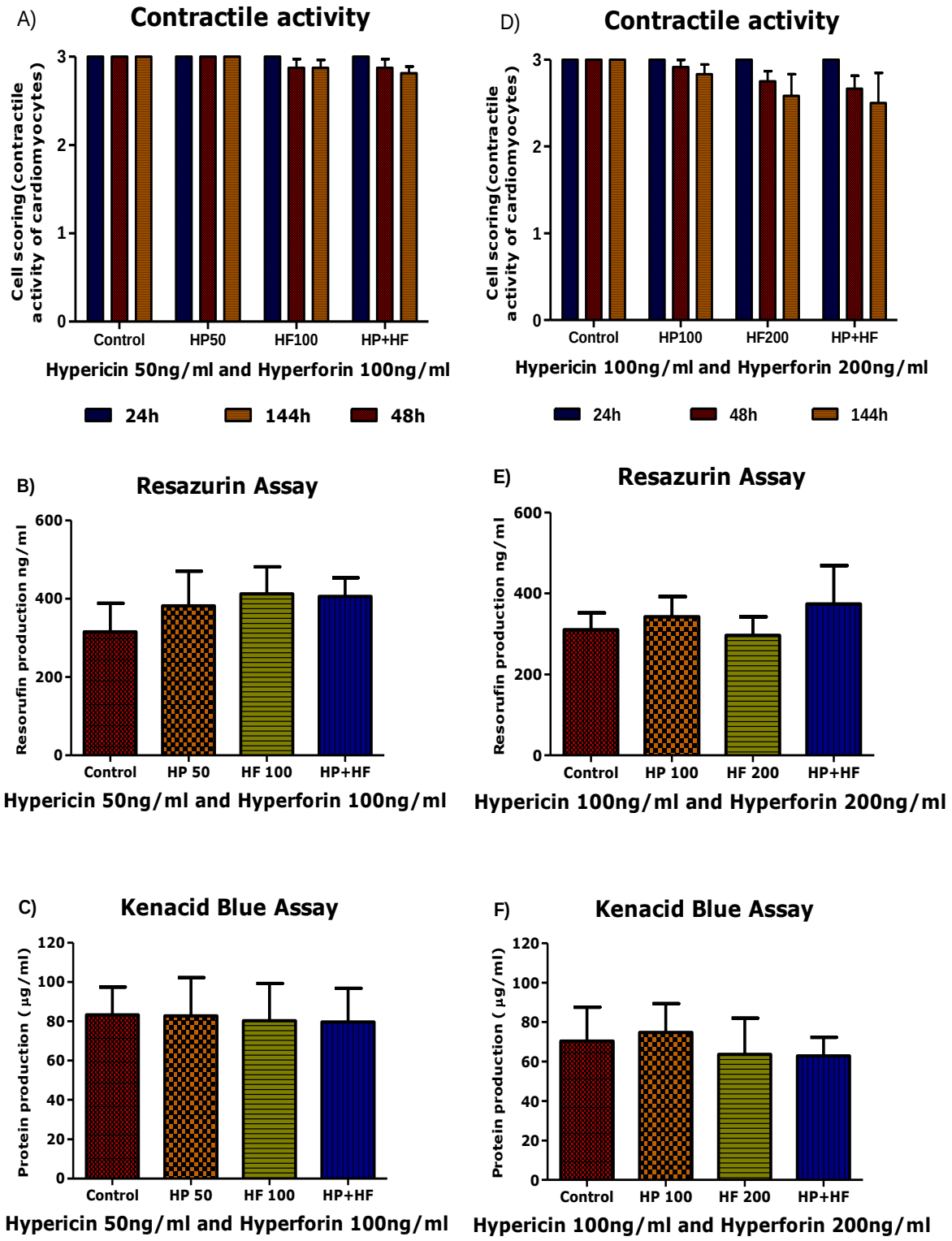


Fig 6.2 The effects of HP and HF on cardiomyocytes in the MM system. A) Effects of HP 50ng/ml and HF 100ng/ml on contractile activity, B) effects of HP 50ng/ml and HF 100ng/ml on cell viability (resazurin assay), C) effects of HP 50ng/ml and HF 100ng/ml on total protein content (kenacid blue assay), D) effects of HP 100ng/ml and HF 200ng/ml on contractile activity, E) effects of HP 100ng/ml and HF 200ng/ml on cell viability (resazurin assay), F) effects of HP 100ng/ml and HF 200ng/ml on total protein content (kenacid blue assay).

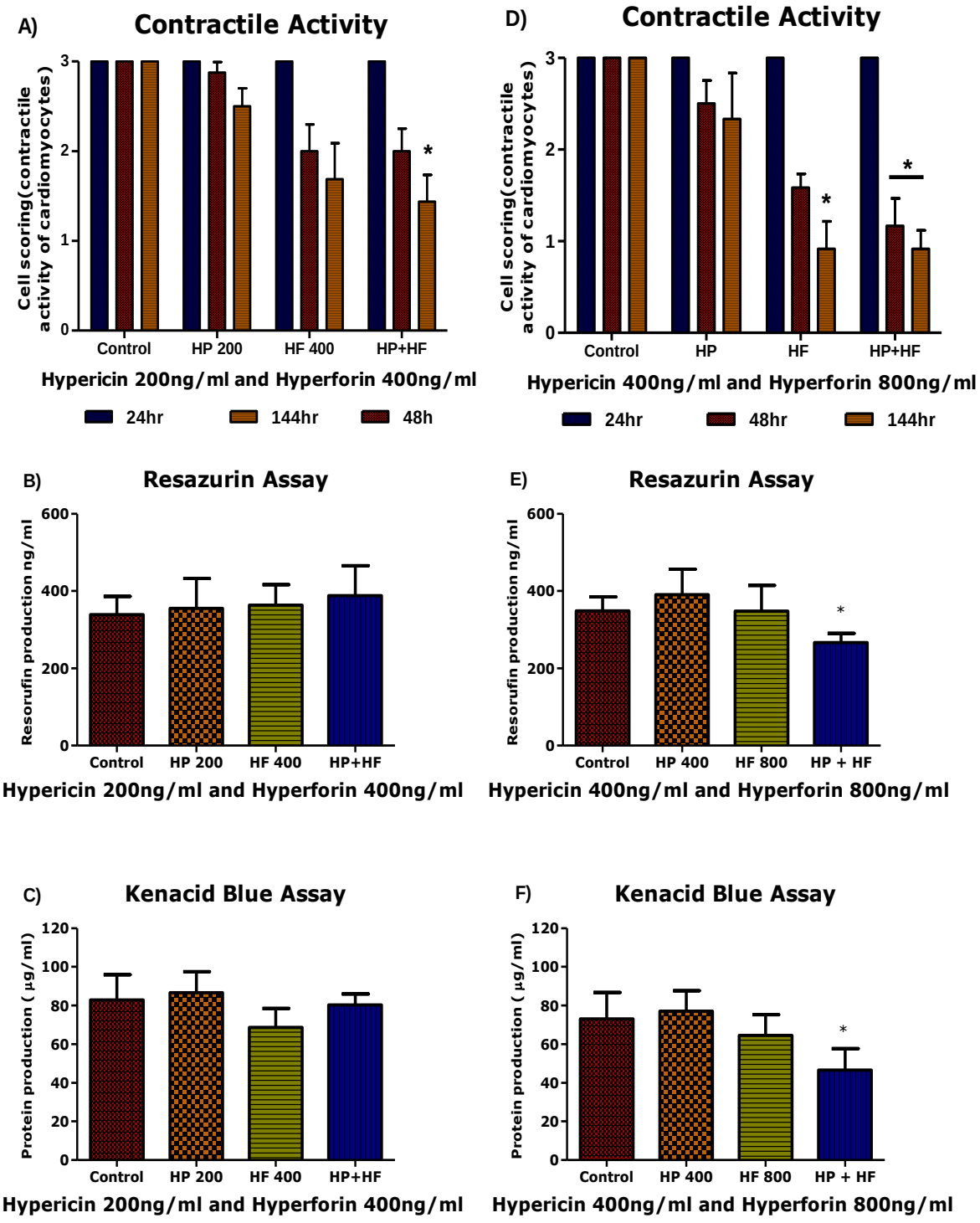


Fig 6.3 The effects of HP and HF on cardiomyocytes in the MM system. A) Effects of HP 200ng/ml and HF 400ng/ml on contractile activity, B) effects of HP 200ng/ml and HF 400ng/ml on cell viability (resazurin assay), C) effects of HP 200ng/ml and HF 400ng/ml on total protein content (kenacid blue assay), D) effects of HP 400ng/ml and HF 800ng/ml on contractile activity, E) effects of HP 400ng/ml and HF 800ng/ml on cell viability (resazurin assay), F) effects of HP 400ng/ml and HF 800ng/ml on total protein content (kenacid blue assay). * represents a significant difference from control.

6.5 Discussion

Increased depression during pregnancy complicates the pregnancy by altered behaviour and increased morbidity rates in the developing embryo (Bellantuono *et al.*, 2007). These increased psychopathological symptoms during pregnancy may directly affect development or the effect may be indirectly mediated by increased cigarette and alcohol consumption due to abnormally depressed behaviour (Bonari *et al.*, 2004a). Untreated depression during pregnancy leads to spontaneous abortion, low birth weights, small head circumference, low Apgar score, neonatal retardation, and may lead to hypertension (Bonari *et al.*, 2004a) and preeclampsia which may develop into eclampsia (Kurki *et al.*, 2000).

Complementary or alternative medicine use has increased markedly during the 1990's. The ease of availability and the general perception of their harmless nature, due to natural ingredients, have played a critical role in the increased popularity (Klepser *et al.*, 2000; Kober *et al.*, 2008). Unfortunately patients often do not disclose the use of herbal medicines or practitioners do not give it importance while recording the medical history (Klepser *et al.*, 2000). Due to a lack of comprehensive studies on SJW effects in pregnancy, and the complication produced by depression, it is difficult to define whether any effects are the results of drug toxicity or depression itself. Further, this dilema is augmented by the presence of a number of pharmacologically active compounds in SJW, which makes it difficult to identify the particular toxic compound.

In this study the active antidepressant constitutents of SJW extract, hypercin and hyperforin, were evaluated separately and in combination for their synergistic contribution using the chick embryonic *in vitro* micromass cardiomyocyte system. Contractile activity, which is a cardiomyocyte intrinsic property, when evaluated with hypericin, showed no significant effect at all doses, although the hypericin 400ng/ml dose was much higher than serum therapeutic levels. The same observation of no altered trends compared to control was found with cell viability and cellular protein content which reflects the effect on cellular proliferation. Antiproliferative effects and decreased cell survival were expected with hypericin (da Conceição *et al.*, 2010).

However, at low doses the toxic effects of hypericin are mainly dependent on light activation and in this experiment the hypericin was protected from light. This confirms the toxic effects of hypericin are probably due to the production of singlet oxygen or ROS, which may disturb different cellular processes by binding to proteins and DNA (Karioti & Bilia, 2010; Theodossiou *et al.*, 2004; Wurglics & Schubert-Zsilavecz, 2006). Low ROS production leads to apoptosis even cell survival, but at high doses it induces necrosis (Kober *et al.*, 2008). The other drug hyperforin at higher doses significantly decreases the cardiomyocytes contractile activity at 400 and 800ng/ml, with some small effects on cell viability and cell proliferation, but these differences were not significant compared to the control. The effect on contractile activity at higher doses may involve some interference with the contraction mechanism or related proteins with no effects on cellular proliferation and viability. Cellular proliferation in the HF treated group does not look very different from the control and this may indicate antiproliferative signalling was not triggered at this dose or in cardiomyocytes compared to the other cell line observed by Hostanka *et al*, 2003. The synergistic effects of hypericin and hyperforin on cell proliferation were already shown by Hostanka *et al*., 2003, and a similar decrease in cell protein and cell viability was also observed in these experiments. The combination of drugs, hypericin with ROS production properties (Theodossiou *et al.*, 2004; Wurglics & Schubert-Zsilavecz, 2006) and hyperforin caspase activation (Hostanska *et al.*, 2003) may contribute to the toxic mechanism. The combined drugs effects on contractile activity were more likely caused by hyperforin alone. It may be that inhibitory effects on contractile activity are purely from hyperforin but antiproliferative effects are due to some synergistic antiproliferative mechanism. In view of above results it can be concluded that the active constituents of SJW, hypericin and hypericin, neither alone nor in combination, at serum therapeutics levels do not produce severe toxic effects on embryonic cardiomyocytes in cell culture. But the drug dose above serum therapeutic level may produce some toxic effects even in the absence of light.

Chapter 7

Effects of selected teratogens on connexin43 expression and distribution in micromass and ES derived cardiomyocytes

7.1 Intercellular communication

The complex events underlying development and homeostatic balance require a flow of information between cells and tissue systems (Levin, 2002; Vinken *et al.*, 2006). The cells exchange information either by release of chemicals or signalling molecules through extracellular spaces or intercellularly between contiguous cells via specialized cell membrane nexus aggregates (gap junctional plaques) (Dhein, 1998; John *et al.*, 2003).

7.2 Intercellular communication via gap junctions

Intercellular communication via gap junctions mediates important regulatory mechanisms in embryonic development, cellular growth control, differentiation, apoptosis, electrical coupling, tissue homeostasis and metabolic transport (Dhein, 1998; Leithe & Rivedal, 2004a; Solan & Lampe, 2005). Gap junctions are specialized cell membrane spanning conduits (channels), which allows the direct exchange of small molecules (<1KDa) including second messengers, nucleotides, ions and other metabolites between adjacent cell cytoplasm (Leithe & Rivedal, 2004a; Ruch *et al.*, 2001; Solan & Lampe, 2005). This extremely versatile system allows rapid synchronized spread of action potentials in excitable cells by ion diffusion (electrical coupling) and of small molecules and second messengers in non-excitable cells (metabolic coupling) (Goodenough & Paul, 2003; Michael, 2007; van Veen *et al.*, 2001). Mutation in gap junction genes or disturbance in gap junction intercellular communication (GJIC) results in a number of diseases including tumour growth (Ruch *et al.*, 2001).

In vertebrates, gap junctions are encoded by the connexin gene family, while in invertebrates it is innexins (Lo, 2000; Michael, 2007; Wei *et al.*, 2004). To date, 20 connexin genes in the mouse genome and 21 in the human genome have been identified (Söhl & Willecke, 2004; Wei *et al.*, 2004). Two nomenclature systems are used to designate connexins; one based on molecular mass, for example 43KDa connexin protein is Connexin43 or Cx43; the second system divides the connexins into subclasses (α, β, γ, or δ) according to sequence similarity and cytoplasmic domains (Vinken *et al.*, 2006; Wei *et al.*, 2004). Connexins are fairly ubiquitous,

with Cx43 the most widespread (Laird, 2005). The connexins are cotranslationally integrated in the endoplasmic reticulum (ER), assembled into connexons in the *trans* Golgi network (TGN), then trafficked and docked head to head in the plasma membrane (PM) to form a junction with another connexon supplied by the adjacent cell or they connect the cell with extracellular milieu (non-junctional) (Solan & Lampe, 2005).

7.3 Gap junction's composition (Topology)

Gap junctions are dodecameric bipartite structures, composed of one hexameric subunit (connexon) from each communicating cell (Dhein, 1998; Vinken *et al.*, 2006), which bridge a 2nm gap between the plasma membrane of adjacent cells (Dhein, 1998). Each connexon is an assembly of six connexin proteins and the overall gene and protein structure among various connexins isoforms is largely conserved (van Veen *et al.*, 2001). Each connexin protein has 9 principal domains: the four transmembrane domains (TM1-TM4), two extracellular loops (E1 and E2), the C-terminus (CT), the N-terminus (NT) and the cytoplasmic loop (fig 7.01A) (Dhein, 1998; van Veen *et al.*, 2001; Vinken *et al.*, 2006). The C-terminus amino acids sequence length determines the molecular mass of the particular connexin, while the extracellular loops and four transmembrane domains are highly conserved among species and connexins isoforms (Dhein, 1998; van Veen *et al.*, 2001). The four transmembrane domains (M1-M4) exhibits an α-helical structure, while the extracellular domains form a tight seal to prohibit substance leakage between the channel lumen and the extracellular milieu (Wei *et al.*, 2004). Three conserved cysteine residues in extracellular loops E1 and E2 help in connexon head-to-head docking by forming a disulphide bond with the opposing hemichannels. (Dhein, 1998; van Veen *et al.*, 2001). Connexins may express different isoforms (Martin & Evans, 2004) by forming homomeric (six identical connexins) or heteromeric (more than one connexins isotype) connexons, which in turn may bond with either the same (homotypic) or different (heterotypic) connexons from the adjacent cell (fig 7.01B) (Dhein, 1998; Wei *et al.*, 2004).

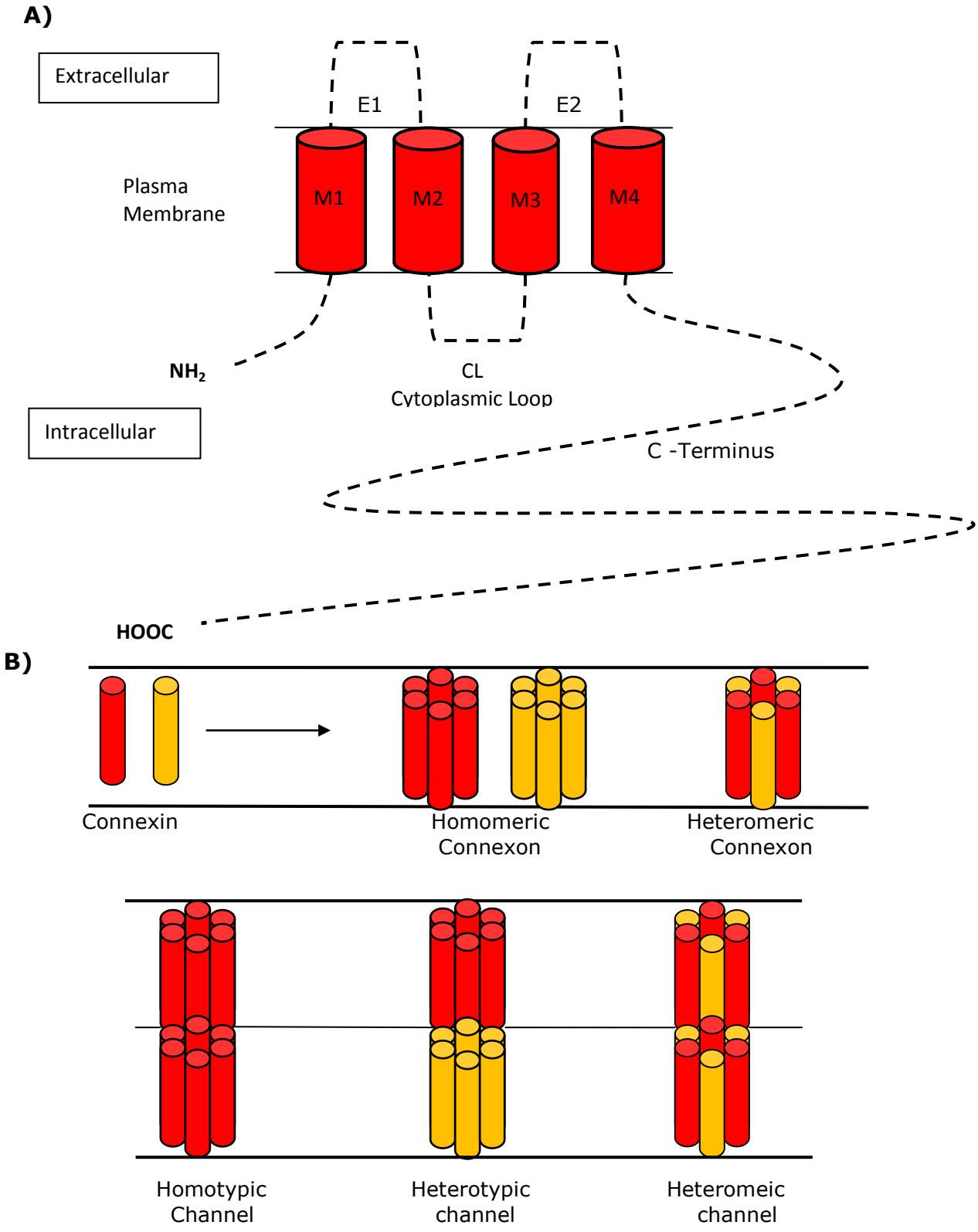


Fig 7.1A Connexin structure adopted from Sohl *et al*., 2004; **fig 7.1B** schematic diagram representing the six connexin protein assembly to form homo or hetero connexon. These connexon may form either homo or heterotypic channels at the plasma membrane.

7.4 Gap junction regulation and phosphorylation

Regulation of GJIC from gene transcription to degradation makes a substantial contribution in number of physiological and pathological situations (Laing *et al.*, 1997; Vinken *et al.*, 2006). Gap junctions have a half-life of 2-5h; therefore continuous turnover is critical for GJIC (Solan & Lampe, 2005). Investigations have shown that connexin protein biogenesis mainly occurs in ER membrane bound ribosomes, and they oligomerize into hexameric connexons as they are transported through the TGN, followed by their trafficking along the secretory pathways to fuse into the PM (Lauf *et al.*, 2002; Martin & Evans, 2004). In the PM the randomly dispersed connexons cluster at new or pre-existing gap junction plaques for functional coupling (Chipman *et al.*, 2003; Lampe & Lau, 2004). A number of studies confirm the presence of non-junctional connexins in PM that connect cells to the extracellular milieu (Lauf *et al.*, 2002). Molecular chaperones assist in stable protein folding in ER, and upon exit the properly folded proteins pass through the ERGIC prior to entering TGN (Laird, 2006), while misfolded proteins are subjected to degradation (Berthoud *et al.*, 2000). The precise location for oligomerization is unclear, it differs with connexin and cell type but the disruption of the Golgi apparatus and microtubule network prevents Cx43 trafficking (Martin & Evans, 2004). An exception is Cx26 which oligomerizes before reaching the Golgi apparatus and is transported via Golgi independent pathways (Laird, 2006; Thomas *et al.*, 2005). Therefore it is slightly affected by Golgi disturbance, while highly sensitive to microtubule inhibitors (Martin & Evans, 2004). Closed connexons are delivered to the PM to prevent uncontrolled exchange (Laird, 2006), where cadherin based adherens junctions may act as a prerequisite for gap junction formation and cell-cell coupling (Wei *et al.*, 2004). The Cx43 independent binding domains in CT interacts with cell junction proteins like ZO-1 (which links connexin to the cytoskeleton actin) thereby generating scaffolds for recruiting other regulatory proteins in gap junctions (Chipman *et al.*, 2003; Martin & Evans, 2004). Beside this a number of Cx43 protein binding partners including α/β-catenin, p120-catenin, ZO-2, caveolin-1, cadherins, α-actinin and microtubules participate in signalling pathways that regulate cell

adhesion, cell motility, and the actin cytoskeleton. (Laird, 2006; Wei *et al.*, 2004). Gap junction gating is regulated by a number of mechanisms including Ca^{2+}, pH, free radicals, transjunctional and transmembrane voltage and phosphorylation (Chipman *et al.*, 2003; Vinken *et al.*, 2006; Wei *et al.*, 2004).

Both ubiquitin-proteasomal and lysosomal pathways are involved in connexin degradation. The proteasome degrades most cytosolic and nuclear proteins and removes misfiled and disaggregated proteins from the ER, while the lysosome degrades integral membrane proteins and receptor mediated internalized proteins (Berthoud *et al.*, 2000; Laing *et al.*, 1997; Qin *et al.*, 2003). Gap junction internalization occurs via the formation of an annular junction, which is internalized into one of the two cells and degraded (Jordan *et al.*, 2001).

Transient changes in the GJIC signalling cascade appear necessary for normal cell cycling (Solan *et al.*, 2003). The connexin gap junctions undergo various biochemical and post translation modifications, but among these phosphorylation has acquired the most attention (Solan & Lampe, 2007). Connexin phosphorylation regulates or alters the normal functioning of gap junctions. Altered phosphorylation induces changes in the connexin protein structure, which translocate the protein to the cytoplasm instead of forming GJ within the cell membrane (Musil & Goodenough, 1990). Connexin phosphorylation is apparently not required for functional channel formation, but appears to efficiently regulate channel gating, assembly and turnover (Martin & Evans, 2004; Solan *et al.*, 2003).

The majority of connexin proteins are phosphoproteins (Solan & Lampe, 2005), with the exception of Cx26, which is probably due to a short CT (Lampe & Lau, 2000; Musil & Goodenough, 1991). Phosphorylation mostly occurs on the CT of the protein, while NT phosphorylation has not been reported (Solan & Lampe, 2005). Connexin phosphorylation predominantly occurs on serine residues, but has been reported also on threonine and tyrosine residues of CT (van Veen *et al.*, 2001). Cx43 CT is involved in channel voltage gating and phosphorylation and dephosphorylation of CT may affect this (Moreno & Lau, 2007). Difference in unitary conductance of the channel has been attributed to the phosphorylation state (Moreno, 2004).

Several kinases are known to phosphorylate connexins, including MAPK, PKC, PKA, PKG, CK1, v-Src, p130gag-fps (Lampe & Lau, 2000; Musil & Goodenough, 1991; van Veen *et al.*, 2001; Vinken *et al.*, 2006). Little is known about connexin dephosphorylation that maintains the equilibrium (Vinken *et al.*, 2006).

Cx43 upon phosphorylation showed reduced migration on polyacrylamide gel electrophoresis (SDS-PAGE) (Solan *et al.*, 2003). Western blot analysis of Cx43 indicates three bands ranging from 41-46KDa molecular weight: P_o the non-phosphorylated state, the P_1 phosphorylated state and the P_2 the highly phosphorylated state (van Veen *et al.*, 2001). These phosphorylated species are sensitive to phosphoserine specific phosphatases (Qin *et al.*, 2003).

Reagents that activate PKC inhibit GJIC (Lampe & Lau, 2000). The phorbol ester TPA was the first tumour promoter identified as a GJ inhibitor (Loch-Caruso *et al.*, 2004). TPA hyperphosphorylates the Cx43 on serine368 by direct stimulation of PKC, which down regulates Cx43 trafficking, intercellular coupling and GJIC (Cruciani & Mikalsen, 2002; Solan *et al.*, 2003; van Veen *et al.*, 2001). Cx43 assembly is blocked and the half-life is shortened on TPA exposure (Lampe, 1994). During key stages of the cell cycle where gap junctional assembly is reduced, increased Ser368 phosphorylation was observed (Solan *et al.*, 2003). Phosphorylation at Ser255 accelerates internalization and degradation, while tyrosine phosphorylation reduces GJ. Growth factors like EGF PDGF, IGF and FGF decrease GJ by phosphorylation of Cx43 (van Veen *et al.*, 2001).

The PKC activator TPA promotes tumorigenesis, increases gap junction phosphorylation and decreases GJIC in a number of cell types (Lampe & Lau, 2000; Ruch *et al.*, 2001). TPA mimics diacylglycerol. DAG is produced along with IP3 via PLC mediated cleavage of PIP2. The IP3 induces calcium release while DAG activates PKC (Leithe & Rivedal, 2004b; Nishizuka Y, 1986). TPA mimics DAG structure and activates PKC. The TPA phosphorylates Cx43 at Ser368 via PKC activation and inhibits gap junction assembly and modifies the channel properties (Lampe & Lau, 2000; Ruch *et al.*, 2001). After prolonged exposure to TPA the cells become refractory to the inhibitory effects, as had been frequently observed in other cells.

This may be due to down regulation of PKC, a major cellular target for TPA (Oh *et al.*, 1991).

cAMP has been found to increase GJIC (Lampe & Lau, 2000) and several studies have indicated that activation of the PKA pathway via dibutryl-cAMP increases serine364 (Ser364) phosphorylation, which leads to an increase in GJIC and the number of Cx43 positive plaques (TenBroek *et al.*, 2001). However some cell types do not alter the phosphorylation status of Cx43 in response to cAMP. Recent evidence indicated that the cAMP dependent PKA pathways did not phosphorylate Cx43 directly, but enhanced assembly of Cx43 GJIC and was totally dependent upon the basal phosphorylation of Ser364 by unknown kinases (TenBroek *et al.*, 2001).

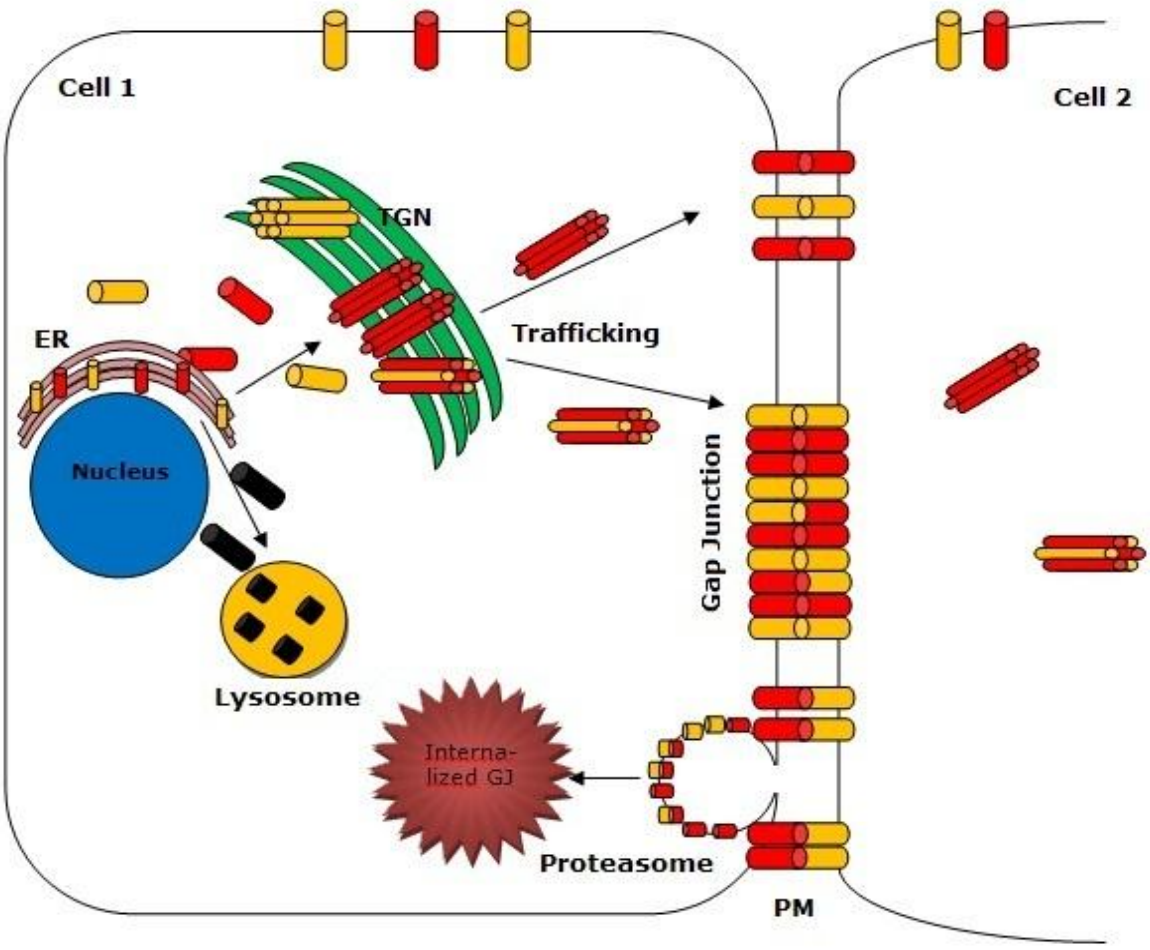


Fig 7.2 Schematic diagram representing connexin biogenesis in the ER and assembly at TGN to form connexon hemichannels. The black misfolded proteins are degraded by the lysosome. The Connexin migrates to PM to form gap junction. The junctional plaque is internalized and degraded via proteasomal pathways.

7.5 Gap junctions in the cardiovascular system

Connexins are not only structural precursors of gap junctions (Vinken *et al.*, 2006), but also they play a critical role in development by forming morphogen gradient compartments, which regulate cell growth, patterning and differentiation (Wei *et al.*, 2004). In the heart, connexins are involved in electrical impulse propagation, development and cardiomyocyte volume regulation (Gros *et al.*, 2005). The non-junctional channels control NAD and ATP release in the extracellular spaces, which are involved in Ca^{2+} wave propagation between cells. These channels remain closed most of the time and are tightly regulated, otherwise they may cause cell death (Vinken *et al.*, 2006).

Cardiac tissues are arranged phenotypically, in different compartments, and the gap junction channels connect and electrically synchronize these compartments (Levin, 2002; Vink *et al.*, 2004). Gap junctions in the heart typically co-exist with adhesion junctions at ingeniously orchestrated intercalated discs, which facilitate the propagation of low resistance electrical impulses and mechanical attachment between cardiomyocytes (Gourdie, 1995; Martin & Evans, 2004). Cx43 is the predominant isoform expressed in the heart, others include Cx40, Cx45, Cx37 and Cx50 (Vink *et al.*, 2004). Cx43 is widely expressed in all atrial and ventricular myocardium cells at all stages of development and is principally responsible for electrical synchrony in the heart (Martin & Evans, 2004; van Veen *et al.*, 2001; Wei *et al.*, 2004). Cx45 is in the SA and AV node, Cx40 is coexpressed with Cx43 in atrial myocardium (Martin & Evans, 2004; van Veen *et al.*, 2001), Cx37 is confined to endothelial tissue and Cx50 is only in cardiac valves (Vink *et al.*, 2004). Cx40 generates channels with high conductance and Cx45 forms voltage-sensitive channels with very low conductance (Wei *et al.*, 2004). This compartmentalized connexin expression pattern in the heart is expected to provide for the orderly sequential spread of activation potential from the atrial to ventricular chambers (Wei *et al.*, 2004). An increase in Cx43 expression is observed immediately prior to labour (Lampe & Lau, 2000; Solan & Lampe, 2005). The gene targeting studies have further explored the specific role of connexin channels in heart conduction and

morphogenesis. Cx43 disturbance interferes with the working myocardium, and slow conduction and partial AV block is observed in Cx40 knockout, while Cx45 knockout mice exhibit conduction block and endocardial cushion defects in gestation (Wei *et al.*, 2004). Cx40 knockout mice die during gestation with AV septation defects or outflow tract malformations, while Cx45 is required for endocardial cushion development. Cx43 plays an essential role in development of the coronary arteries and outflow tract morphogenesis (Wei *et al.*, 2004) and mice with Cx43 disruption exhibit right ventricular outflow tract obstruction, causing cyanosis and death at birth (Vinken *et al.*, 2006). Despite this abnormality, however, the hearts of these Cx43 deficient animals still beat rather rhythmically, though not synchronously throughout the tissue, suggesting that gap junction proteins in addition to Cx43 may partially compensate for the loss of intercellular connections (Vink *et al.*, 2004). In Cx43 knockout mice, dysregulation of neural crest cell migration during embryonic development results in heart malformation (Giepmans, 2004). In $Cx40^{-/-}$ mice, the most common abnormalities are double outlet right ventricle, bifid atrial appendages, tetralogy of Fallot, ventricular septal defects, aortic arch abnormalities and partial endocardial cushion defects. These results indicate that Cx40 role in the septation process (Gros *et al.*, 2005).

7.6 Gap junctions in disease and teratogenicity

The GJIC is regarded as an important mechanism in coordinating growth and development and tissue compartmentalization during embryogenesis (Goodenough *et al.*, 1996). Communication between cells is very important during development; small morphogens can pass through the channels and may control or affect normal functioning. Abnormal connexin expression is involved in cancer, cardiac ischemia and cardiac myopathy (Kumar & Gilula, 1996). The processes of mitotic cell division and apoptosis exhibit high levels of GJIC at the start which decreases as the event progress, this correlates the GJIC and cellular growth (Vinken *et al.*, 2006). Tumour cells have dysfunctional GJIC, which may be due to post-translational modification or decreased degradation (Leithe & Rivedal, 2004a). Connexin overexpression in tumour cells act as a tumour suppressor, which decreases cell proliferation and

increases cell death (Vinken *et al.*, 2006).Mutation in connexin proteins results in 'channelopathies' which negatively regulate various processes at cellular levels and may affect development (Levin, 2002; Solan & Lampe, 2005). The connexin C-terminus tails possess multiple sites for phosphorylation and this regulates channel assembly, trafficking, gating and turnover (Lampe & Lau, 2000). Cx43 is ubiquitously expressed during embryo development in various tissues. In Cx43 knockout mice, dysregulation of neural crest cell migration during embryonic development results in heart malformation (Giepmans, 2004). Cx43 knockout mice die due to malformation of the conotruncal region of the right ventricle (Lampe & Lau, 2000). Mutation in Cx43 from serine to proline at Ser364 results in visceral atrial heteroataxia in humans and Xenopus (Levin, 2002; Solan & Lampe, 2005). Cx43 pharmacological inhibitors, blocking GJC, affect cellular proliferation (Giepmans, 2004). C-terminus tail lacking mutants of Cx43 and Cx32 result in inhibition of cell proliferation (Giepmans, 2004), while Cx43 mutation results in developmental abnormalities of the face, eyes, limbs and dentition called oculodentodigital dysplasia (ODDD) (Laird, 2006; Wei *et al.*, 2004). Mutation in Cx26, Cx30 and Cx31.1 genes result in non-syndromic deafness and skin disorders (Martin & Evans, 2004), Cx32 results in demyelinated Schwann cells causing Charcot-Marie-Tooth syndrome (CTMX), Cx26 and Cx45 knockout mice die in utero, Cx37 female mice are infertile (Lampe & Lau, 2000). Cx50 and Cx46 mutation is implicated in cataract formation (Lampe & Lau, 2000; Martin & Evans, 2004).

7.7 Material and Methods:

The immunohistochemistry and Western blot analysis materials (Appendix 1) and methods are discussed in detailed in chapter 2.

7.7.1 Statistical analysis

For Western blot analysis three repeat results were statistically analysed using one way ANOVA followed by post hoc test.

7.8 Controls

7.8.1 Positive Control

Day 9 chick hearts were used as a positive control for Cx43 protein immunolocalization and Western blot analysis. Heart tissue has been shown to express Cx43 with increased intensity with development (Kirchhoff *et al.*, 2000; Wiens *et al.*, 1995).

7.8.2 Negative Control-TPA

The PKC activator TPA (12-O-tetradecacanoylphorbol-13-acetate) was used as a negative control. TPA decreases the Cx43 gap junction communication by hyperphosphorylation in a number of cell types (Lampe & Lau, 2000). TPA increases the phosphorylation of Cx43 at Ser368 in the C-terminus tail. Increased phosphorylation results in decreased communication between cells by decreasing GJ assembly (Lampe & Lau, 2000; Ruch *et al.*, 2001). The TPA was diluted in DMSO to a stock solution of 50μM; the stock solution was further diluted to 100nM in culture medium and exposed to the cells for 2h.

7.8.3 Loading control for Western blot

Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in glycolysis and catalyzes the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate. It has also been implicated in several non-metabolic cell processes (Tarze *et al.*, 2006). The GAPDH gene is stable and constitutively expressed at high levels in almost all tissues and considered as a housekeeping gene. The housekeeping genes are used as a loading control to normalize the protein in molecular assays (Barber *et al.*, 2005). The Western blot analysis exhibits the GAPDH band at approximately 36-40KD.

7.9 Results

The connexin43 Western blot analysis exhibits multiple bands (Green color bands). Indicating a lower non-phosphorylated P_0 and higher phosphorylated P_1 band between the molecular weight 40-50KDa. The non-phosphorylated isoform showed increased migration on gel compared to the high molecular weight phosphorylated isoform of Cx43. The phosphorylated isoform of Cx43 at Ser368 was labelled (red band) by the phospho-Ser368 Cx43 specific antibody between the Cx43 bands. With Cx43 WB a number of non-specific band were observed on the blot that may represent some fluorescent proteins labelled by primary or secondary antibodies (Fig 7.3A). In immunolocalization the Cx43 protein was found appositionally at the cell membrane or the intracellularly in the cytoplasm (Fig 7.3B).

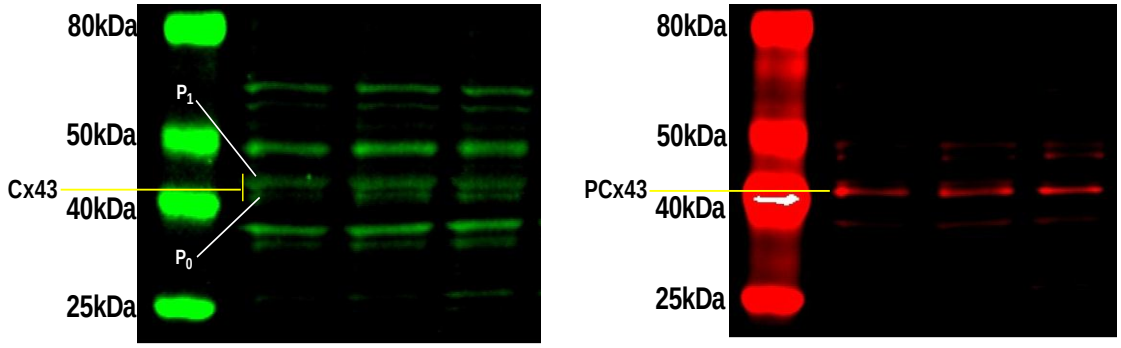


Fig 7.3A Cx43 and PCx43 western blot analysis. Exhibiting the **Cx43** P_0 and P_1 isoforms and **PCx43** bands.

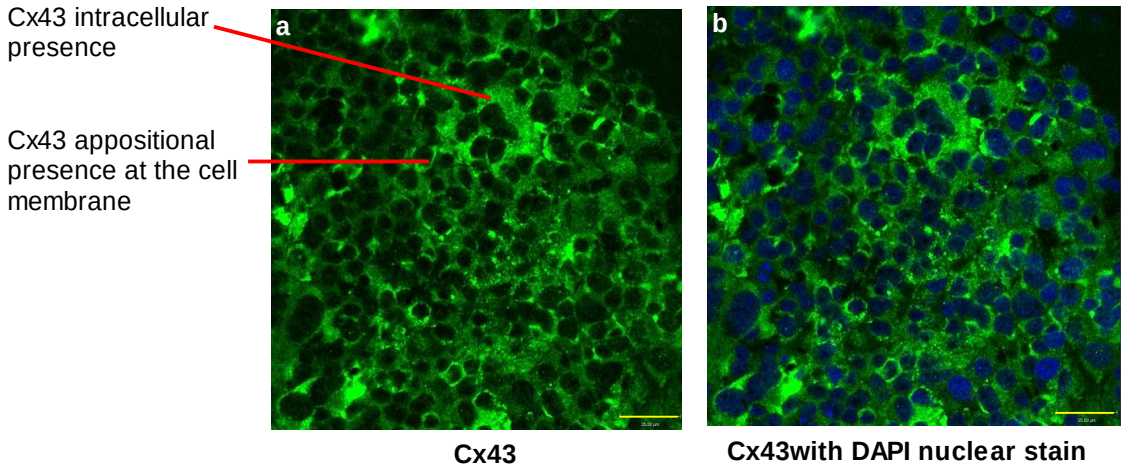


Fig 7.3B Cx43 immunolocalization intracellularly and at the cell membrane. a) The **line** indicates the **Cx43** protein presence intracellularly and at the cell membrane, b) **Cx43** protein with **DAPI** nuclear stain. (Scale bar 33µm).

7.9.1 Evaluation of Cx43 antibody

The connexin 43 immunolocalization was studied in both systems and day 9 chick hearts. The control showed no non-specific binding with secondary antibody only with the cardiomyocytes (fig 7.5A). The cryosectioned day 9 chick heart staining with monoclonal anti-connexin 43 showed the presence of Cx43 protein (fig 7.5B). In the micromass system the cultured embryonic cardiomyocytes exhibit appositional (cell membrane) and intracellular (cytoplasm) presence of Cx43 (fig 7.5C). The ES derived contractile cardiomyocytes also exhibit the presence of Cx43 in the cell cytoplasm and at the membrane connecting with other cells (fig 7.5D).

Western blot analysis for the quantification of Cx43 and PCx43 in culture cardiomyocytes and day 9 hearts exhibits a protein band between molecular weight marker 40 and 50kDa (fig 7.4A). The green bands of Cx43 represent different phosphorylated states of the protein with different migration potential on the gel (fig 7.4A). The red band at or above Cx43 represent the CT Ser368 phosphorylated isoform of Cx43 tagged with Phospho-Cx43 (PCx43) antibody (fig 7.4A). The house keeping gene GAPDH band was observed on the blot below the 40KDa molecular marker and was used to normalize the protein (fig 7.4B).

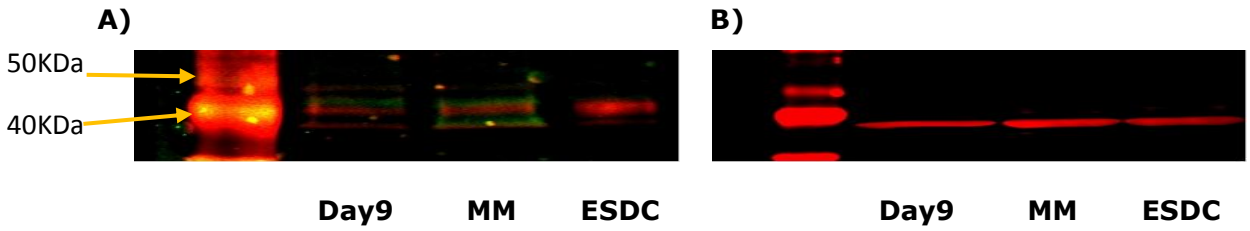


Fig 7.4 Western blot analysis of Cx43 and phospho-Cx43 proteins. A) Represents **Cx43** and **PCx43** bands, B) the red band below 40KDa marker represents **GAPDH**.

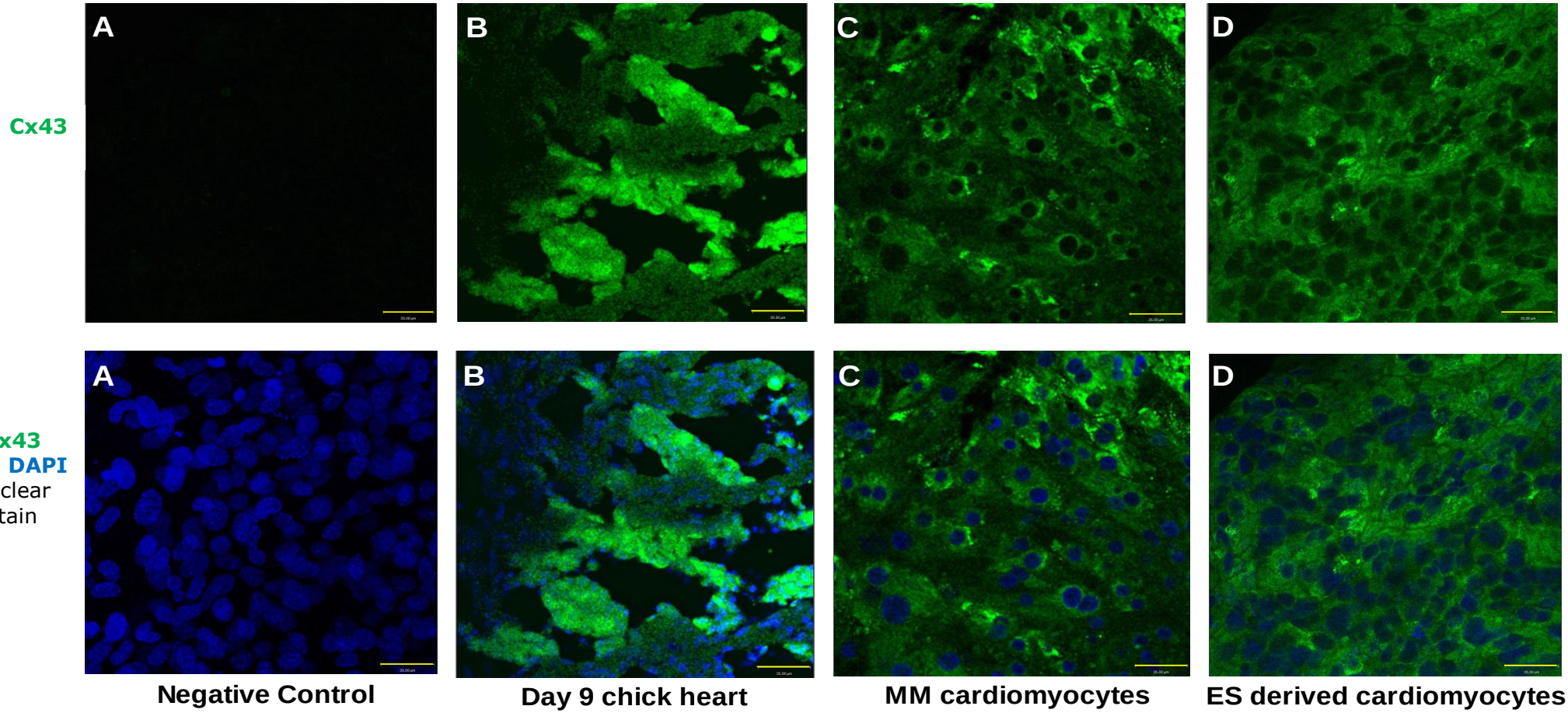


Fig 7.5 **Cx43** immunolocalization in day 9 chick heart, MM and ES derived cardiomyocytes. A) Negative control, B) Day 9 chick heart, C) micromass cardiomyocytes and D) ES cell derived cardiomyocytes (Green color represents **Cx43** while Blue is **DAPI** nuclear staining). (Scale bar 33μm).

7.9.2 Effects of VPA on Cx43 immunolocalization in the MM and ESDC systems

In the MM system, the Cx43 monoclonal antibody tagged with green fluorescent Fluo488 secondary antibody, showed the presence of Cx43 proteins intracellularly and at the cell membrane in the cultured cardiomyocytes (fig 7.6A). The negative control of TPA (100nM) treatment for 2h reduced the protein expression compared to the control (fig 7.6B). When Cx43 immunolocalization in VPA treated cardiomyocytes was compared with the control, increasing drug dose resulted in an overall decrease in protein expression (fig 7.6). At VPA 200μM, the effect on total protein expression was not very obvious (fig 7.6C). When the drug dose was doubled, Cx43 expression decreased with some disturbance in protein localization compared to control (fig 7.6D). These effects continued at higher doses and reduced protein expression was observed in cardiomyocytes after VPA 800μM treatment (fig 7.6E).

In the ESDC no treatment group, anti-Cx43 antibody showed the protein presence inside the cell and at the cell membrane, connecting with other cell gap junctions (fig 7.7A). With TPA treatment in ES cells, a clear decrease in protein expression was recorded with some intracellular disturbed localization (fig 7.7B). VPA treatment with same doses reduced the Cx43 protein overall expression in cardiomyocytes. At 200μM the VPA treated cells exhibited protein expression at the cell boundary, which was found to be disturbed in the 400μM treated group and was further affected with 800μM VPA treatment (fig 7.7C, D and E).

7.9.3 Western blot analysis of VPA treated cardiomyocytes in MM and ESDC system

The Western blot analysis of Connexin43 and the form of Connexin Phosphorylated at Ser368 in the cytoplasmic tail showed the positive presence of protein at its specific molecular weight of 43KDa. In the micromass system the VPA and negative control TPA treated cardiomyocytes results were compared with the control. The TPA showed a decrease in overall Cx43 protein expression and an increase in Cx43 phosphorylation when normalized using the housekeeping gene GAPDH. When the VPA results were compared with the control a significant decrease in Cx43 protein expression was observed with increased dose (fig 7.8B). Increased Cx43 phosphorylation was detected which was elevated to significant levels at the 800μM drug dose (fig 7.8C).

In the ESDC the results were more or less similar to the MM system. The TPA treatment showed some increase in the cellular phosphorylated isoform of Cx43 and decrease in total Cx43 compared to control. When the VPA treated ESDC were subjected for the quantification of cellular Cx43 and PCx43 levels the cells showed an effect on protein expression with increased VPA dose. A significant decrease in Cx43 level was observed with VPA treatment compared to control (fig 7.8E), while the Cx43 phosphorylation was increased after VPA treatment compared to the control (fig 7.8).

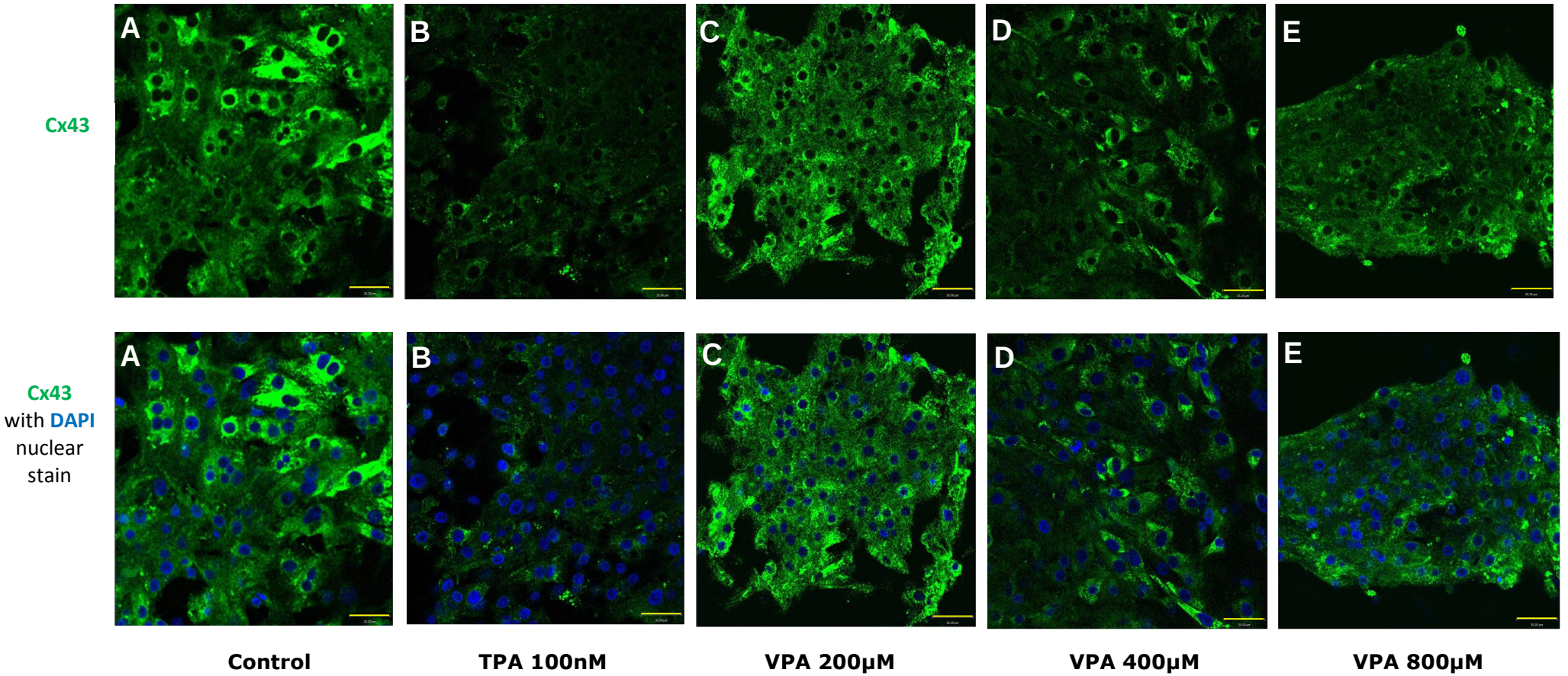


Fig 7.6 **Cx43** expression in VPA treated chick cardiomyocytes in MM system. A) Control, B) TPA 100nM, C) VPA 200µM, D) VPA 400µM and E) VPA 800µM (Green color represents **Cx43** while Blue is **DAPI** nuclear staining). (Scale bar 33µm).

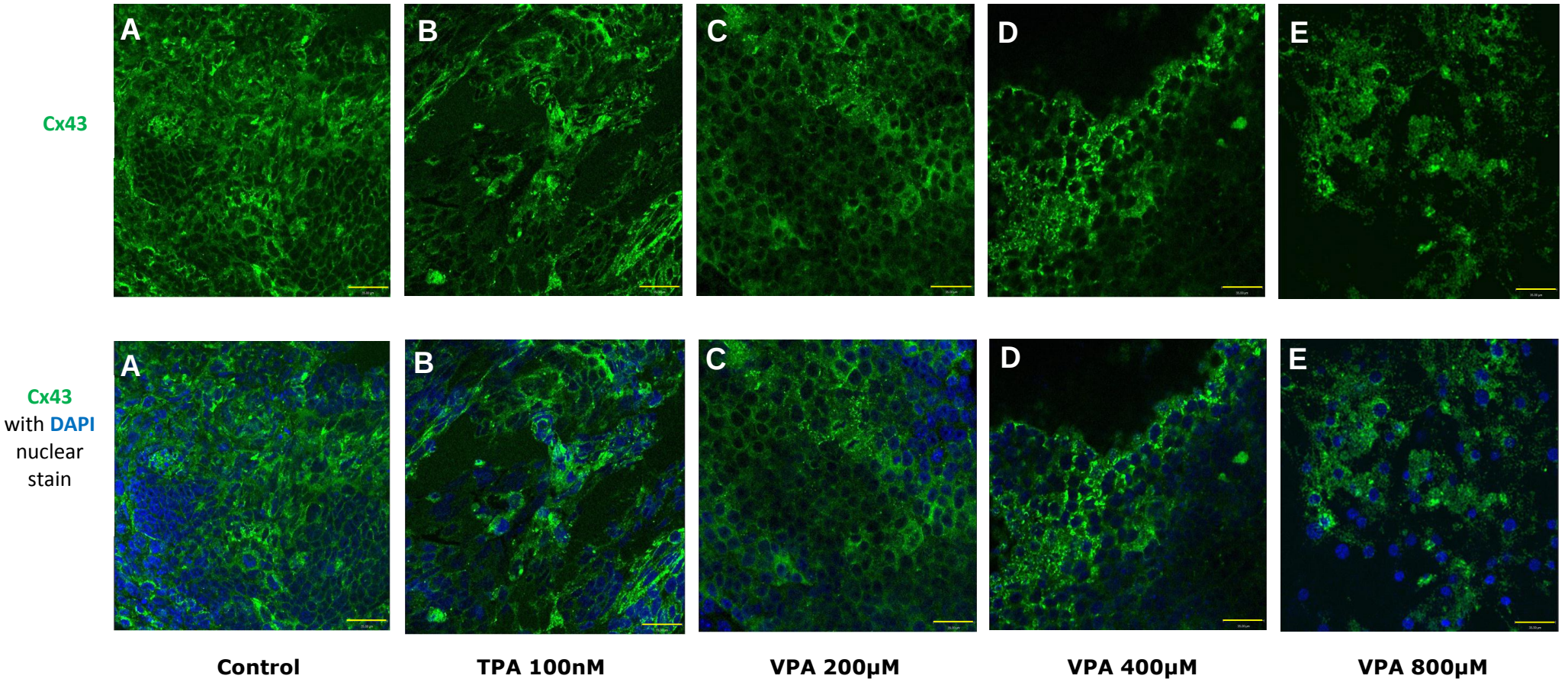


Fig 7.7 **Cx43** expression in VPA treated ES derived cardiomyocytes. A) Control, B) TPA 100nM, C) VPA 200µM, D) VPA 400µM and E) VPA 800µM (Green color represents **Cx43** while Blue is **DAPI** nuclear staining). (Scale bar 33µm).

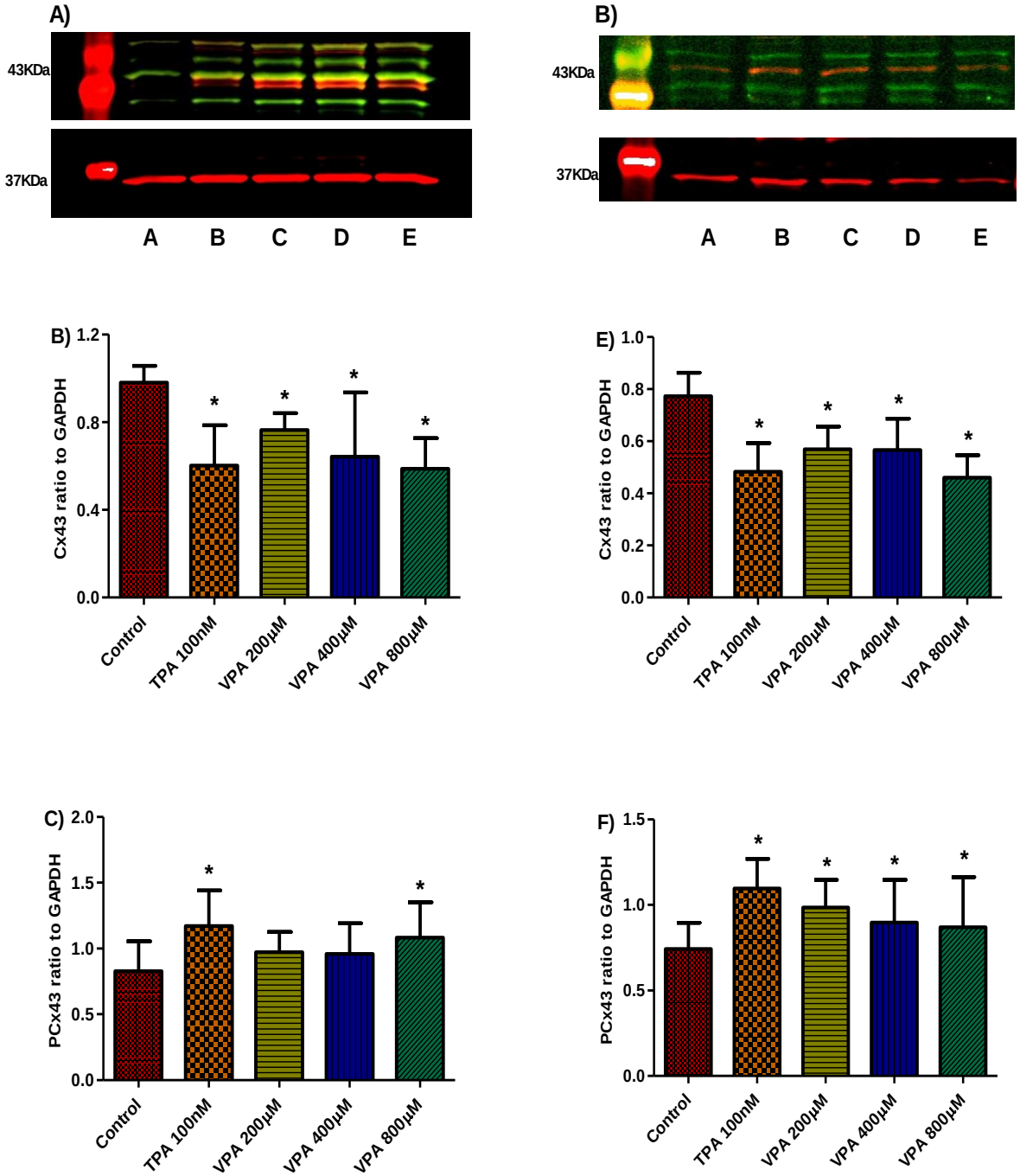


Fig 7.8 Western blot analysis of VPA effects on Cx43 and PCx43 in the MM and ESDC systems A) Cx43, PCx43 and GAPDH blot in the MM system (A=Control, B=TPA100nM, C=VPA200µM, D=VPA400µM and E=VPA800µM)., B) VPA effects on Cx43 in the MM system, C) VPA effects on PCx43 in the MM system (A=Control, B=TPA100nM, C=VPA200µM, D=VPA400µM and E=VPA800µM)., D) Cx43, PCx43 and GAPDH, blot in the ESDC system E) VPA effects on Cx43 in the ESDC system and F) VPA effects on PCx43 in the ESDC system. * represents the significant difference from control.

7.9.4 Effects of CBZ on Cx43 immunolocalization in the MM and ESDC systems

As before, control groups in the MM system showed Cx43 positive staining in cardiomyocytes expressing green color (fig 7.9A). The negative control TPA showed decreased cellular Cx43 levels after 2hr of treatment (fig 7.9B). When the cultured cardiomyocytes in the MM system were treated with CBZ for 5 days, the drug affected the Cx43 protein. The 100 and 200μM treated groups showed decreased and disturbed Cx43 localization compared to the control (fig 7.9C and D). With an increased dose of 400μM the decreased protein expression continued with disturbed assembly compared to control (fig 7.9E).

In the ESDC the control and negative control groups continued to produce the expected results (fig 7.10A and B). In CBZ 100 and 200μM treated ESDC the Cx43 expression was not very much affected (fig 7.10C and D). The cells showed weak protein expression but the cellular localization was like the control. ES cell differentiation in the presence of 400μM CBZ resulted in reduced cell proliferation and the ability to spread on the surface after attachment (see section 4.4.2.3). The cells did not form connections between them and reduced assembly of Cx43 at the cell membrane was observed (fig 7.10E).

7.9.5 Western blot analysis of CBZ treated cardiomyocytes in MM and ESDC system

In the MM system, CBZ affects Cx43 protein as observed using western blot analysis, showing reduced levels of Cx43 protein with increased drug dose (fig 7.11B). After 5 days of treatment with CBZ at 200 and 400μM the chick embryonic cardiomyocytes showed reduced levels of CX43 protein, which became significant compared to the control at 400μM CBZ dose. The negative control TPA continued to affect the cellular levels of Cx43 after 2h treatment. With PCx43 antibody the results showed a different picture; the cellular levels with CBZ treatment were unaffected even at high dose, while TPA increased the overall phosphorylation of connexin43 at Ser368 (fig 7.11C).

With the ESDC the results after CBZ treatment were like the MM system. Increase in CBZ dose resulted in a decrease in CX43 levels in the differentiating cells. With 100 and 200μM CBZ dose the Cx43 protein does not drop down much compared to the control, but with CBZ 400μM the cardiomyocytes exhibited a significantly low level of Cx43 protein (fig 7.11E). The effects on Cx43 phosphorylation showed a reduced protein phosphorylation at the Ser368 cytoplasmic tail residue compared to the control. At 100 and 200μM the values appeared to be decreased compared to control group but failed to attain statistically significant levels. While at 400μM the cells exhibited significantly reduced protein phosphorylation compared to the control (fig 7.11F).

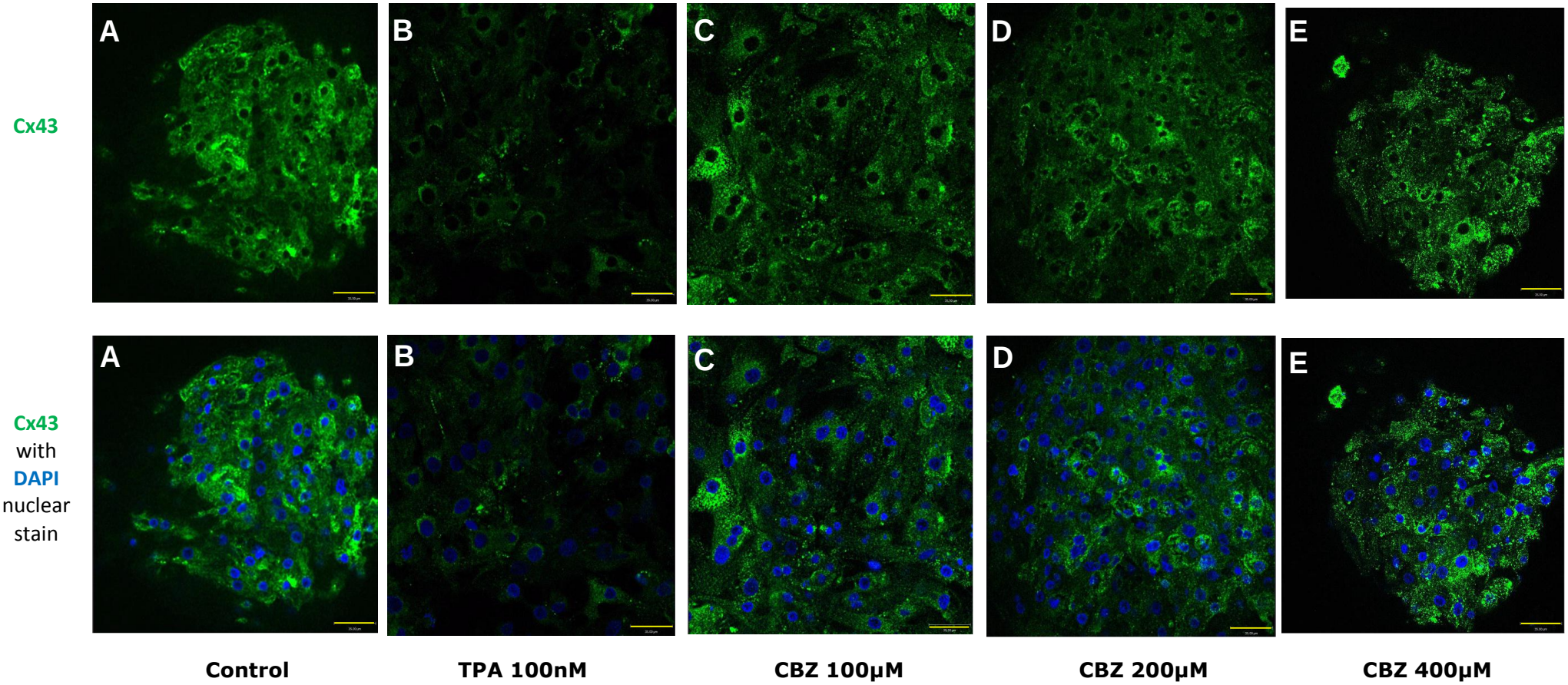


Fig 7.9 **Cx43** expression in CBZ treated chick cardiomyocytes in the MM system. A) Control, B) TPA 100nM, C) CBZ 100µM, D) CBZ 200µM and E) CBZ 400µM (Green color represents **Cx43** while Blue is **DAPI** nuclear staining). (Scale bar 33µm).

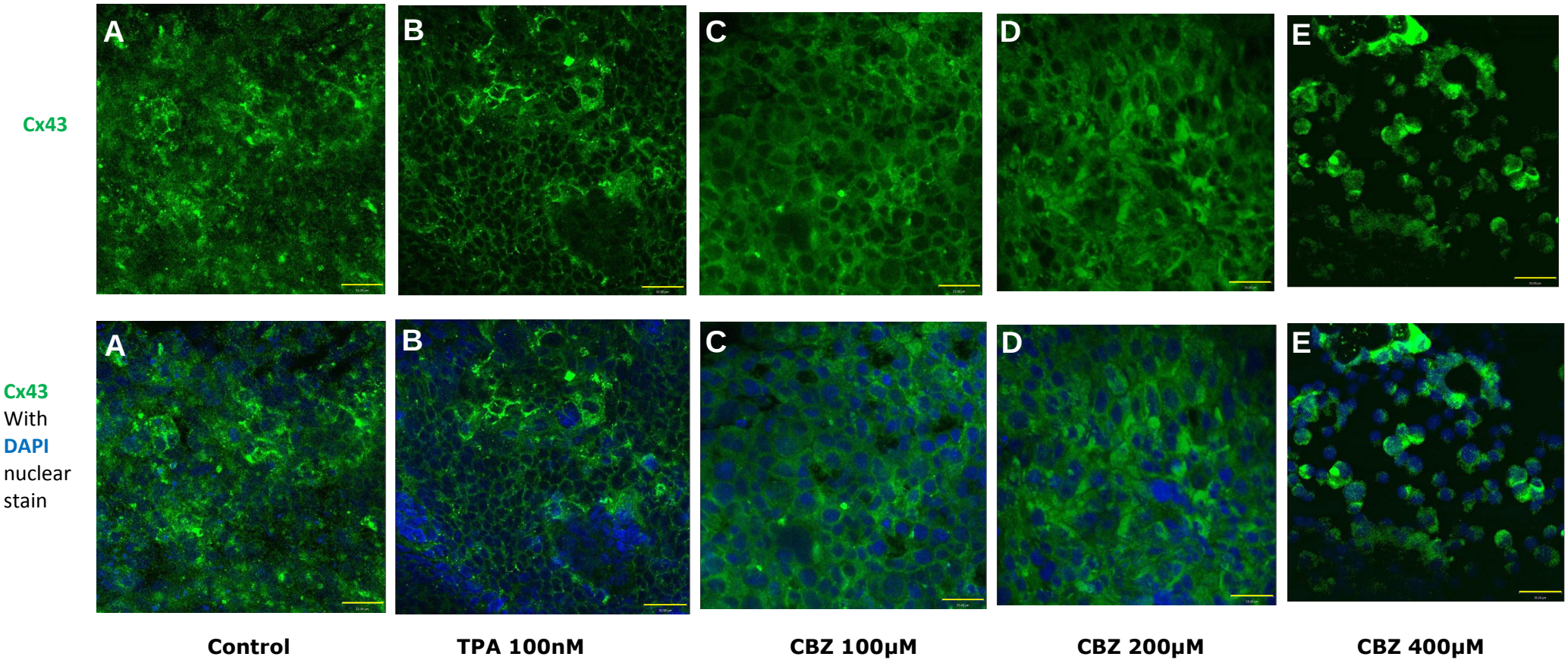


Fig 7.10 **Cx43** expression in CBZ treated ES derived cardiomyocytes. A) Control, B) TPA 100nM, C) CBZ 100µM, D) CBZ 200µM and E) CBZ 400µM (Green color represents **Cx43** while Blue is **DAPI** nuclear staining). (Scale bar 33µm).

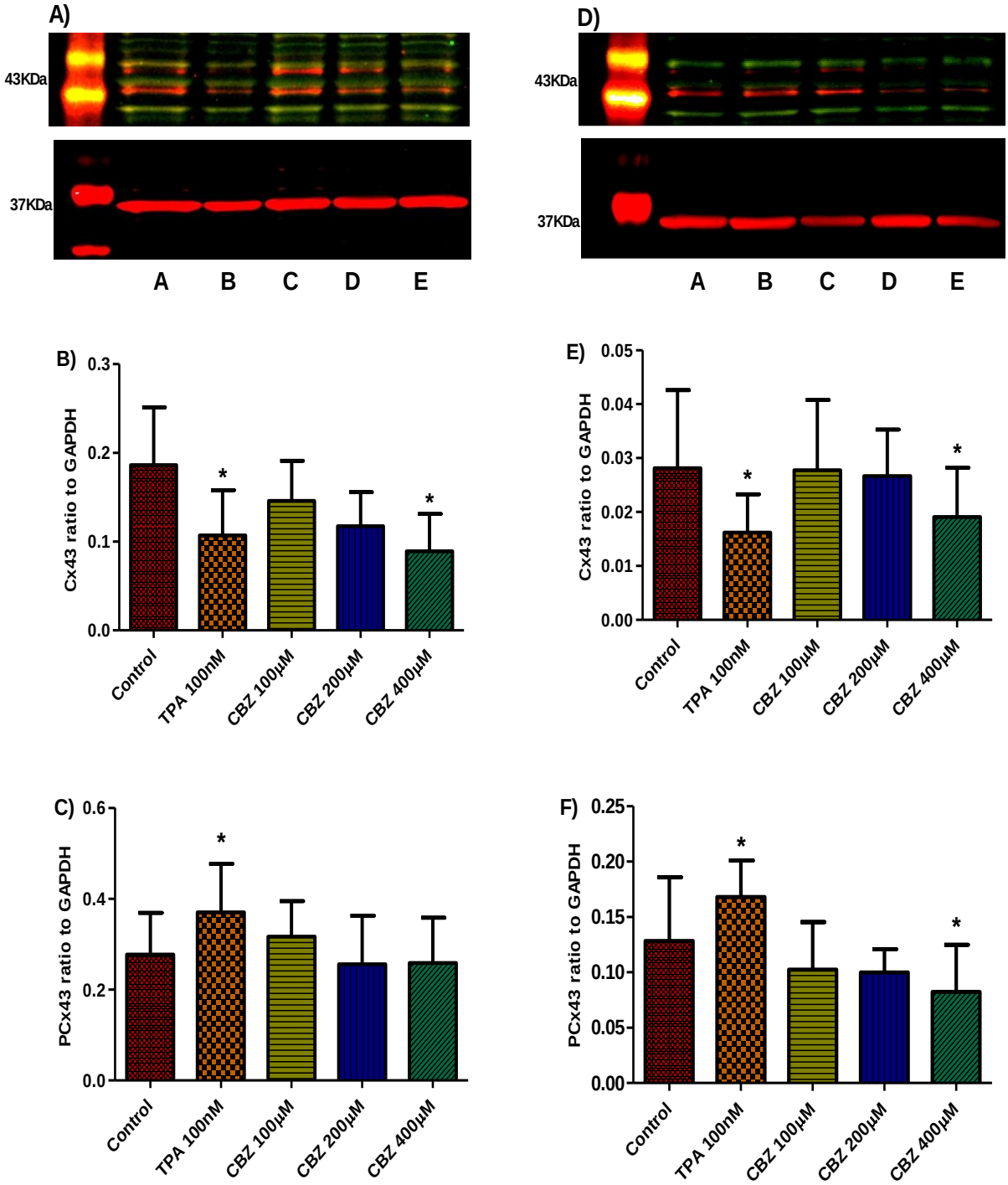


Fig 7.11 Western blot analysis of CBZ effects on Cx43 and PCx43 in the MM and ESDC systems. A) Cx43, PCx43 and GAPDH blot in the MM system (A=Control, B=TPA 100nM, C=CBZ100µM, D=CBZ200µM and E=CBZ 400µM), B) CBZ effects on Cx43 in the MM system, C) CBZ effects on PCx43 in the MM system, D) Cx43, PCx43 and GAPDH blot in the ESDC system (A=Control, B=TPA100nM, C=CBZ100µM, D=CBZ200µM and E=CBZ 400µM), E) CBZ effects on Cx43 in the ESDC system and F) CBZ effects on PCx43 in the ESDC system. * represents the significant difference from control.

7.9.6 Effects of BPN on Cx43 immunolocalization in the MM and ESDC systems

BPN doses in the MM system decrease and disturb Cx43 protein expression. With an increase in drug dose from 50 to 200μM a gradual decrease in protein level was observed (fig 7.12). In the BPN 50μM treated (fig 7.12C) group the cardiomyocytes exhibit disturb protein localization compared to the untreated group. At higher doses this continues with reduced expression. At 200μM BPN the Cx43 expression further declined and protein distribution and assembly as seen in the control was hardly observed (fig 7.12E).

When the BPN effects on ESDC were compared, the drug showed reduced expression but no significant disturbance in protein distribution (fig 7.13). As the dose of BPN increased from 50-200μM effects of reduced Cx43 expression were observed but they did not drastically change. The protein localization looked very much unaffected when compared to the results with TPA treated group.

7.9.7 Western blot analysis of BPN treated cardiomyocytes in ES and ESDC system

With increasing doses of BPN a gradual decrease in Cx43 protein levels in the micromass system was seen compared to the control. The low dose (BPN 50μM) analyzed with Western blotting showed a Cx43 protein level close to control, while at 100μM the Cx43 protein dropped down and continued further with 200 μM (fig 7.14C). The phosphorylated Cx43 isoform showed no variation after BPN treatment in the MM system (fig 7.14D), while TPA treatment exhibited high levels of PCx43. In ESDC the BPN showed no significant variation in Cx43 levels after treatment. The high doses of 100 and 200μM BPN after 12 days of treatment appeared to reduce the Cx43 levels to some extent but this failed to attain the statistical significance (fig 7.14E). The phosphorylated protein exhibited reduced expression with increase in dose but again the variation was not enough to induce a statistically significant reduction (fig 7.14F).

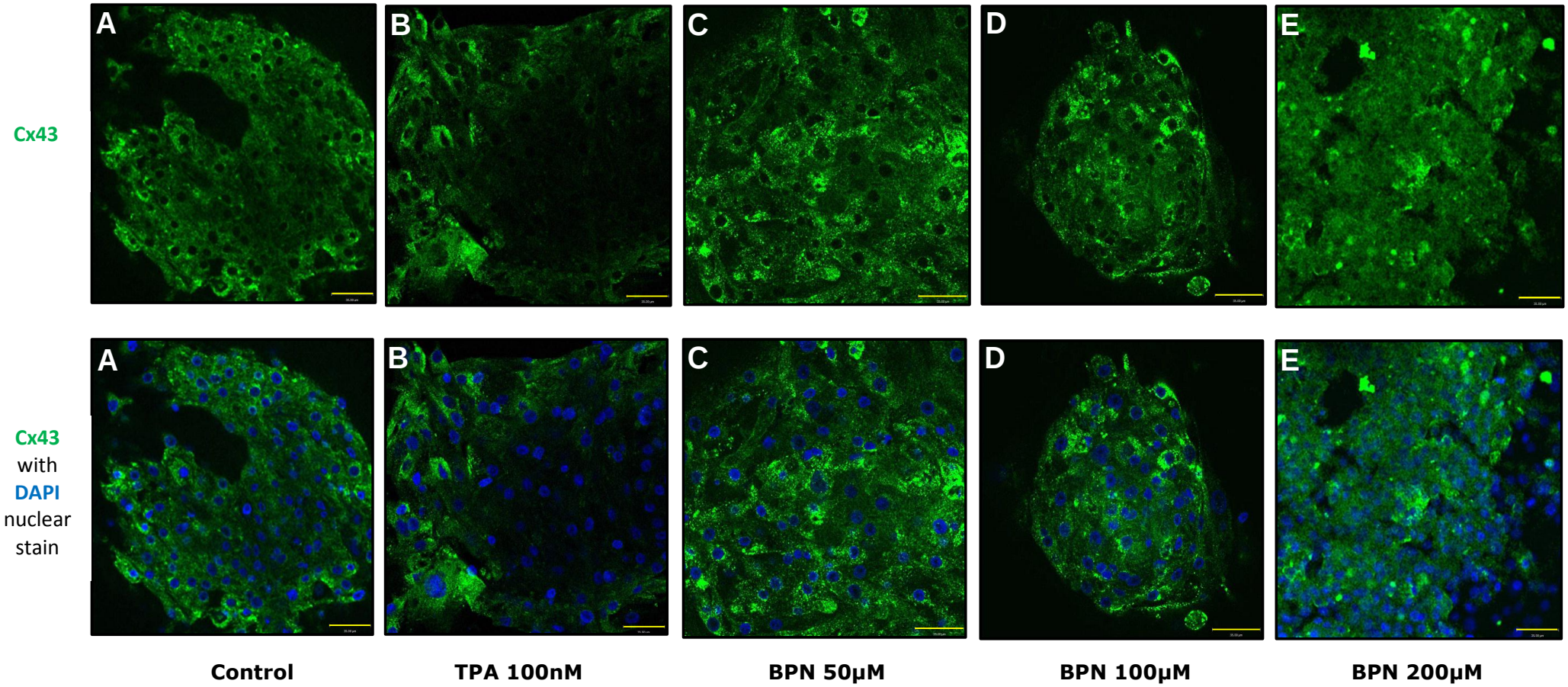


Fig 7.12 **Cx43** expression in BPN treated chick cardiomyocytes in the MM system. A) Control, B) TPA 100nM, C) BPN 50µM, D) BPN 100µM and E) BPN 200µM (Green color represents **Cx43** while Blue is **DAPI** nuclear staining). (Scale bar 33µm).

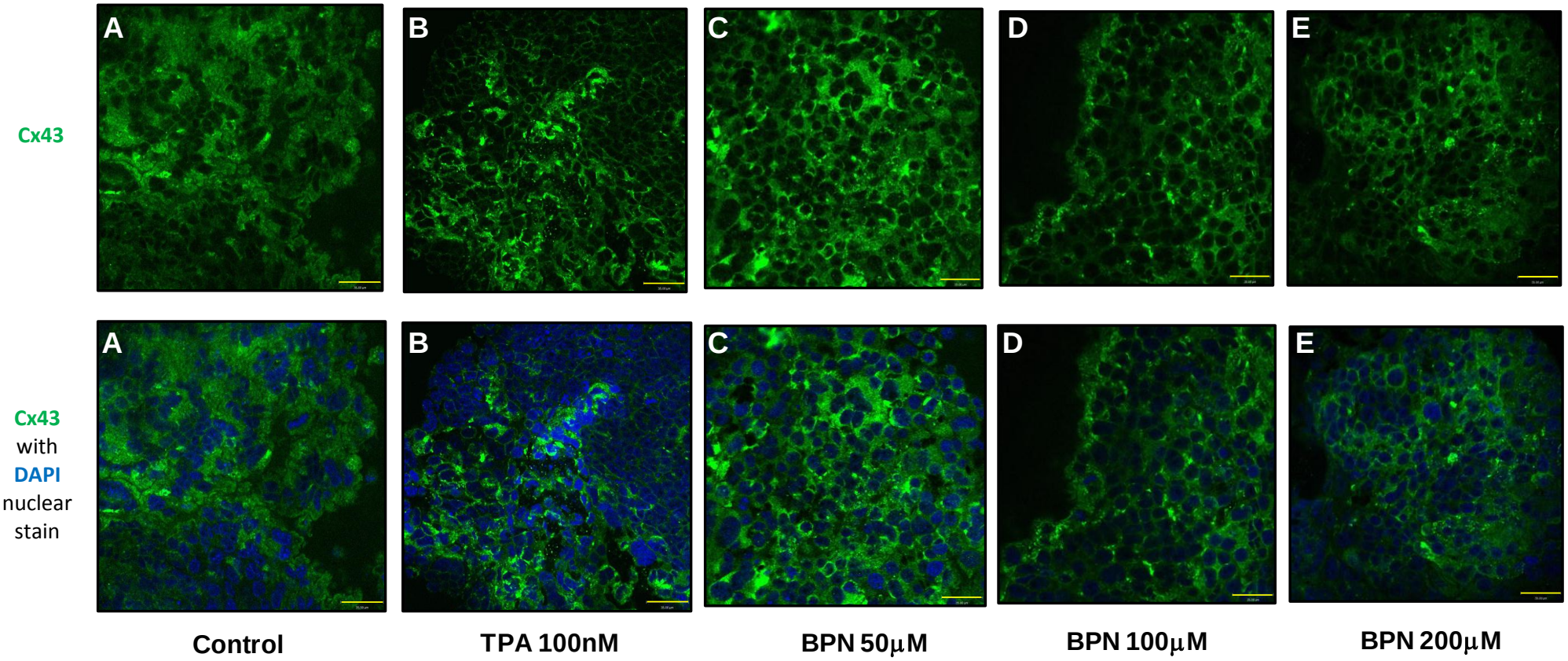


Fig 7.13 **Cx43** expression in BPN treated ES derived cardiomyocytes. A) Control, B) TPA 100nM, C) BPN 50µM, D) BPN 100µM and E) BPN 200µM (Green color represents **Cx43** while Blue is **DAPI** nuclear staining). (Scale bar 33µm).

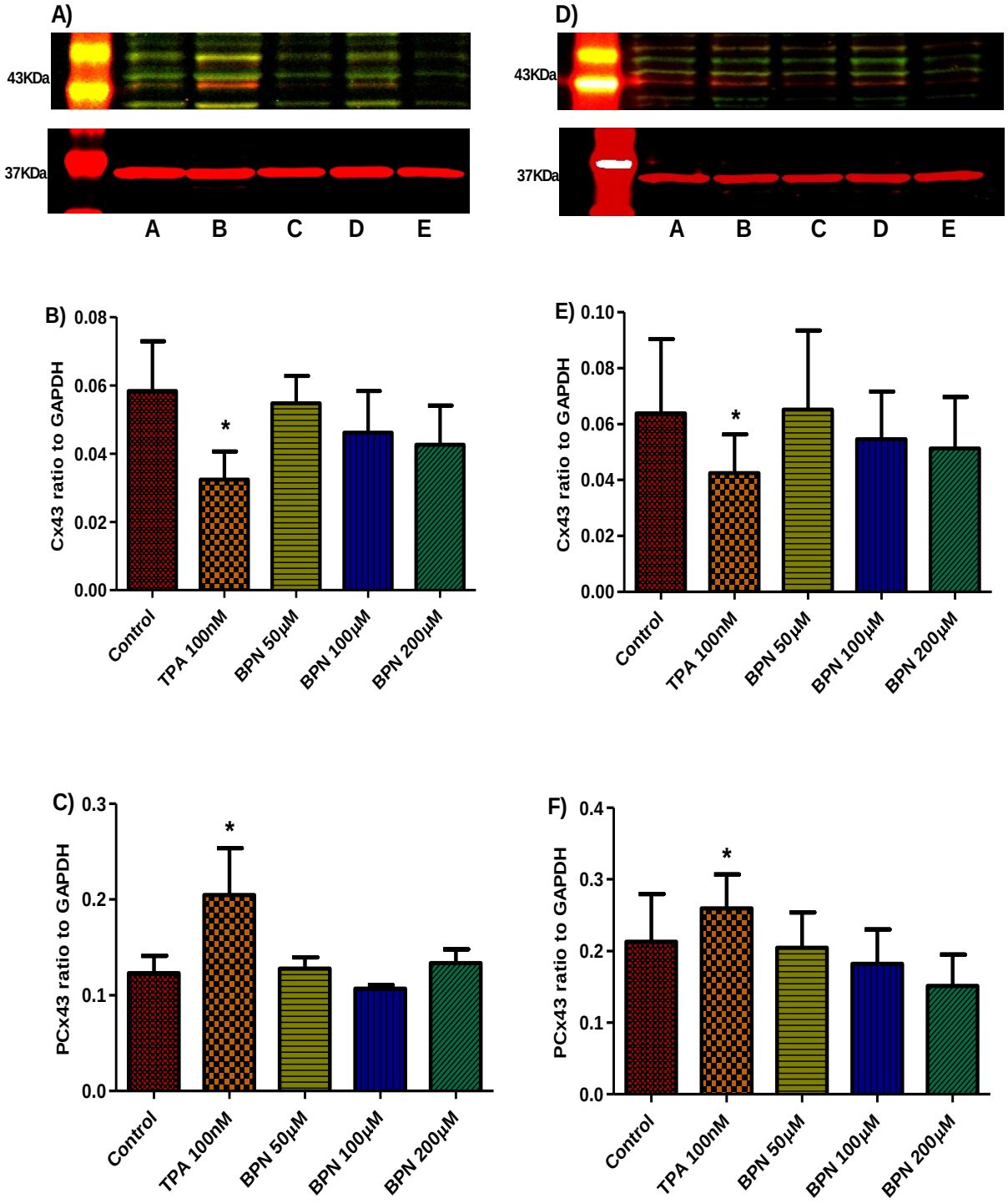


Fig 7.14 Western blot analysis of BPN effects on Cx43 and PCx43 in the MM and ESDC systems. A) Cx43, PCx43 and GAPDH blot in MM system (A=Control, B=TPA100nM, C=BPN50µM, D=BPN100µM and E=BPN200µM), B) BPN effects on Cx43 in MM system, C) BPN effects on PCx43 in MM system, D) Cx43, PCx43 and GAPDH blot in ESDC system (A=Control, B=TPA100nM, C=BPN50µM, D=BPN100µM and E=BPN200µM), E) BPN effects on Cx43 in ESDC system and F) BPN effects on PCx43 in ESDC system. * represents the significant difference from control.

7.9.8 Effects of Li_2CO_3 on Cx43 immunolocalization in the MM and ESDC systems

In the MM system Li_2CO_3 at therapeutic serum levels showed effects on cardiac Cx43 expression, which further became prominent with increase in drug dose (fig 7.15). Li_2CO_3 800μM effects on Cx43 protein localization and turn over were comparable to control (fig 7.15C). As the drug dose was increased to 1500μM, disturbed gap junction assembly was observed (fig 7.15D). The Li_2CO_3 2000uM dose further reduced the overall expression of Cx43 protein. (fig 7.15E).

In ESDC, the 800μM treated group exhibited no effect on Cx43 protein expression compared to higher doses (7.16C). At 1500μM (fig 7.16D) the drug slightly reduced the protein expression with low amount of fluorescent green color localization. With 2000μM the Cx43 expression was not much affected compared to TPA (fig 7.16E).

7.9.9 Western blot analysis of Li_2CO3 treated cardiomyocytes in ES and ESDC system

In Western blot analysis lithium carbonate treatment at 800-2000μM showed reduced level of Cx43 protein in the MM system (fig 7.17B). The dose of 800μM reduced the protein expression near to those observed with TPA. The significantly reduced protein levels continued with increase in drug dose. In ESDC treatment with lithium carbonate failed to produce any effects on cellular Cx43 levels (fig 7.17E). The drug effects on phosphorylated Cx43 in the MM system showed an apparent reduced cellular level at all doses analyzed, but only at 2000μM reached a statistically significant difference (fig 7.17C), while in ESDC no alteration or variation in PCx43 levels was observed compared to the control (fig 7.17F).

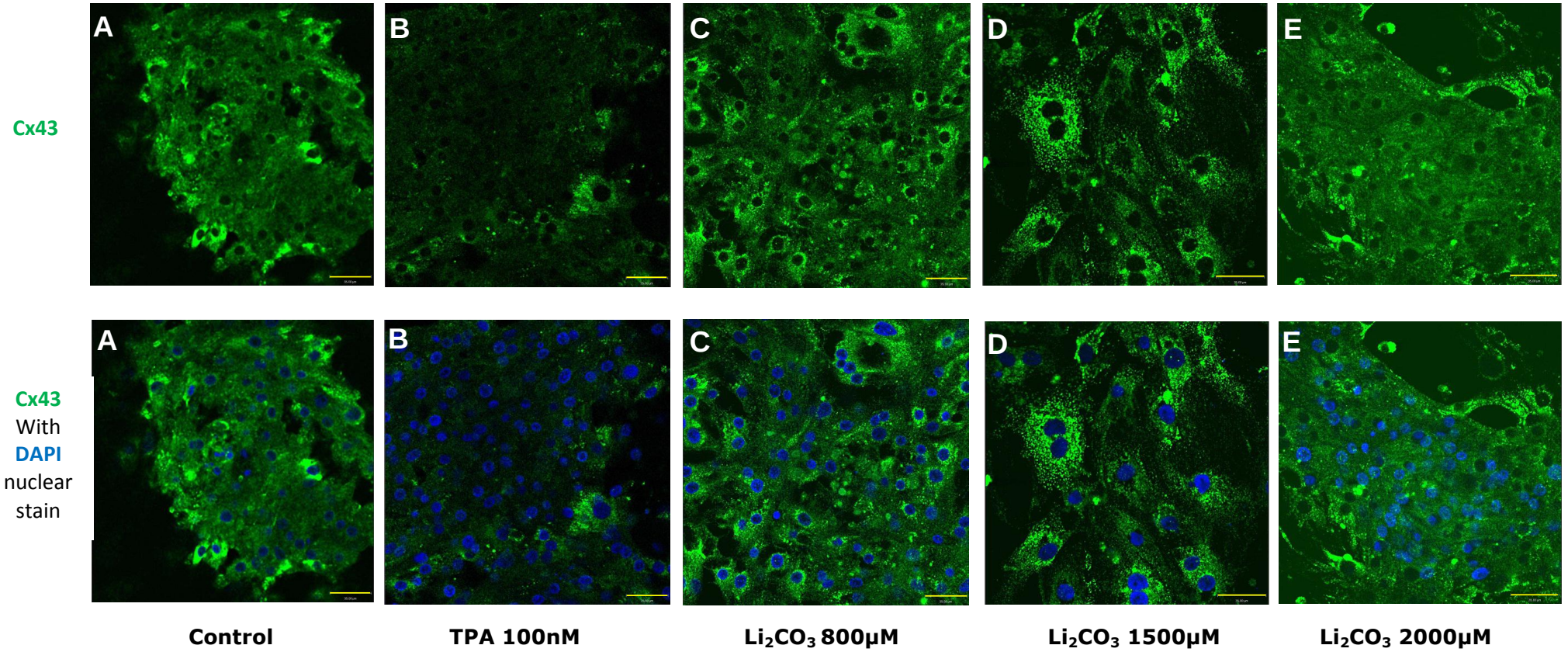


Fig 7.15 **Cx43** expression in Li_2CO_3 treated chick cardiomyocytes in the MM system. A) Control, B) TPA 100nM, C) Li_2CO_3 800µM, D) Li_2CO_3 1500µM and E) Li_2CO_3 2000µM (Green color represents **Cx43** while Blue is **DAPI** nuclear staining). (Scale bar 33µm).

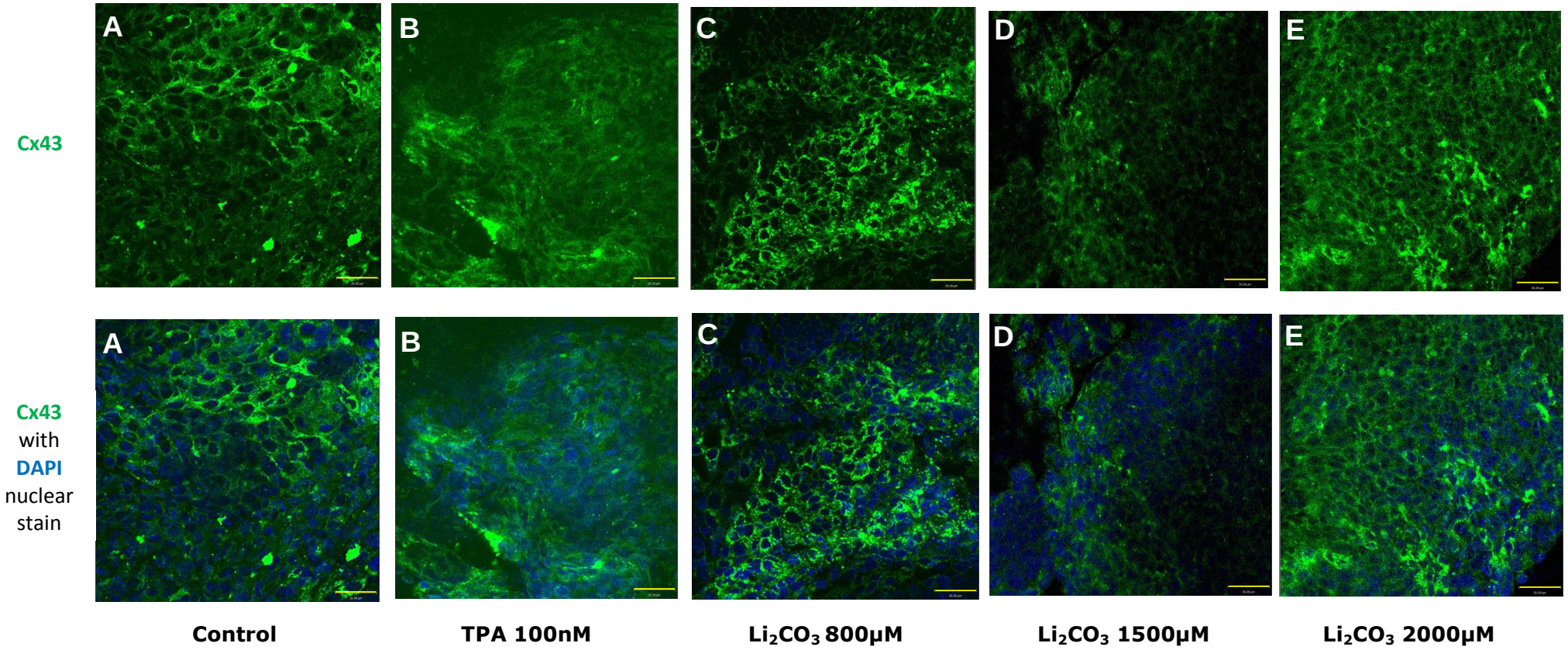


Fig 7.16 **Cx43** expression in Li_2CO_3 treated chick cardiomyocytes in the ES system. A) Control, B) TPA 100nM, C) Li_2CO_3 800µM, D) Li_2CO_3 1500µM and E) Li_2CO_3 2000µM (Green color represents **Cx43** while Blue is **DAPI** nuclear staining). (Scale bar 33µm).

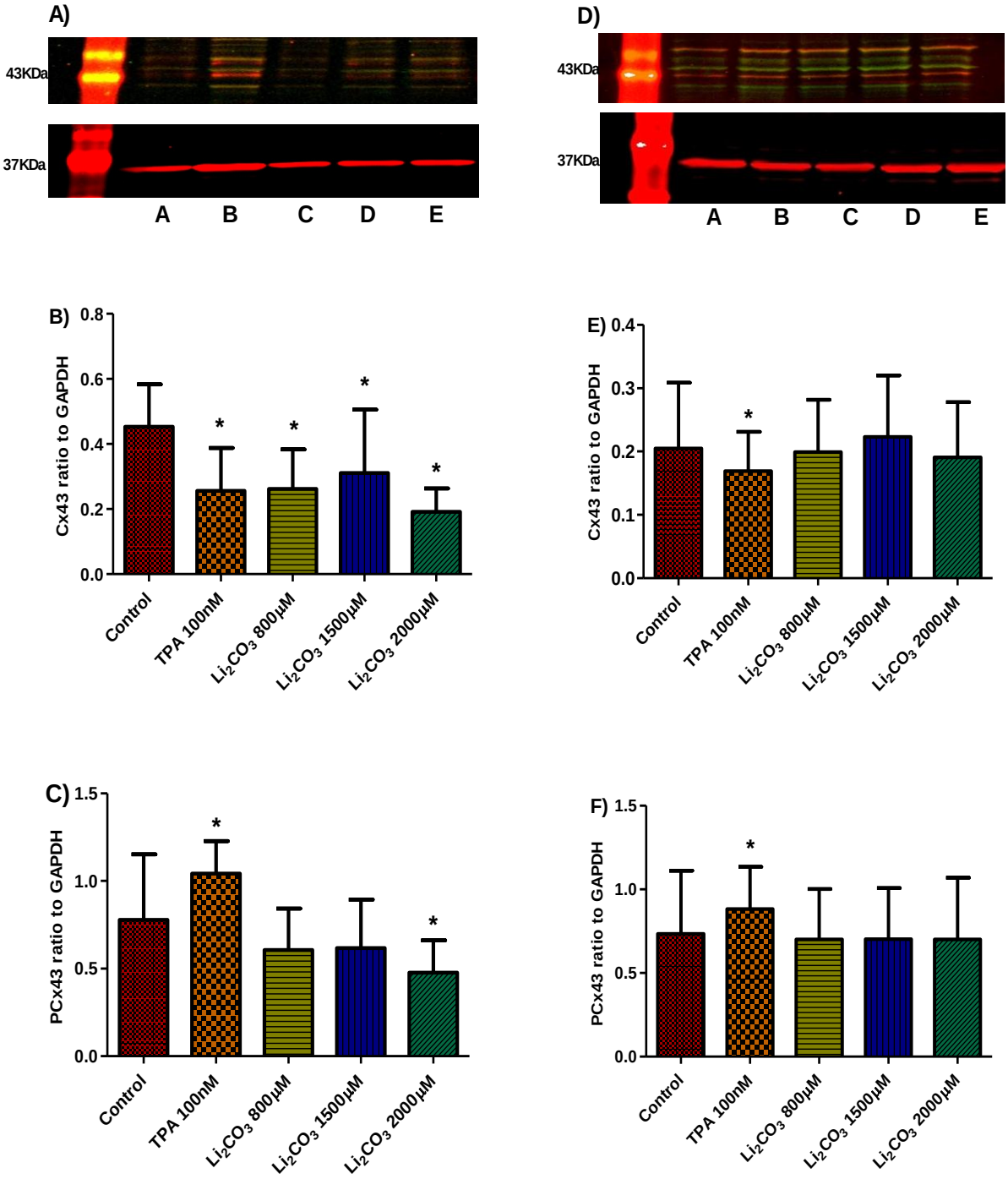


Fig 7.17 Western blot analysis of Li_2CO_3 effects on Cx43 and PCx43 in the MM and ESDC systems. A) Cx43, PCx43 and GAPDH blot in MM system (A=Control, B=TPA100nM, C=$Li_2CO_3$800µM, D= $Li_2CO_3$1500µM and E=Li_2CO_3 2000µM), B) Li_2CO_3 effects on Cx43 in MM system, C) Li_2CO_3 effects on PCx43 in MM system, D) Cx43, PCx43 and GAPDH blot in ESDC system (A=Control, B=TPA100nM, C=$Li_2CO_3$800µM, D= $Li_2CO_3$1500µM and E=Li_2CO_3 2000µM), E) Li_2CO_3 effects on Cx43 in ESDC system and F) Li_2CO_3 effects on PCx43 in ESDC system. * represents the significant difference from control.

7.10 Discussion

This chapter aims to evaluate the possible role of connexin 43 in the etiology of drug induced effects on differentiation and contractile activity of cardiomyocytes. The developing embryo exhibits highly orchestrated molecular and cellular events involving cell signaling, transcription and the flow of information between cells (Lanza, 2006; Lanza, 2004) that first differentiates the embryonic stem cells into germ layers (Lanza, 2006; Lanza, 2004). With further development the mesodermal germ layer predominates in the EBs and differentiates it towards a cardiac lineage with synchronized rhythmical phases of relaxation and contraction. The continuous flow of information between differentiating cells is important for development, growth and regulation (Levin, 2002; Vinken et al., 2006). Small molecules can flow directly through gap junction channels between adjacent cells (Leithe & Rivedal, 2004a). This phenomenon is important, especially in tissue compartmentalization and electrical coupling of individual myocytes which mediate rapid synchronized contraction (Coppen *et al.*, 2003; Levin, 2002; Vink *et al.*, 2004; Wei *et al.*, 2004). In vertebrates, gap junctions are encoded by the connexin gene family (Lo, 2000; Michael, 2007; Wei et al., 2004), with Cx43 ubiquitously expressed in almost all cells (Laird, 2005) and essential for the normal development and function of the heart (Zhaowei *et al.*, 2000). The connexin protein, from gene transcription to degradation, contributes substantially in a number of physiological and pathological processes (Laing et al., 1997; Vinken et al., 2006). Connexin turnover and regulation is very important for the formation of functional channels and due to its short half-life a continuous regulation of protein synthesis, assembly, trafficking, formation of functional channels and degradation is required (Solan & Lampe, 2005). Any disturbance in connexin regulation or mutation results in a loss of communication between cells (Ruch *et al.*, 2001), but there are reports that different connexins in the heart may compensate for loss of function of other connexins (Vink *et al.*, 2004). In the heart, mutation or disturbance in Cx43 exhibits various cardiac developmental defects with fetal deaths in some cases (Vinken *et al.*, 2006). The connexins undergo various biochemical and post translational modifications, with

phosphorylation regulating various gap junction functions. (Martin & Evans, 2004; Solan *et al.*, 2003). Phosphorylation at the Cx43 Ser368 cytoplasmic tail has been shown to be involved in trafficking and channel gating (Lampe *et al.*, 2000). Any disturbances in phosphorylation affects connexin regulation (Solan *et al.*, 2003) as altered phosphorylation induces changes in connexin structure, which translocate it to the cytoplasm instead of forming GJ within the cell membrane (Musil & Goodenough, 1990).

The control group cardiomyocytes in both the MM and ESDC systems tagged with anti-connexin43 exhibit intracellular and appositional localization of protein. The positive control, the growing chick heart on day 9 of development, as expected exhibits high levels of protein, which was further confirmed by imunoblot analysis of the protein. No information regarding the use of phospho-Cx43 antibody for immunohistochemical analysis was provided by the supplier and when tested the antibody produced only non-specific binding (data not shown). Cx43 migration on gels exhibits different bands as observed in earlier studies. The unphosphorylated band due to its low molecular weight compared to its phosphorylated isoform showed increased migration on the gel (Loch-Caruso *et al.*, 2004; Ruch *et al.*, 2001), while the red PCx43 band was found in between the green Cx43 band. TPA showed reduced Cx43 expression after 100nM treatment for 2hours, at the lower dose of 50nM the TPA effects were not prominent in immunolocalization and immunoblot (data not shown here). TPA acts by hyper phosphorylating the Cx43 at Ser368 through PKC stimulation, which results in gap junction closure and internalization (Loch-Caruso *et al.*, 2004; Ruch *et al.*, 2001), which was evident in immunoblot analysis, when low levels of Cx43 were detected with increased phosphorylation of the total connexin present at Ser368.

The sodium valproate treatment of cardiomyocytes reduced the Cx43 level in both the systems, which was further confirmed by immunohistochemical analysis of VPA treated cardiomyocytes. From the results of Western blotting it can be concluded that VPA increases the phosphorylation of Cx43 at Ser368 and affects protein regulation. As stated earlier Ser368 phosphorylation regulates some important

events in connexin turnover (Lampe & Lau, 2000) and translocates the protein into the cytoplasm with increased internalization, which was evident from the results of Cx43 immuno analysis (Loch-Caruso *et al.*, 2004; Musil & Goodenough, 1990; Ruch *et al.*, 2001). When the results of Cx43 staining were compared for the VPA treated cardiomyocytes they clearly show that increased drug dose results in Cx43 hyperphosphorylation, which affects Cx43 regulation. In turn this interferes with the passage of ions, which is necessary to establish contractile activity. The potential mechanism for the altered effects could be HDAC inhibition (Phiel *et al.*, 2001) or increased ROS production (Na *et al.*, 2003) as observed earlier with VPA treatment which may affect the protein. The VPA effects on PKC and Erk1/2 (Gotfryd *et al.*, 2011; Gurvich *et al.*, 2004) could be another source of inducing disturbance in Cx43 protein regulation.

The FDA category C teratogenic drug carbamazepine (Ornoy, 2006), induces anomalies in the developing heart (Murabe *et al.*, 2007b). Immunohistochemistry of the CBZ treated group in the MM system showed effects on the Cx43 cellular distribution. The results of Western blot analysis confirm the reduced turnover of Cx43 protein with increase in CBZ dose. These CBZ effects completely support the idea that reduced cell contractions observed in the MM system are the result of drug effects on Cx43. The decreased Cx43 expression observed with TPA treatment due to hyperphosphorylation at Ser368 affects the protein turnover (Cruciani et al., 2002; Solan et al., 2003; van Veen et al., 2001). But Western blot analysis of CBZ treated chick cardiomyocytes do not support this idea in the MM system, and the PCx43 levels after CBZ treatment were comparable to the control. It may involve some mechanism other than phosphorylation at Ser368 (TenBroek *et al.*, 2001; van Veen *et al.*, 2001). In ESDC the cellular expression of Cx43 protein at 100 and 200μM CBZ was comparable to control. The cellular Cx43 distribution was unaffected but in ESDC inhibited contraction was observed with 100μM treatment and no contraction at the 200μM dose. Western blot analysis supports the immunohistochemistry result, with unaffected Cx43 levels with 100 and 200μM CBZ treatment, although some apparent low levels of PCx43 were observed after these dose treatments. The

decreased contraction might have been attributed to altered phosphorylation at Ser368, which is involved in many connexin regulation processes, especially in channel gating (Lampe & Lau, 2000). It is possible that carbamazepine affects channel gating by decreasing phosphorylation, which results in the formation of non-functional channels. With 400μM CBZ, Cx43 expression as shown by immunohistochemistry exhibited reduced levels and GJs ability to form connections between cells. That might be the result of reduced cell proliferation due to formation of monopolar spindles with 400μM CBZ treatment (Perez Martin *et al.*, 2008). The immunoblot analysis showed reduced Cx43 with CBZ 400μM treatment which may affect cell proliferation and differentiation because connexins plays an important role during these processes (Dhein, 1998; Leithe & Rivedal, 2004a; Solan & Lampe, 2005). The other mechanism could be HDAC inhibition by CBZ which may alter the protein expression at the gene level (Beutler *et al.*, 2005; Ornoy, 2009). However, Khan found HADC inhibition increased Cx43 levels in neural progenitors with decreased phosphorylation of Cx43 (Khan *et al.*, 2007), while in hepatocytes HDAC inhibitors negatively affect the Cx43 protein levels (Vinken *et al.*, 2007). These effects in part agree with the result observed in our lab. Increased ROS production with CBZ treatment may affect protein regulation, especially in case of the MM system where disturbed regulation was observed with increased dose compared to the control. Treatment with antioxidants may protect the Cx43 protein from ROS induced damage.

The antidepressant molecule Bupropion in the MM system confirms the drug's ability to affect the Cx43 protein levels in cultured chick cardiomyocytes. The Western blot and immunohistochemistry results with anti-Cx43 indicate reduced protein expression. Increase in drug dose seems to affect both the protein levels and cellular distribution. The 200μM BPN treated cardiomyocytes showed disturbed Cx43 cellular organization and assembly at the cell membrane compared to the control. In the MM system the BPN affects the cardiomyocyte contractile activity but the effects are only observed at 200μM after 5 days of exposure. This may mean that BPN affects Cx43 levels after chronic exposure at high doses but at low doses although some

disturbance in protein localization is recorded, other connexins may compensate for the effects on Cx43 and so the cells continue to contract (Vink *et al.*, 2004). At high doses the significant decrease in Cx43 may cause effects on contractile activity and cell proliferation, which involves some mechanism other than Ser368 hyperphosphorylation in the MM system. In ES cells the BPN does not affect the cellular Cx43 protein levels after 12 days of exposure, but the cardiomyocytes contraction was affected after BPN treatment. The other parameter of connexin43 phosphorylation showed reduced levels compared to the control which may affect different processes regulated by connexin phosphorylation and in turn may alter channel properties and affect contraction. The biphasic property of BPN produces inhibition of calcium release which may have the possibility of reducing contractile activity (Cremers *et al.*, 2003).

With the antidepressant drug lithium carbonate, the chick cardiomyocytes exhibit low levels of Cx43 which was observed in immunolocalization and Western blot analysis of the protein. Increased drug doses showed an effect on protein turnover but in the MM system the cardiomyocytes contractile activity was not significantly reduced at 2000μM. When the drug effects on phosphorylation status was analyzed through immunoblots, a reduced Cx43 phosphorylation at Ser368 residue was observed. The disturbed PCx43 may have affected multiple processes in Cx43 regulation which might have affected the cellular turnover of Cx43. Blood serum concentrations of around 2000μM Li_2CO_3 are considered toxic. In the MM system Cx43 levels and distribution are altered at this dose, but the possibility of compensation by other connexins may mean no effect on the contractile activity. In the ES cells the effects on Cx43 were less prominent in immunostaining at the same dose. The drug showed no effect on protein level after 12 days of treatment. Cx43 was not affected by the toxic dose of 2000μM. The protein phosphorylation status also showed no variation compared to control. When the result of immuno analysis was compared with contractile activity at 1500 and 2000μM lithium, treated ES cells do not exhibit contractile activity. The decreased contractile activity might be the result of drug effects on inositol recycling in the cells (Giles & Bannigan, 1999).

Earlier studies confirm that Li^+ by accumulating β-catenin induces Cx43 mRNA and protein expression. Also Wnt signaling is an important modulator of Cx43 dependent intracellular coupling in the heart and lithium mimics Wnt signaling (Zhaowei *et al.*, 2000).

The effects of various drugs on Cx43 levels and distribution confirm the role of Cx43 in contractile activity and cell differentiation. The regulation of Ser368 phosphorylation in the formation of GJ is also likely. Beside this, the drugs do not rely solely on hyperphosphorylation as a potential mechanism to interfere with GJ regulation.

Chapter 8

Chick Neural Stem Cell isolation, characterization, differentiation and effects of teratogens on neuronal differentiation

8.1 Introduction

The central nervous system is a complex organ of the body comprising of the brain and spinal cord. CNS cells - neurons, astrocytes and oligodendrocytes - form intricate connections to facilitate electro-chemical signalling. These cells form trillions of connections, which are made more complex by neuronal types with distinct morphology, connectivity and neurotransmission (Ahmed *et al.*, 2009). During neurogenesis, neurotoxin exposure may result in neural dysfunction and abnormal neural development. In humans the brain continues to develop beyond the second trimester of pregnancy, with postnatal maturation continuing through adolescence. Neurotoxins have many opportunities to interfere with brain development and function during pregnancy and postnatally. The developing brain is much more susceptible to toxic insult compared to the adult brain and therefore neurotoxin exposure during pregnancy provides a potential threat to induce malformation. Neurodevelopmental defects in humans are usually recognised after large scale prospective epidemiological studies (Suñol, 2010). With the advent of *in vitro* teratogenicity testing, neurotoxins can be identified at an early stage of screening and the related risk may be avoided or rectified.

8.1.2 Neural Stem cells

Stem cells are primitive cells that have the ability to proliferate and self-renew, with differentiation into major cell lineages. The stem cell property of self-renewal with regeneration is an important phenomenon in development and tissue homeostasis (Ahmed *et al.*, 2009; Taupin *et al.*, 2000). During neurogenesis CNS cells are derived from neural tube cells. These mitotically active cells are neural progenitors, precursors or stem cells (Reh, 2002). Neural stem cells (NSC) are relatively quiescent and are present in specific regions in the developing and adult nervous system. Like all stem cells NSCs have the ability to proliferate, self-renew and generate the primary phenotype of the nervous system (Kashem *et al.*, 2009; Ma *et al.*, 2006; Wang *et al.*, 2006). The NSC culture system for the isolation, expansion and differentiation into CNS progeny provides a unique and powerful *in vitro* model to study and elucidate the molecular mechanisms involved in nervous system

development, plasticity and regeneration (Louis & Reynolds, 2005) and may help to ameliorate neurodegenerative diseases (Bez *et al.*, 2003; Price & Williams, 2001).

8.1.3 Neurogenesis

The CNS is composed of four major types of cell: neurons, oligodendrocytes, astrocytes and the ependymal lining of the central lumen. These cells arise from common neuroepithelial cells in the neural tube that produce neurons first then glial cells at later stages (Kintner, 2002). Neurogenesis was first thought to be limited to during prenatal development, however it occurs throughout adulthood in various region of brain such as the subventricular zone (SVZ) and subgranular zone (SGZ) of the dentate gyrus (Taupin *et al.*, 2000). After fertilization, the epiblast cells in the blastocyst differentiate into three germ layers. The primitive streak elongates with the formation of the anterior and posterior axis and embryo regionalization and differentiation starts. These highly orchestrated processes are controlled by cyclic expression of various signalling molecules, growth factors and an extracellular matrix gradient (Bottenstein, 2003). The early neural tube formed at the anterior end of primitive streak is composed of homogenous proliferating cells known as neuroepithelial cells (Sommer & Rao, 2002). These cells proliferate and elongate in response to the mitogen Fibroblast growth factor-2 (FGF2) to form the neural plate and undergo a series of morphological changes with the formation of a closed neural tube. Posterior mesenchymal cells canalize and extend the spinal cord into the lumbosacral region (secondary neurulation). The number of neuroepithelial cells reduces during development as they transform into neural and glial precursors. These precursors start to express appropriate receptors for additional growth and differentiation, notably EGF receptors, which increase the NSC population in the neuroepithelial cells (Bottenstein, 2003). These neural stem cells differentiate in response to specific signals and migrate to target regions to contribute to brain development (Sommer & Rao, 2002). Mitogens like FGF and EGF promote NSC and progenitor cell proliferation *in vivo* and *in vitro* (Taupin *et al.*, 2000).

8.1.4 Control of Stem cell behaviour and differentiation: Growth factors and signalling molecules

Neurogenesis involves first the generation of lineage committed progenitors followed by their terminal differentiation into brain cell types (Ahmed et al., 2009). The stem cell microenvironment regulates progenitor self-renewal and differentiation, via several intrinsic and extrinsic determinants *in vitro* and *in vivo* including Wnt, Notch and, Sonic hedgehog (SHH) (Scott *et al.*, 2010; Shi *et al.*, 2008). These trigger pathways are mediated through various growth factors and molecules which act at different stages of stem cell development and differentiation and sculpt the whole brain. The early NSC respond to FGF which acts as a mitogen and increases the pool of NSC - decrease in FGF receptors results in decreased NSC proliferation (Sommer & Rao, 2002). Later-stage NSC respond to EGF along with FGF (Bottenstein, 2003; Sommer & Rao, 2002). Therefore the mitogens FGF and EGF play an important role in neural stem cells proliferation and self-renewal. These growth factors modulate extracellular matrix component secretion and composition such as laminin B1 and B2 chains, which up-regulate the NSC proliferation. Along with these mitogens and adhesion molecules the NSC express various integrins, extracellular components and neurotransmitters which may regulate NSC numbers (Sommer & Rao, 2002). Various signalling pathways like cAMP, PKA and calcium may also regulate NSC proliferation. Despite continued exposure to proliferative signals the cells respond to signalling molecules for a specific period of time only and undergo differentiation, apoptosis or senescence (Sommer & Rao, 2002). During neurogenesis the Wnt/β-catenin pathway plays an important role in NSC proliferation and self-renewal by acting on cyclin D1 (Ahmed *et al.*, 2009; Shi *et al.*, 2008). It controls progenitor expansion and differentiation balance, and depending upon the stage of development it may promote NSC proliferation or neuronal differentiation. β-catenin has been shown to promote neural differentiation, but in the presence of FGF-2 it inhibits neuronal differentaition. Mutation in β-catenin results in midbrain boundary cell elimination while overexpression results in hyper-cellularity of the brain (Ahmed *et al.*, 2009). Besides this, notch and sonic hedgehog signalling have been implicated

in regulating key pathways in the balance between neural progenitor expansion and differentiation balance. It can be concluded that Wnt, Notch and Shh signalling appears to be essential for NSC maintenance and differentiation (Ahmed *et al.*, 2009). FGF-2 has both mitogenic and neurotrophic action (Vescovi *et al.*, 1993). Low levels of FGF promote neuronal differentiation, while higher levels promotes glial differentiation (Bottenstein, 2003). *In vitro* FGF-2 is used in the presence of heparin, which is shown to enhance FGF-2 induced NSC proliferation, while also reducing the number of astrocytes differentiating. Insulin has also been found to enhance the proliferative effects of FGF-2 (Kelly *et al.*, 2003). Neurotrophins like NGF, BDNF and NT3 receptors are expressed early in neural progenitor cell formation. Addition of BDNF to the culture of neurospheres obtained from E-15 mouse brain promotes differentiation of multipolar neurons. *In vitro* BDNF increased the hippocampal stem cells survival and differentiation. While NT3 promotes NSCs differentiation by increasing the length of G1 phase of cell division and decreasing cyclin D2. Intraventricular infusion of NT3, like BDNF, increased the number of neurons in the cortical plate, and both NT3 and NT4 increased neuronal survival rather than neuronal commitment in human neurospheres (Bottenstein, 2003). Addition of B27 and N2 supplements to the medium promotes NSC survival and differentiation (Svendsen *et al.*, 1995). Recently it has been demonstrated that BDNF promotes the differentiation of neural crest cells along a sensory neuron lineage, and that BDNF and NT-3 promote the maturation of embryonic chick sensory neurons *in vitro* (Ghosh & Greenberg, 1995).

8.1.5 Neural stem cells *in vitro* isolation and differentiation

The earlier evidence of adult neurogenesis was presented by Joseph Altaman and Gopal Das in the 1960's (Altman, 1962; Altman & Das, 1965), which totally contradicts the long held dogma of no new neurons after birth proposed originally by Cajal (Ming & Song, 2005). In a landmark paper Reylond and Weiss in 1992 further disapproved the earlier belief that neurogenesis in adults CNS does not occur and first isolated the adult mouse NSC and established the *in vitro* method of neurosphere formation (Reynolds & Weiss, 1992). The embryonic brain contains a

large population of NSC which are restricted to certain regions in the adult brain (Taupin *et al.*, 2000). The neurosphere formation assay has been applied to both embryonic and adult tissues (Ahmed, 2009). The isolated NSCs are grown *in vitro* in a serum free medium, where these cells form aggregates or neurospheres. These neurospheres are mostly round shaped consisting of 3D aggregates of cells, containing a heterogeneous population of progenitor cells along with neuronal and glial lineage cells at different stages of differentiation (Ahmed, 2009; Wang *et al.*, 2006). The cells in the neurosphere are multipotent and differentiate into major CNS lineages (Lim *et al.*, 2007). The idea of the neurosphere-formation assay was based on the fact that the cells which form neurospheres are NSC, and these cells proliferate, self-renew and differentiate *in vitro* upon provision of appropriate microenvironmental cues (Ahmed, 2009; Gobbel *et al.*, 2003). *In vitro* these NSCs are maintained in the presence of mitogens FGF and EGF with serum replacement supplements like N2 and B27 which expands their number without the loss of differentiation potential (Gobbel *et al.*, 2003). These neurospheres enlarge and become darker at the centre representing increased apoptotic and necrotic events due to an insufficiently nourished inner core and hypoxic conditions (Bez *et al.*, 2003). These neurospheres are enriched in integrin, cadherin and EGF receptors. NSC survival in neurospheres critically depends on the growth conditions and supplements in the *in vitro* system. High density cultures of NSC exhibit neurosphere aggregation. To date there are no definite markers for NSC; the formation of neurospheres *in vitro* is still considered the indicator of NSC presence (Ahmed, 2009). However, the intermediate filament protein nestin (Storch *et al.*, 2003), transcription factor SOX2 and transmembrane glycoprotein CD133 are used to identify NSC presence (Ahmed *et al.*, 2009). When the mitogens are removed the NSC differentiate *in vitro* on a PDL coated substrate (which promotes neurite growth and cell viability) (Ahmed, 2009; Gobbel *et al.*, 2003). In the presence of serum/laminin these cells differentiate into predominantly astrocytes followed by neurons and then oligodendrocytes. The addition of growth and neurotrophic factors such as BDNF, NT3, NT4, bFGF, IGF-1 and retinoic acid significantly increases

neuronal differentiation, while PDGF promotes oligodendrocytes and NGF peripheral neurons and astrocytes. Thus from the earlier studies it is observed that neurosphere derived cells are multipotent and the media composition and growth factors regulates their differentiation into specific lineage cells (Ahmed *et al.*, 2009; Gobbel *et al.*, 2003; Ren *et al.*, 2009).

8.1.6 Neural stem cells characterization

The neural stem cell has the ability to proliferate, self-renew and differentiate into nervous system progeny. Various markers have been used *in vivo* and *in vitro* to characterize the stem cells including transcription factors that maintain neural progenitor cells (Graham *et al.*, 2003). The NSC ability to form neurospheres *in vitro* is frequently used to identify stem cells (Ahmed, 2009). The intermediate filament nestin is used as a marker of NSC but it is also expressed in immature neurons (Brazel *et al.*, 2005; Miyagi *et al.*, 2006). Beside this Notch1, Sox2 and c-Myc are also used to characterize NSC presence (Kerosuo *et al.*, 2008; Miyagi *et al.*, 2006; Wang *et al.*, 2004). The transcription factor SOX2 is expressed in epiblast cells as well as embryonic, neural and trophoblast stem cells but not in the differentiated cells (Miyagi *et al.*, 2006; Scott *et al.*, 2010). In NSC SOX2 is expressed both in adult and embryonic progenitor cells (Ahmed *et al.*, 2009). SOX2 appears to be a common marker for all cells within these heterogeneous population in neurospheres (Miyagi *et al.*, 2006). SOX2 along with Nanong and Oct4 through co-operative interaction maintain the stem cell phenotype (Ahmed *et al.*, 2009; Rodda *et al.*, 2005). Its down regulation results in neural progenitor pool depletion with induction of neuronal differentiation (Ahmed *et al.*, 2009; Graham *et al.*, 2003). In mature neurons SOX2 expression is absent (Komitova & Eriksson, 2004). SOX2 knockout mouse die at around the implantation stage (Miyagi *et al.*, 2006; Uchikawa *et al.*, 2011).

8.1.7 Neuronal marker-Neurofilament

Neurofilaments (NF), along with various other cytoskeleton proteins, form the basic neuronal structure. NFs are most abundant, ubiquitous neuron specific filaments and

are used as marker of neurons (Grant & Pant, 2000; Schwartz *et al.*, 1992). NF are composed of three major subunits NF-L (68KDa), NF-M (120KDa) and NF-H (200KDa) (Bottenstein, 2003). They are distributed in neuronal axons, dendrites and perikaryons (Hashimoto *et al.*, 2000). NF mediates radial growth and determines axonal diameter, which directly regulates the rate of nerve conduction velocity. NF-L loss results in axonal diameter reduction, While its accumulation results in disturbed transport and neurodegenerative diseases (Bottenstein, 2003; Dale & Garcia, 2012). Neurofilament-L was chosen in this work because it is abundant in neurons compared to its high molecular weight subunits (Bottenstein, 2003; Jung, 2007).

8.2 Methodology

8.2.1 Chick NSC Isolation

For the isolation of chick neural stem cells, eggs were incubated at 37C, 100% humidity for the required days. On the day of explantation, embryos were dissected in 2% glucose solution in cold PBS (Ca^{2+} and Mg^{2+} free) under the dissecting microscope (Nikon, UK). The embryos were decapitated first then the brain was removed using fine sterilized forceps and micro scissors. The meninges were removed carefully and the brain tissues were transferred into a 15ml universal tube containing cold PBS with 2% glucose on ice. The brain was allowed to settle down and the glucose solution was removed after centrifuging at 400rpm for 5 minutes. Tissues were suspended in 1ml (for 12 brains) complete NSC culture medium and dissociated manually by pipetting up and down, first using a 1ml pipette tip then 200µl tip until a single cell suspension was obtained. The tissues were allowed to settle down and the supernatant was removed. The undissociated tissues were suspended again in 1ml medium and gently triturated to obtain a single cell suspension. The cell density and cell viability was determined using a haemocytometer with a 0.4% Trypan blue solution (see appendix 5). Viable cells at a $0.3\text{-}0.5\times10^{6}$ $cells ml^{-1}$ density were suspended in 10ml NSC culture medium in a low attachment T-25 flask. The cells start to form floating neurospheres within 2-3 days, frequent shaking helps the neurospheres to detach from the surface. On day 4

the medium was changed by centrifuging the culture flask at low speed 400rpm for 5 min and removing the supernatant containing dead floating cells. The neurospheres were re-suspended in 10ml NSC culture medium.

8.2.2 Sub culturing the Neurospheres

When the neurospheres were grown to considerable size (more than 100µm) they were passaged to avoid cell necrosis. The culture medium containing the neurospheres was collected in a universal flask. The T-25 flasks were washed with warm PBS (Ca^{2+} and Mg^{2+} free) to detach the attached neurospheres. The neurospheres were centrifuged at 900-1000rpm for 5 min and the supernatant was removed. The neurospheres were washed in PBS followed by centrifugation at the same speed. The collected neurospheres were dissociated using 1ml 0.1% trypsin solution in Ca^{2+} and Mg^{2+} free PBS and incubated for 5 min at 37°C. After 5 min the neurospheres were dissociated again by gentle trituration (50 times) using a 200µl pipette tip and incubated again for 3 min. 5ml culture medium was added to the trypsinized cells and centrifuged at 900-1000rpm for 5 min. The pellet was suspended in 2ml medium, and cell density and cell viability was determined using a haemocytometer (see appendix 5). $0.3\text{-}0.5\times10^{6}$ viable cellsml^{-1} were suspended again in culture medium in a T-25 flask and labelled with passage number and date.

8.2.3 NSC differentiation

8.2.3.1 PDL coating

100µgml^{-1} poly-D-Lysine (PDL) solution was prepared in distilled water. The 96-well plates were coated overnight with 100µl$well^{-1}$ PDL solution at room temperature. The solution was aspirated and wells were rinsed with sterile distilled water to remove the excess PDL. The plates were dried at room temperature and stored at 4-8°C for future use. In the case of coverslip coating, the coverslips were first washed in alcohol followed by 3 washes in distilled water and then autoclaved. Overnight PDL coating was followed by a rinse and drying. The coverslips were transferred to 24-well plates using sterile forceps.

8.2.3.2 NSC differentiation into neurons

After preparing a single suspension of neurospheres (see section 8.2.2), the cells were either passaged or differentiated into neurons. For differentiation the cells were suspended in a complete differentiating medium (see appendix 2.4) and cell density and cell viability was determined (see appendix 5). A 40µl aliquot of 0.75×10^6 cellsml^{-1} was placed at the centre of the PDL coated 96-well plates and 100µl aliquot containing 10^4 cells on the PDL coated coverslips. The cell were allowed to attach for 4h and flooded with more differentiating medium. In the case of drug effects evaluation, the medium containing the required drug dose was added after 4h of cell seeding (control cultures received carrier medium only). The cell viability was determined using LDH (see section 8.3.2) and resazurin assays (see section 2.5.2.1) on day 6 of differentiation followed by Kenacid blue total protein content estimation (see section 2.5.3.1).

8.3 End Points

Three end points were used in this study to estimate the effects of drugs on NSC differentiation into neurons.

8.3.1 Resazurin and Kenacid blue assay

For the resazurin (see section 2.5.2.1) and kenacid blue assay (see section 2.5.3.1) the same methods were applied as stated in section 2.5. In the resazurin assay the time of dye incubation was reduced to 45 min as it was found that NSC are fast growing cells and they readily reduced the dye to resorufin.

8.3.2 Lactic dehydrogenase (LDH) assay

Measurement of the release of LDH into the media is used as a marker of dead cells. For measurement of LDH in the medium a Sigma-Aldrich, UK LDH detection kit was used. In brief the cells were grown as described in section 8.2.4.2. On the 6th day of NSC differentiation the culture medium from the 96-well plate was transferred to another 96-well plate in a laminar flow hood. The plate containing the medium was centrifuged at 250×g for 4 min and then used for enzymatic analysis. The LDH assay mixture was prepared prior to use by mixing an equal volume of LDH assay

substrate solution, LDH assay dye solution and 1× LDH assay cofactor preparation. 75µl centrifuged culture medium was transfer into a flat bottomed 96-well plate and twice the amount of LDH assay mixture (150µl) was added. The plate was protected from light and incubated at RT for 20-30 min. 23µl of 1N HCl was added to each well to terminate the reaction. The absorbance was recorded at wavelength 490nm with 690nm background. The background absorbance wavelength was subtracted from dye absorbance and the results were expressed as the percentage of controls.

8.3.3 Material and method for Western blot and Immunohistochemistry

The materials (appendix 1) and methods for Western blot (WB) (see section 2.8) and immunohistochemistry (IHC) (see section 2.7) are discussed in detail in chapter 2.

8.3.4 In cell Western blot

For the neurofilament-L protein analysis in differentiated neurons, the 'In cell' Western blot method was used. 0.1×10^6 cellswell^{-1} were grown in black walled clear bottomed 96-wells plates. On the 6th day of differentiation the cells were washed in PBS first followed by 20 min fixation in 4% PFA (see appendix 3). The cells were washed again and permeabilized for 30 min using 0.15% triton solution in TBST (see appendix 3). Using 3% Fish skin gelatin (FSG) solution in TBST the cell were blocked for 30 min and incubated overnight with mouse monoclonal Neurofilament-L primary antibody (1:300) and rabbit GAPDH (1:3000) prepared in 3% FSG solution. The next day cells were washed three times with PBS and incubated with secondary antibodies (green mouse 1:3000 and red rabbit 1:3000 (see appendix 4.3)) for 1h at 37^{o}C. The cells were washed again with PBS and plate was scanned using an Odyssey scanner at resolution 84µm keeping the offset at 4.

8.4 Results

8.4.1 Determination of chick NSC isolation day

The exact chick NSC isolation day was determined using the NSC marker SOX2 (Sigma-Aldrich, UK). The undifferentiated mouse embryonic stem cells were used as a positive control because of their ability to exhibit cellular SOX2 presence (Yamanaka *et al.*, 2008). 4-8 day old chick brains were isolated (see section 8.2.1)

and Western blot (WB) samples were prepared as described earlier in section 2.8.4.2. The SOX2 antibody band, according to supplied information is observed between 34-37kDa. The results of SOX2 WB indicate a green band below 40KDa molecular weight marker, while the housekeeping gene GAPDH (red band) with a molecular weight 37KDa was observed above it (fig 8.1B). When the results were analysed (fig 8.1A), the SOX2 ratio to GPADH clearly shows the positive presence of SOX2 at days 4-8 of chick brain development along with ES cells as a positive control. At day 4 the expression was low which increased with further development on day 5 and remained more or less constant up to day 7 and started to decline after day 7 (fig 8.1A). The day 5 chick embryo brains were selected for the neural stem cell isolation assay because of the early stage of development and high levels of SOX2 which represent a NSCs pool.

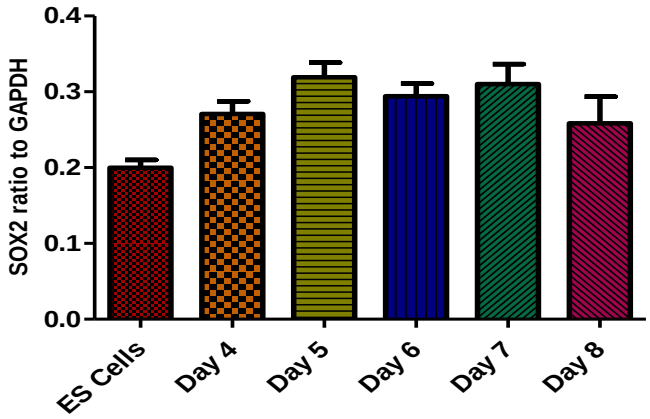


Fig 8.1A The graphical representation of Western blot analysis of SOX2 expression in positive control embryonic stem cells (ES cells) and in embryonic day 4-8 chick brains. The graph shows the presence of the stem cell marker SOX2 in ES cells, while the SOX2 expression increased after day 4 of development in chick brain and was maintained until day 7. The day 8 brain showed some decreased SOX2 values compared to earlier days. The embryonic day 5 brain was selected for NSC assay because of increased SOX2 expression at earlier days of development.

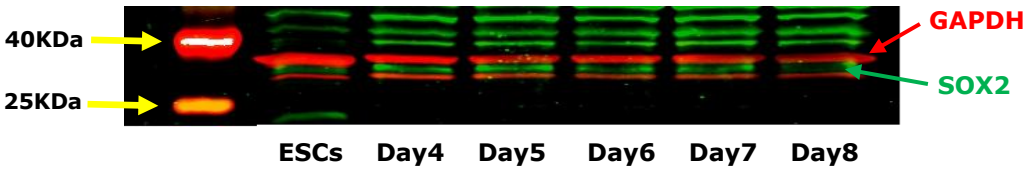


Fig 8.1B Western blot picture of anti-SOX2 staining of fig 8.1A. The **SOX2** (mol.wt 34-37KDa) protein band is seen below **GAPDH** (mol.wt 37KDa). The SOX2 protein was normalized using GAPDH to calculate the amount of SOX2 present in total number of cells.

8.4.2 NSC characterization

The chick neurospheres were characterized either using the NSC marker SOX2, or by their intrinsic property to form floating neurospheres (Ahmed, 2009), resembling the D3 ES cell embryoid bodies (see section 2.4.2.4). The cells when cultured formed floating neurospheres after 3 days (see section 8.2.2), the size and number of spheres formed depended upon the initial cell seeding density (fig 8.2A and B). When the neurospheres become considerably enlarged the central region becomes dark (fig 8.2C) and neurospheres start clumping together (fig 8.2D).

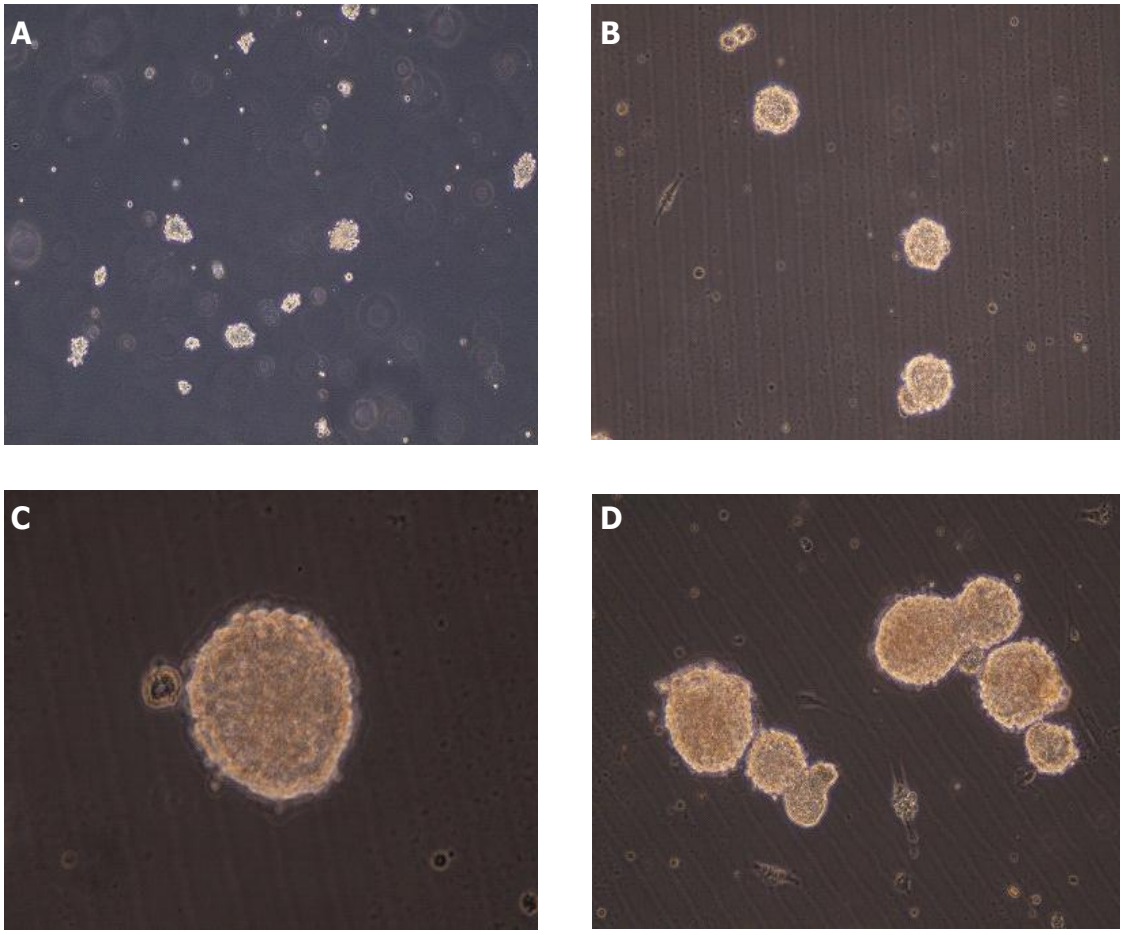


Fig 8.2 NSC neurospheres formation in an *in vitro* system. (A) On day 1 the cells start to come close and communicate with each other to form neurospheres, (B) on day 3 these small cell aggregates (neurospheres) further increase in size (C) black coloured necrotic areas can be seen in enlarged neurospheres (magnification ×25), and D) large neurospheres shows clumping and increased dark areas (magnification ×20).

The neurospheres were stained with the NSC marker SOX2 using IHC techniques (see section 2.7.2) to confirm the presence of neural stem cells. Under the fluorescent microscope the neurospheres showed the positive presence of SOX2 protein in the neurosphere cells (fig 8.3A).

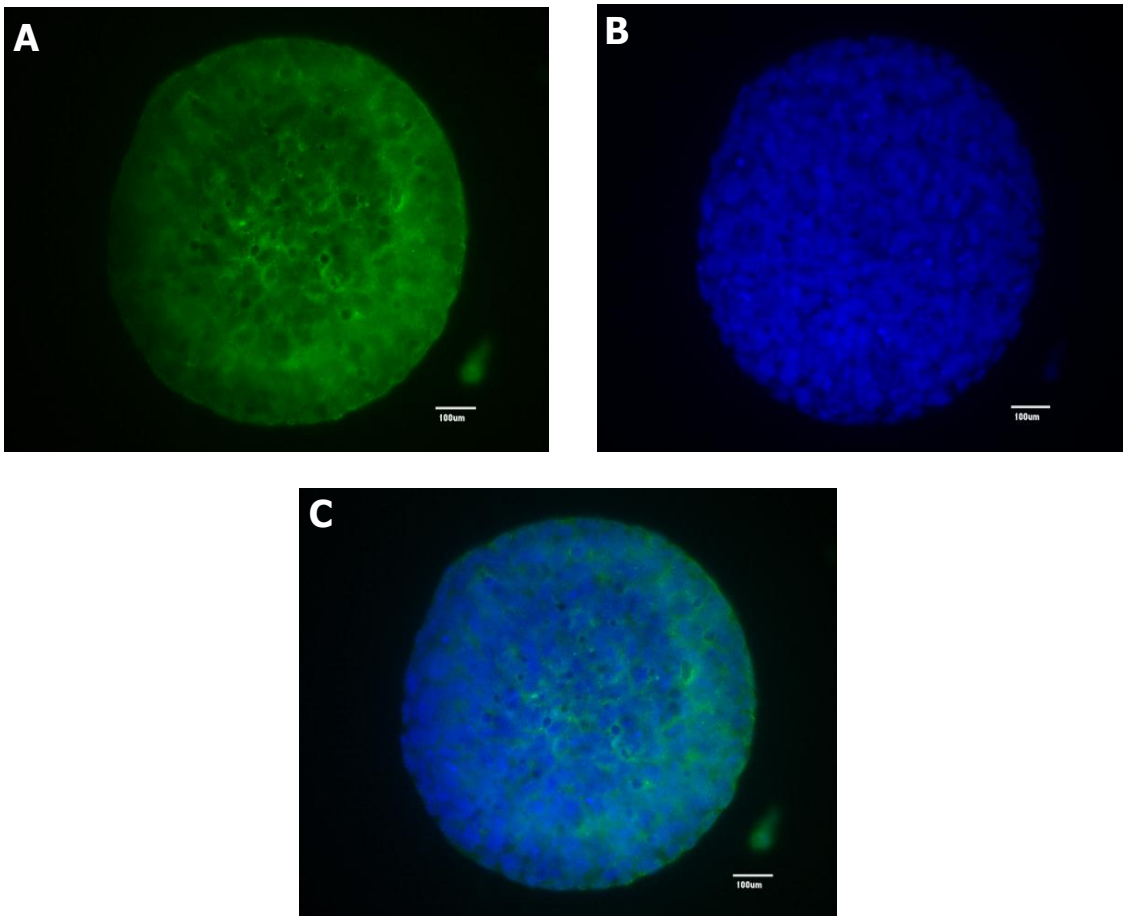


Fig 8. 3 NSC characterization with SOX2 immunostaining of neurospheres. A) **SOX2** staining indicating the NSC presence in the neurosphere, B) **DAPI** nuclear staining of cells present in the neurospheres and C) **SOX2** and **DAPI** merge.

8.4.3 NSC differentiation into neurons

For the NSC differentiation into neurons two approaches were applied. Firstly neuronal differentiation with addition of low doses of FGF-2 (5ngml^{-1}) compared to no FGF-2; secondly the use of different concentrations of chicken serum. The extent of neuronal differentiation was analysed using the β-Tubulin III (1:1000) marker which is expressed in differentiating neuronal cells (Bottenstein, 2003), while Glial fibrillary acidic protein (GFAP) (1:15000) was used for astrocytes (Bottenstein, 2003). The Western blotting (WB) procedure is described earlier in section 2.8.3.

The WB blot results clearly indicate the presence of β-Tubulin III and GFAP proteins with all different combinations (fig 8.4C), but the presence of serum promoted GFAP expression (astrocytes) (fig 8.4B), while low concentration of FGF-2 promoted β-Tubulin III (neuronal differentiation) particularly in the absence of chicken serum (fig 8.4A). NSC differentiation in the absence of chicken serum with low concentration of FGF-2 (5ngml^{-1}) was selected for the neuronal differentiation and estimation of drug effects.

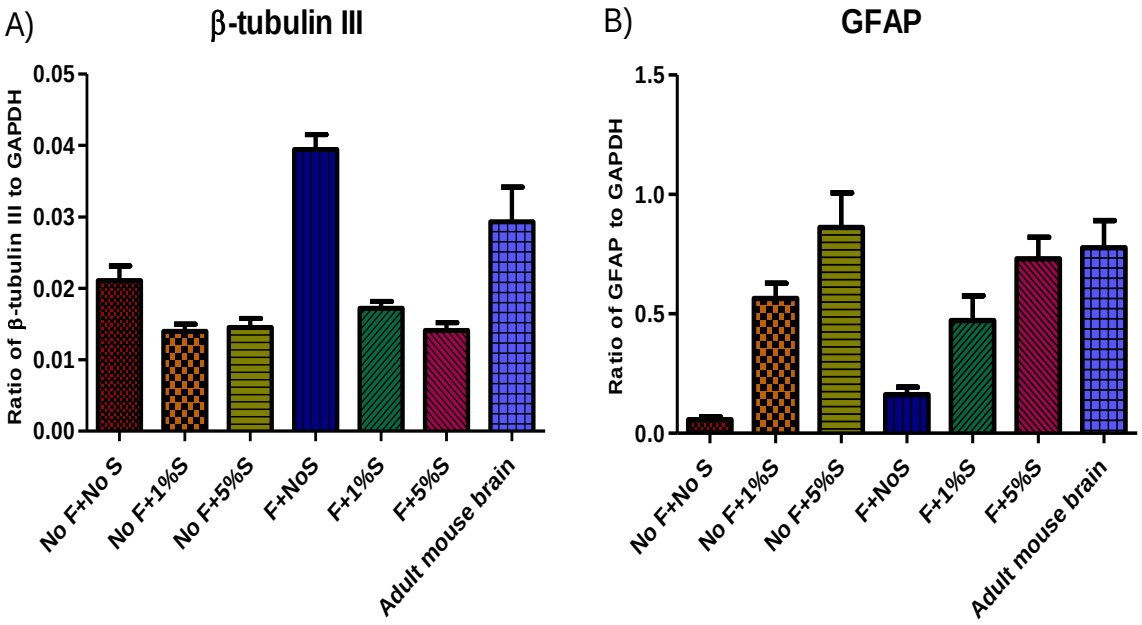
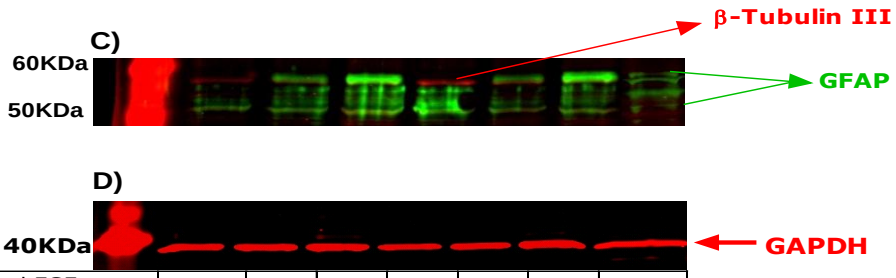


Fig 8.4A and B Result of Western blot analysis of NSC differentiation into neurons in the presence and absence of FGF-2 (F) and chicken serum (S). The adult mouse brain was used as control. A) The bar graph indicates β-tubulin III protein expression in the presence of FGF-2 and absence of chicken serum increases, B) NSC differentiation into astrocytes (GFAP) is promoted by the increase in serum concentration.



5ng/ml FGF	-	-	-	+	+	+	-
Serum	-	1%	5%	-	1%	5%	-
Adult mouse brain	-	-	-	-	-	-	+

Fig 8.4C The Western blot picture of **β-tubulin III** and **GFAP** protein expression in in differentiating NSC in the presence and absence of FGF-2 and chicken serum. The blot indicates an increase in expression of **GFAP** with increased serum concentration while NSC differentiation into neurons (**β-tubulin III**) was found to increase in the presence of FGF-2 with no serum supplements. **Fig 8.4D** Represents the house keeping gene **GAPDH** which was used to normalize the β-tubulin III and GFAP proteins.

Differentiated neurons in the presence of FGF-2 ($5ngml^{-1}$) and absence of serum were immunostained (see section 2.7.2) with the neuronal marker neurofilament-L (NF-L) (see appendix 4.1.1) to confirm the presence of neurons on day 6 of NSC differentiation. The stained cells, when observed under the fluorescent microscope, exhibited the positive presence of neurofilament-L on day 6 of differentiation (fig 8.5C and D).

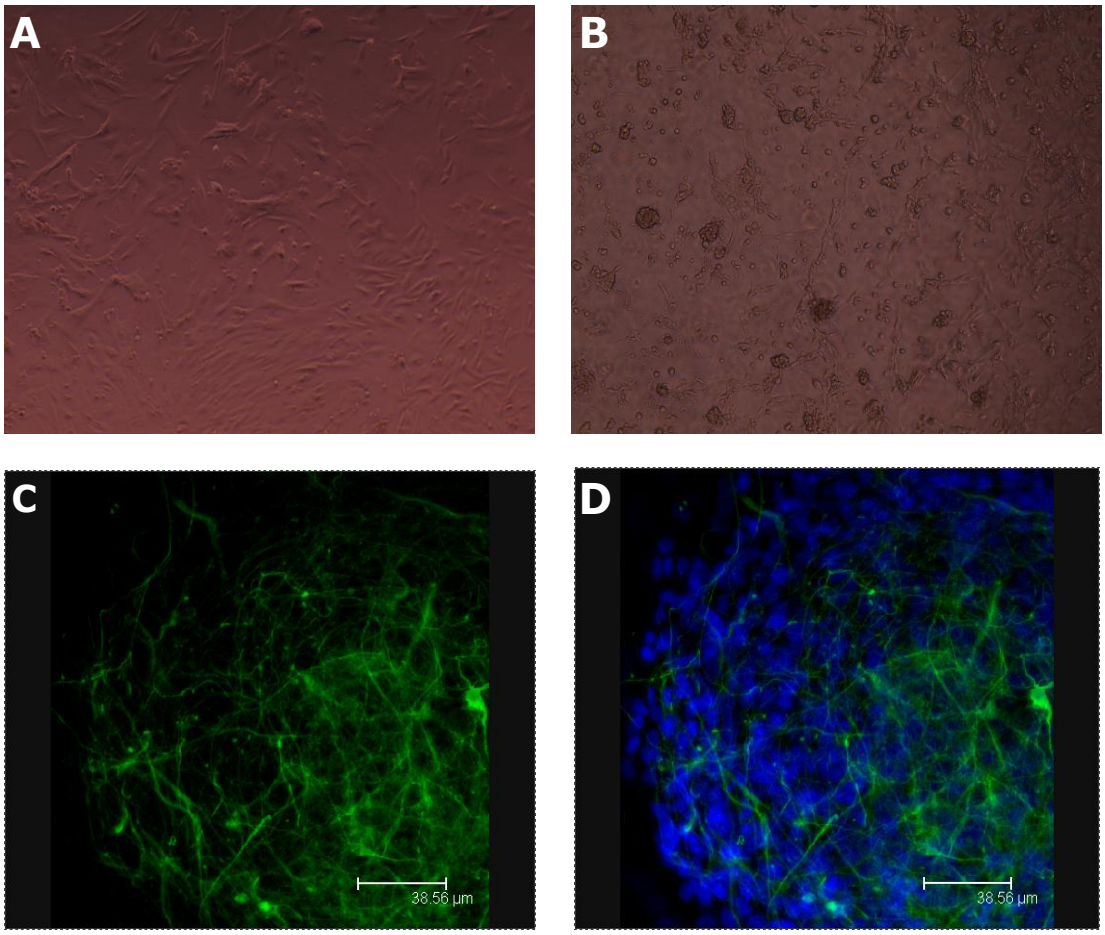


Fig 8.5 Neural stem cell differentiation into neurons in the presence of low FGF-2 concentration (5ngml^{-1}) and absence of serum. Fig 8.5A and B shows differentiated neurons on experimental day 6 (magnification ×20). Fig 8.5C represents the **NF-L** staining of neurons while fig 8.5D represents **NF-L** staining merged with **DAPI** nuclear stain.

8.4.4 Effects of known drugs on NSC derived neurons

NSC differentiation into neurons was tested using known drugs, whose toxic nature is already recorded in the literature. Penicillin G (PG) is a non-teratogenic drug (Anneelieke *et al.*, 2007) which exhibits comparable LDH release in the medium to the control (fig 8.6A). No significant effect on cell viability or protein was observed on neuronal differentiation after 6 days treatment with PG (fig 8.6B and C). The non-teratogenic analogue of 5-Fluorouracil (5-Fu), 6-Methyluracil (6-Mu) (Cumberland *et al.*, 1994) showed some increased percentage of LDH release in the medium compared to control (fig 8.6A), but cell viability and total protein content remained not significantly different to the control (fig 8.6B and C). The teratogenic drug 5-Fu

(Anneelieke *et al.*, 2007) showed a decreased percentage of LDH release in the medium compared to the control (fig 8.6A), and cell proliferation and viability were also significantly reduced after 5-Fu treatment compared to the control (fig 8.6B and C).

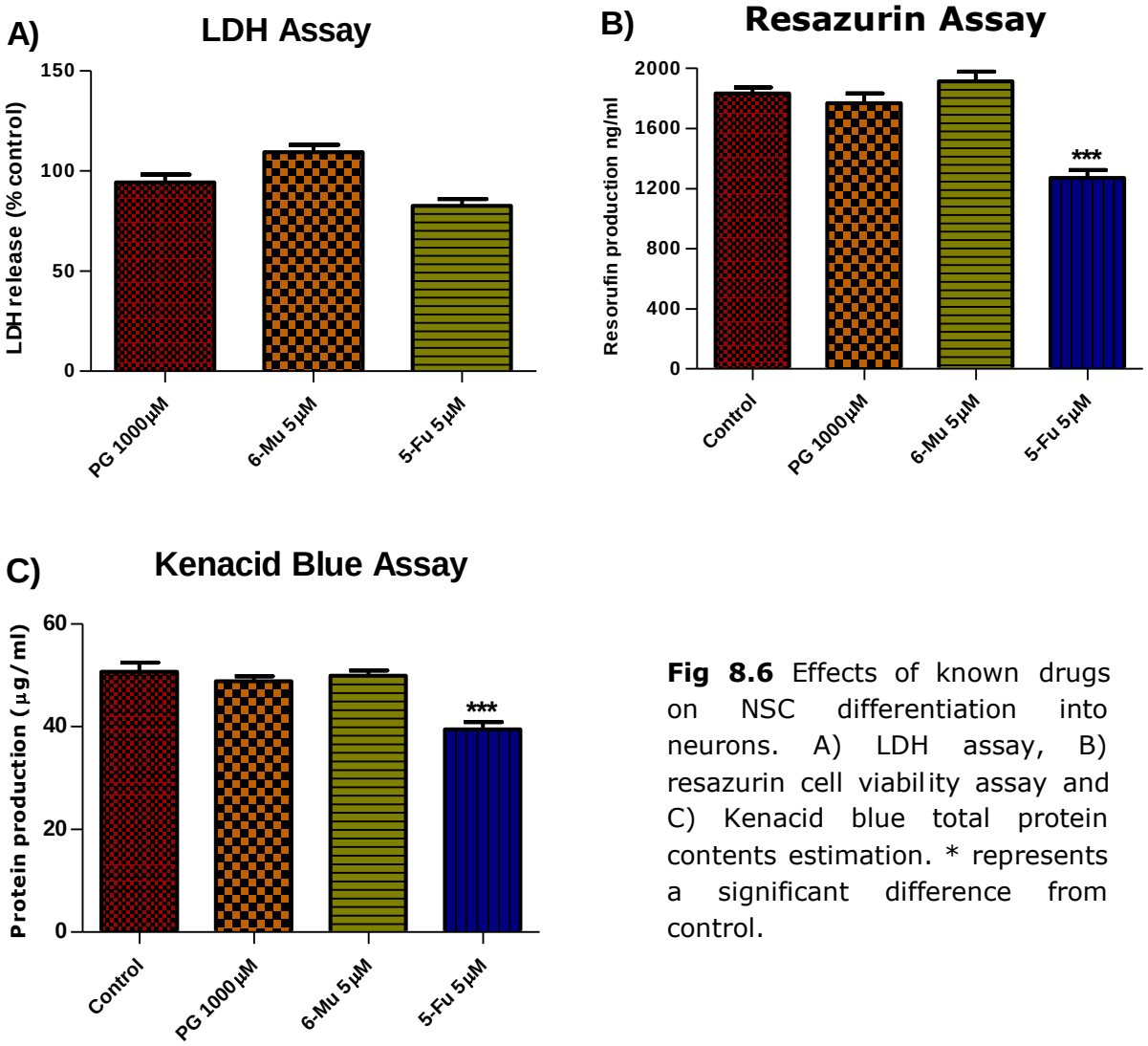


Fig 8.6 Effects of known drugs on NSC differentiation into neurons. A) LDH assay, B) resazurin cell viability assay and C) Kenacid blue total protein contents estimation. * represents a significant difference from control.

8.4.5 Effects of selected teratogens on NSC derived neurons

8.4.5.1 Sodium valproate

The chick NSC derived neurons were treated with 3 doses of VPA ranging from blood serum levels (100μM) to a very high dose (800μM) (fig 8.7A, B and C). After six days of treatment the VPA treated neurons were subjected to LDH release and resazurin cell viability assays. Using the resazurin assay the 5-Fu (which is used as negative control because of its known effects on NSC differentiation (fig 8.6)) affected the neuronal viability to significantly low levels compared to the control ($P<0.05$), while the cell viability of VPA treated neurons remained as in the control even at the 800μM dose (fig 8.7B). At lower doses, VPA100uM treated cells exhibited an apparent increased release of LDH into the medium compared to high doses (800μM) expressed as a percentage of the control values (fig 8.7A). When the same cells were subjected to total protein content estimation the results were comparable with those from resazurin cell viability assay (fig 8.7B and C). The 5-Fu significantly affected the cell proliferation while VPA showed no significant difference from the control (fig 8.7C).

8.4.5.2 Carbamazepine

The second antiepileptic drug (AED) carbamazepine was tested on differentiating neurons at a dose ranging from 50-400μM. The toxic effects on resazurin cell viability were evident with increased dose (fig 8.7E). The 400μM CBZ dose exhibited some significantly reduced cell viability compared to the control. The declining trend was obvious with increased dose, at low doses (50μM) the neurons showed apparent improved cell viability in comparison to the control (fig 8.7E). Using the cell proliferation Kenacid blue assay similar effects were observed showing a decreased proliferation with increased dose, but the control and 50μM CBZ treatment showed comparable protein concentrations (fig 8.7F), while at 200μM it became significantly different to control (fig 8.7F) (fig 8.7E). When the drug effects on LDH release were compared as a percentage of the control, the 100 and 400μM CBZ even with reduced cell proliferation showed high levels of LDH release in the medium (fig 8.7D).

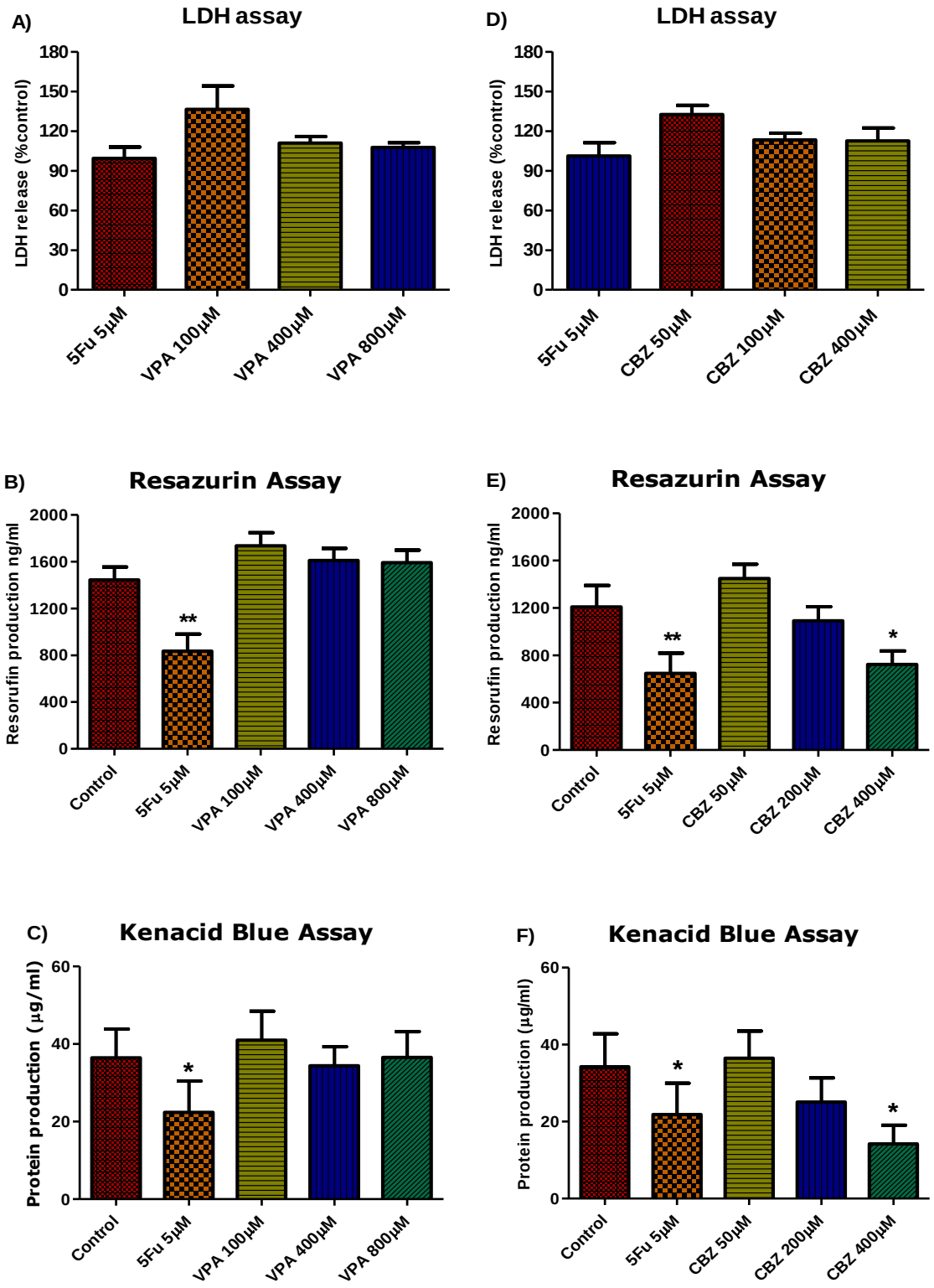


Fig 8.7 Effects of VPA and CBZ on NSC derived neurons. A) LDH assay for VPA, B) resazurin cell viability for VPA, C) Kenacid blue total protein content estimation for VPA, D) LDH assay for CBZ, E) resazurin cell viability for CBZ and F) Kenacid blue total protein content estimation for CBZ. * represents a significant difference from control.

8.4.5.3 Bupropion hydrochloride

The antidepressant molecule bupropion (BPN) showed similar effects to those observed with cardiomyocytes (fig 5.3). Increasing the dose of bupropion decreased cell viability (fig 8.8B). Compared to the control the drug dose effects were significant at 200μM. The drug effects on total protein content indicated reduced cell proliferation. As the drug dose was raised from 25-200μM some significant difference was observed at high doses (200μM) (fig 8.8C). With respect to LDH release (fig 8.8A), an increase in BPN dose resulted in an increase in LDH release in spite of reduced cell number.

8.4.5.4 Lithium Carbonate

When the differentiating neurons were treated with antidepressant molecule lithium carbonate, in the blood therapeutic serum range (600-1200μM), it showed no signs of toxicity or changes in cell viability (fig 8.8E and F). The effect of the drug at doses of 500 and 1000μM were comparable to control, but as the dose was increased above serum therapeutic levels (2000μM) the cell viability decreased, but failed to achieve significant difference from control (fig 8.8E). The effects on protein content showed a similar trend to the cell viability results (fig 8.8 F). No significant changes in cell proliferation were observed at 500 -2000μM compared to control (fig 8.8F). In LDH assay an increased percentage of LDH release in the medium was observed compared to control at 500-2000μM treated groups (fig 8.8D).

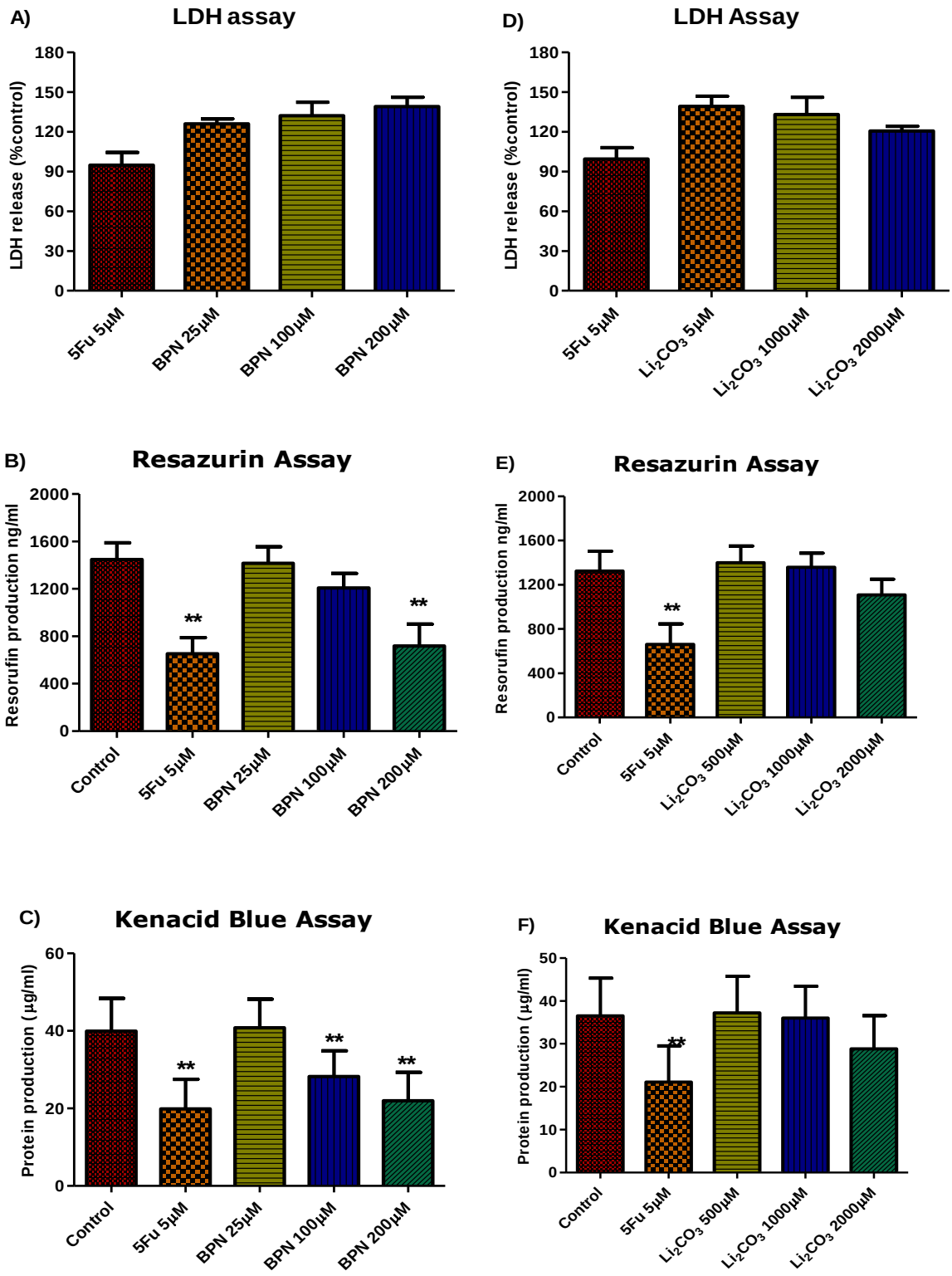


Fig 8.8 Effects of BPN and Li_2CO_3 on NSC derived neurons. A) LDH assay for BPN, B) resazurin cell viability for BPN, C) Kenacid blue total protein content estimation for BPN, D) LDH assay for Li_2CO_3, E) resazurin cell viability for Li_2CO_3 and F) Kenacid blue total protein estimation for Li_2CO_3. * represents a significant difference from control.

8.4.6 Effects of drugs on Neurofilament-L expression in NSC derived neurons

Using 'In cell' Western blot (ICW) techniques the effects of these drugs on neuronal differentiation were evaluated. The neurofilament-L protein levels were normalized from GAPDH representing the total amount of protein (see section 7.8.3). VPA showed no significant effects with increase in drug dose between 100-800μM (fig 8.9A). The negative control 5-Fu also did not alter the NF-L protein levels to significantly different values in differentiating neurons (fig 8.9A). With carbamazepine (50-400μM) no significant alteration in the protein levels were observed in NSC derived neurons when analysed using ICW technique (fig 8.9B).

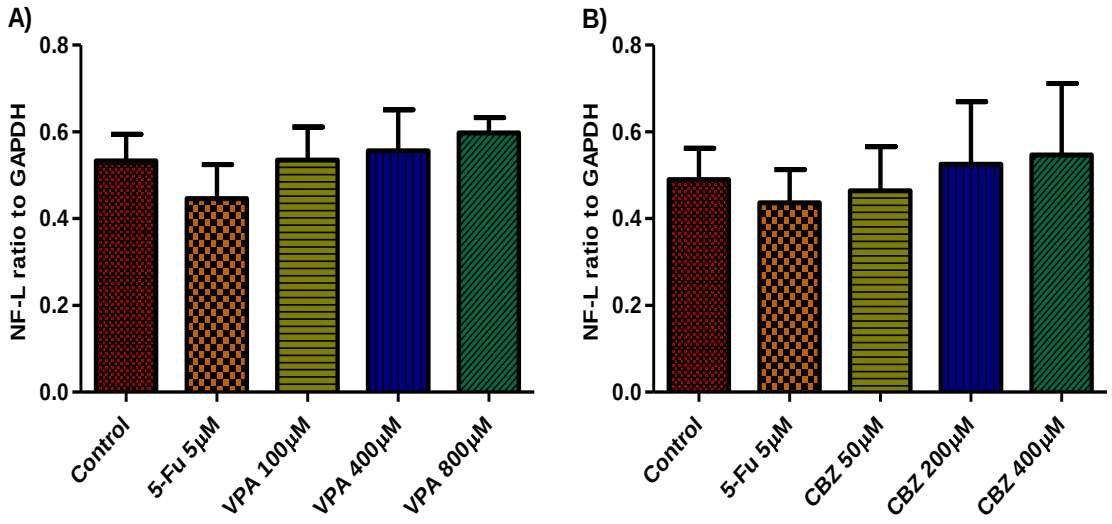


Fig 8.9 'In cell' Western blot results for VPA and CBZ effects on Neurofilament-L expression in NSC derived neurons. A) VPA showed no significant altered levels of NF-L after 6 days treatment, while B) CBZ showed no significant altered levels of NF-L after 6 days treatment.

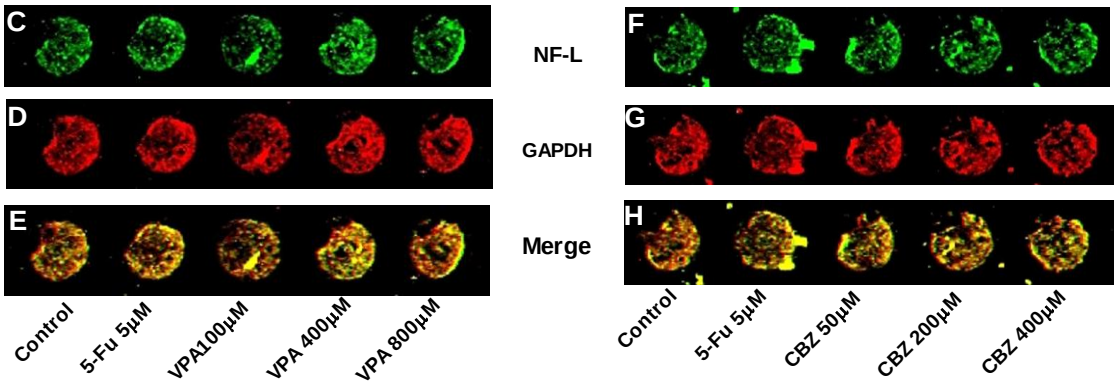


Fig 8.9C-H Represents **neurofilament-L** in an 'In cell' Western blot picture of a scanned plate. **NF-L** expression in differentiated neurons after VPA (fig 8.9C) and CBZ (fig 8.9F) treatment remained no different to control. The **GAPDH** (fig 8.9D and G) was used to normalize the total NF-L protein present in cells, while fig 8.9E and F represents the merge of NF-L and GAPDH.

The antidepressant drug bupropion effects were comparable for protein NF-L levels after 25 and 100μM treatment, the high dose 200μM appeared to reduce protein levels but again failed to achieve statistically significant difference (fig 8.10A). The fourth drug tested in this system was lithium carbonate which exhibited an inconsistent decline in NF-L protein with increase in drug dose, none of which achieved a significant difference (fig 8.10B) from the control.

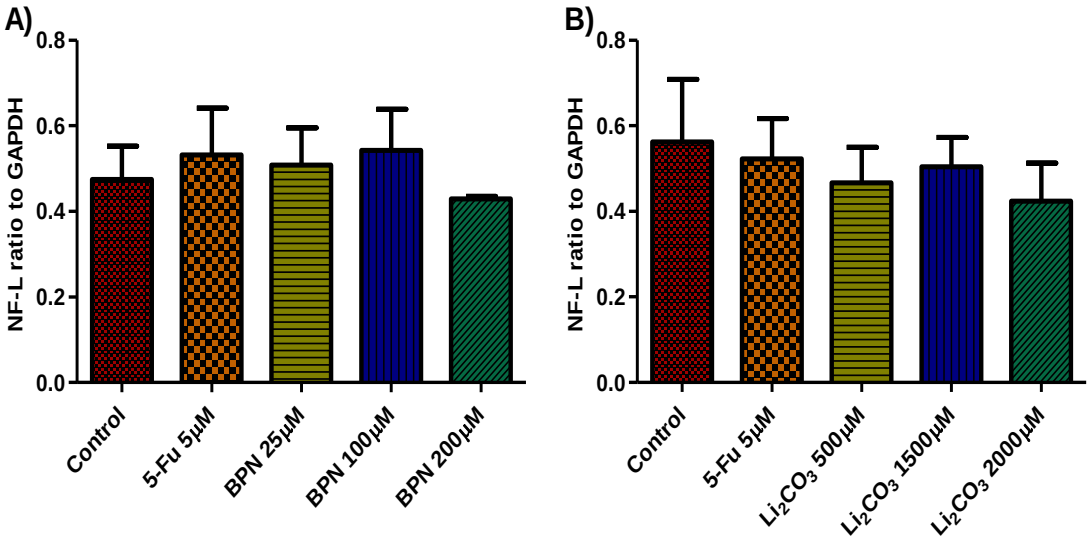


Fig 8.10A and B 'In cell' Western blot results of BPN and Li_2CO_3 effects on Neurofilament-L expression in NSC derived neurons. A) BPN showed no significant difference in NF-L after 6 days treatment with 25 and 100μM, or 200μM drug dose, B) Li_2CO_3 showed no significant difference from the control at any dose.

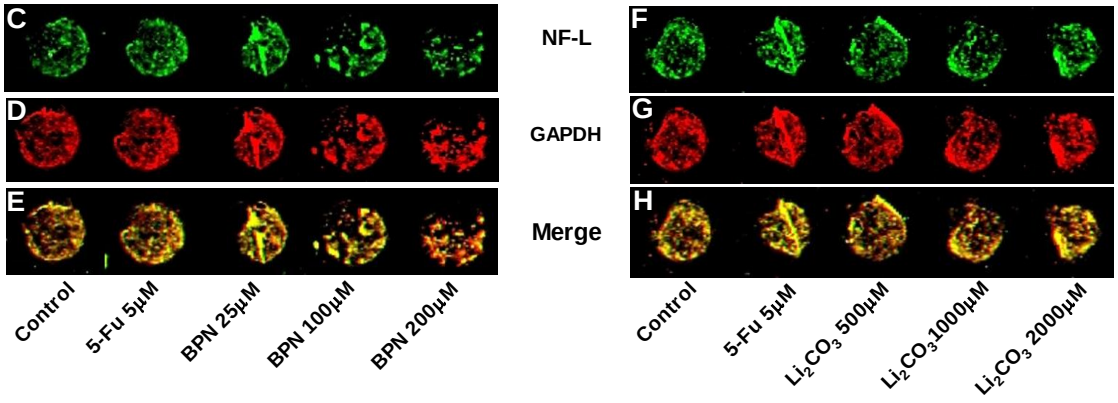


Fig 8.10C-H Represents the **neurofilament-L** 'In cell' Western blot picture of a scanned plate. The **NF-L** expression in differentiated neurons after BPN (fig 8.10C) and Li_2CO_3 (fig 8.10F) treatment remained no different to the control. The **GAPDH** (fig 8.10D and G) was used to normalize the total NF-L protein present in cells, while fig 8.10E and F represents the merge of NF-L and GAPDH.

8.5 Discussion

Neural stem cells in the nervous system provide a unique pool of cells that have the potential to develop into the entire nervous system and maintain tissue homeostasis throughout adulthood (Ahmed *et al.*, 2009; Taupin *et al.*, 2000). The neural stem cells, similar to other stem cells, have the ability to proliferate and self-renew to maintain the primitive cell pool and differentiate into CNS lineages to replace malfunctioning and dead cells (Reynolds & Weiss, 1996). During development NSC are derived from neuroepithelium cells (NEP). These cells upon activation of particular signalling pathways direct their fate to nervous cell lineages - neurons, astrocytes and oligodendrocytes (Sommer & Rao, 2002). Earlier studies by Cajal suggesting no postnatal neurogenesis (Ming & Song, 2005) were disapproved by Joseph Altaman and Gopal Das in the 1960's by exhibiting the presence of dividing cells in the adult central nervous system (Altman, 1962; Altman & Das, 1965). Reynolds and Weiss further confounded this old idea by isolating and culturing NSC from adult brain which proliferated in response to the mitogen EGF (Reynolds & Weiss, 1992). During fetal neurogenesis a large number of NSC are present but as development proceeds these cells migrate and differentiate into their committed cell

types, and in the adult brain the neurogenesis continues only in SVZ and SGZ of the dentate gyrus (Bottenstein, 2003).

The *in vitro* isolation of NSC provides a unique opportunity to elucidate the mechanism at the molecular level. NSC *in vitro,* in the absence of serum, respond to mitogens FGF and EGF and form clonal populations of cells known as 'neurospheres', which represent a heterogeneous progenitor pool at different stages of development and differentiation (Ahmed, 2009; Wang *et al.*, 2006). Providing the requisite growth factors and substrates these cells can be diverted towards different lineages. The use of neurotropic factors NT3, NT4 and BDNF along with low levels of FGF-2 has been shown to promote neuronal differentiation *in vitro* (Bottenstein, 2003; McAllister, 2001). The addition of serum promotes GFAP expression in differentiating NSC compared to β-tubulin III.

As development of the brain proceeds the pool of NSC starts to decrease along with their proliferative potential, with an increase in lineage commitments (Brazel *et al.*, 2005). SOX2 has been shown to be the persistent marker present in fetal and adult NSC and has been used to quantify the presence of NSC. The embryonic Day5 chick brain expressed increased levels of SOX2 compared to earlier days. Later day brains also expressed almost same levels of the NSC marker but these are more developed and it might be that NSC have migrated to particular regions in brain. The decreased expression of SOX2 in chick after day 8 is confirmed by some earlier studies (Uwanogho *et al.*, 1995). Charlotte Scott in earlier studies confirmed the ability of embryonic day 5 chick brain tissue to form neurospheres, but they were relatively scarce in numbers (Scott *et al.*, 2010). In this study it was found that embryonic day5 chick (E5) neural tissues produced a substantial numbers of neurospheres.

In this study the chick neural stem cells were grown and differentiated into a neuronal lineage using growth factors and supplements. The addition of drug to this system might provide a neurotoxic profile for the drug, in particular with respect to neuronal differentiation and the cytoskeletal protein neurofilament-L.

The use of the antiepileptic drug VPA during the first trimester of pregnancy can result in neural tube and CVS defects (Ornoy, 2009). VPA is a HDAC inhibitor and

HDAC inhibition inhibits NSC proliferation and induces differentiation. VPA also shows protection against pro-apoptotic stimuli in neurons like lithium (Gurvich & Klein, 2002). The effects of VPA in NSC were different compared to the embryonic stem cell derived cardiomyocyte (ESDC) system. In ESDC the higher doses (800μM) inhibited cardiogenesis but in NSC the higher dose of 800μM showed no effect on neuronal differentiation. The 100μM VPA, which is a therapeutically relevant dose, result in increased percentage of LDH release in the medium that might be because slight increased cell number (Kenacid blue assay) was observed with VPA 100μM treatment. In 'In cell' WB the NF-L showed no declining trend with increased drug dose, indicating increased VPA dose does not inhibited the neuronal differentiation. These types of result are observed with HDAC inhibitors (Hsieh *et al.*, 2004). The other property of VPA to inhibit GSK3-β might have played role here too, because increased GSK3-β was found to alter neurogenesis while its inhibition promotes neurogenesis. But the VPA mechanism of GSK3-B inhibition is different from lithium (Gurvich & Klein, 2002). The VPA activation of ERK pathway that stimulates neurogenesis cannot be ruled out here (Zhou *et al.*, 2011). In earlier studies VPA was found to promote neuronal cell differentiation and inhibit glial differentiation (Hsieh *et al.*, 2004), but VPA is also associated with neural tube defect production. Analysing the effects of VPA at gene level that promotes neuronal differentiation might provide a better elucidation of its effects. From the results observed here and in some earlier studies it can be concluded that VPA inhibits early NEP which results in NTD, while it does not inhibit the neuronal differentiation.

The antiepileptic drug carbamazepine in the NSC system showed no significant toxic effects at low doses (50μM) on cell viability and protein content but increased percentage of LDH release in the medium was observed. But at higher doses above 50μM a declining trend of proliferation was observed. Similar types of result were observed with ES cells, where the 200 and 400μM doses showed decreased cell proliferation and differentiation. The ability at high doses (400μM) of CBZ to arrest cell division might have played a role here, resulting in directly decreasing the cell viability (Perez Martin *et al.*, 2008). Interestingly the reduced number of cells results

in increases in LDH release in the medium compared to the control; so the drug not only arrests cell division at high doses (400μM) but also affects cell membrane integrity, which ultimately results in cell death. The CBZ induction of apoptosis at high doses (400μM) has already been confirmed in previous studies (Gao *et al.*, 1995). The drug effects on NF-L protein showed a different picture when normalized using GAPDH (see section 7.8.3). The increase in drug dose did not change the NF-L protein levels in the total neurons present. This means carbamazepine at low doses (50μM) does not produce toxic effects on cell proliferation and cell membrane integrity, but at higher doses may arrest cell division, induce cell death and increase LDH release into the medium, while the drug itself does not affects neuronal differentiation. This is might be because of its HDAC inhibition effects which on one side induces apoptosis while also promoting neuronal differentiation (Gurvich *et al.*, 2004).

In differentiating neurons the antidepressant drug bupropion shows strong antiproliferative effects at high doses (200μM) tested, but these doses are much higher than normal serum therapeutic concentrations. As stated earlier in chapter 5 the activation of the caspase pathway in bupropion treatment might have shown consistent results in all systems used in this study (Jang *et al.*, 2011). Increasing the dose to 100 and 200μM showed major effects on total protein levels which represent cell proliferation. The effect on cellular oxidoreductase levels seems to be dependent on the number of cells. But the LDH assay clearly shows an increase in apoptotic activity after drug treatment. The 200μM dose of BPN decreased the protein levels comparable to the effects of 5-Fu, but even the low number of cells released increased amounts of LDH into the medium. This shows the drug effects on cell membrane integrity, which result in increased apoptosis. When the BPN effects on NF-L were estimated the increased in drugs dose did not show any particular trend in protein levels. As neurofilament was used as marker of differentiated neurons, it means the drug is inhibiting cell proliferation but not affecting NSC differentiation into neurons, in particular the protein estimated through 'in cell' western blotting. It would be really interesting to analyse caspase activation after BPN treatment in NSC,

which would provide a better description of its antiproliferative effects on neuronal differentiation.

The drug lithium carbonate acts by two mechanisms, the activation of the Wnt signalling pathway, which results in increased cell proliferation, especially in NSC with neuronal differentiation (Muroyama *et al.*, 2004). The other mechanism of PI cycle inhibition is not clear in neurogenesis but it might have effects through disturbed Calcium regulation (Giles & Bannigan, 1999; King *et al.*, 2010). In this study lithium showed no significant toxic effects on differentiating neurons at therapeutically relevant (600-1200μM) and at toxic doses (2000μM) on cell proliferation and neural differentiation. The lithium carbonate in NSC derived neurons showed an increased percentage of LDH release at therapeutically relevant serum levels but interestingly the cell proliferation was not dropped significantly.

As discussed earlier, Lithium carbonate acts by two mechanisms the PI cycle inhibition and by mimicking the Wnt/GSK3-β pathway. GSK3-β has proapototic activity and its inhibition results in cytoprotection (Leng *et al.*, 2008). The Wnt pathway activation normally results in increased cell proliferation which was found in ESDC but not in the case of neurons. Some earlier studies have shown that lithium has no effects on progenitor proliferation but protects from neurotoxic agents or conditions (Boku *et al.*, 2011; Ishii *et al.*, 2008). The Wnt protein in the presence of FGF-2 inhibits neuronal differentiation, (Bottenstein, 2003) which was not significant in this study. It would be interesting to see how lithium carbonate affects differentiating neurons in the absence and presence of FGF-2 at doses above 2mM.

It can be concluded that the use of the *in vitro* chick NSC culture system provides an easy way to elucidate neurogenesis mechanisms. The chick NSC system is an easy and less expensive model compared to rodents. These neural stem cells are highly plastic and differentiate into various brain cell types and provide the opportunity to screen drug toxic mechanisms before marketing.

For the antiepileptic drugs VPA, HDAC inhibition, GSK3β inhibition and activation of ERK signalling in combination decides the neural stem cell fate with direction either

toward apoptosis or differentiation, while the other AED CBZ does not affect neuronal differentiation but at high doses arrests cell division. The BPN activation of caspase pathways increases cell apoptosis, while the drug apart from inhibiting cell proliferation exhibits no significant inhibitory effects on neuronal differentiation. Lithium carbonate, like VPA, affects many signalling pathways but at drug doses above serum therapeutic levels, mainly activation of the Wnt pathway and in the presence of FGF-2 it may negatively regulate neuronal differentiation.

With the addition of different growth factor combinations and differentiation to different neuronal and other CNS cell types, plus the identification of appropriate end points and molecular markers, the chick NSC system could be a reliable *in vitro* model to estimate teratogenic effects.

Chapter 9

General Discussion

9.1 General Discussion, Conclusion and Future Work

The study of embryo development is one of the most fundamental and fascinating areas of modern science (Levin, 2002), since it involves intricate signalling, transcription, differentiation, assembly and regulation. This complex web of events requires a continuous flow of information between cells and tissue systems (Levin, 2002; Vinken *et al.*, 2006). Any disturbance in normal regulation and information sharing could lead to a disastrous developmental outcome. The heart is the first mesodermal organ that starts function because of the high demand of nutrient supply in other developing regions. Any abnormal regulation at earlier stages of heart development directly affects the oxygen and nutrient supply to other proliferating and differentiating cells. Neural developmental defects lead to cognitive, motor and intellectual dysfunctions. Nervous and cardiovascular system patterning and migration during development are tightly regulated by various factors whose increased expression promotes one lineage and can inhibit others (Murashov *et al.*, 2005). Potential contact during pregnancy either by medication, food or by environmental factors affects normal development, which by disturbed regulation of signalling molecules may promote one lineage and affect others, as observed in the case of VPA and Li_2CO_3. Interestingly the drug's effects are not only restricted to lineage but also vary with the stage of development.

In this study two lineages were targeted during development. Firstly the cardiac lineage, for which the chick embryonic cardiomyocyte micromass system has been used, which represents early embryonic heart cells (Ahir & Pratten, 2011) which have acquired the cardiac lineage but are anatomically underdeveloped (Bellairs & Osmond, 1998). Also mouse embryonic stem cells were differentiated into cardiomyocytes. The use of the ESDC system in tandem with a fibroblast cell line has been accepted and validated by ECVAM as a potential screening method for teratogenicity testing. However, in this study the ESDC protocol of differentiation was used without estimating effects on the fibroblast cell line which does not actually represent the in vivo maternal condition (Anneelieke *et al.*, 2007; Marx-Stoelting *et al.*, 2009). The third system is a newly developed chick Neural Stem Cell isolation

and differentiation of neurons. The chick NSC isolation system is easy and cheap compared to mouse, but there remains a problem with molecular markers in the chick system. The brain tissues isolated were grown in neurospheres, which represent the pool of neural stem cells at different stages of development and differentiation (Wang *et al.*, 2006). Providing the requisite growth factors and supplements, the NSC cells were diverted into the neuronal lineage. In all three systems drugs were applied to estimate their effects at the cellular and molecular level. A number of endpoints have been used in *in vitro* teratogenicity testing. In this study the resazurin cell viability assay was preferred because of its ease of use in these systems and the same cells can be used for protein content estimation using the Kenacid blue assay. As the chick NSC is a newly developed method, LDH release in the medium was also estimated as a marker of cell membrane integrity after drug treatment. In cardiomyocytes, contractile activity was used as a marker to confirm the extent of cardiac differentiation and any drug effects. Two types of drug groups were selected based on the in vivo data available and the FDA pregnancy category: Anti-Epileptic Drugs and Anti-Depression Drugs. These drugs and their disease states during pregnancy present a conflict in drawing a clear conclusion on results, but in vivo data have confirmed the teratogenic nature of these drugs (Brewer *et al.*, 2003; Kallen *et al.*, 2006).

Most of the antiepileptic drugs are shown to perturb normal development (Kaneko *et al.*, 1999). AEDs affect Neural tube integrity, the CVS and show similar effects on facial development (Ornoy, 2009). Some common teratogenic mechanisms have been observed between AEDs, such as oxidative stress through the production of highly reactive epoxide intermediates. Their detoxification depends upon the enzymes present, which lead to different degrees of drug effect with strain and animal type. Low levels of antioxidant enzymes are detected in embryos, which may easily put the embryo on oxidative stress. Using the undifferentiated embryonic stem cells, no altered levels of ROS production were observed. This may mean the early stage pluripotent cells are not equipped with a metabolic system to produce ROS and induce oxidative stress. This may partly explain the lack of earlier embryopathies,

while during organogenesis (cardiogenesis) the differentiated cells shows stage dependant effects on ROS production. Secondly AED effects on Folic Acid metabolism or intestinal absorption have been suggested as possible mechanisms (van Gelder *et al.*, 2010). Differences between the drug mechanisms for inhibition of FA metabolism might be the key to the diverse effects in the *in vitro* systems. For example, VPA inhibits FA metabolism, an effect for which there is efficient protection by adding FA supplements, while CBZ impairs FA absorption, which is an effect that cannot be seen in the in vitro system, consequently the addition of FA supplements failed to show protective effects. Phenytoin, which can decrease FA levels most compared to other AEDs, does not induce NTD to the extent of VPA or CBZ. There is a relation between AEDs, FA and NTD but the mechanism is not fully understood. The third possible mechanism observed with VPA and CBZ is HDAC inhibition which also promotes DNA methylation and induces epigenetic changes at the DNA level (Detich *et al.*, 2003; Murabe *et al.*, 2007b). Beside these mechanisms, drugs may act on various signalling pathways and on cellular biochemistry.

Valproic acid is already known to produce toxic effects in the MM and ESDC system (Ahir, 2009; Murabe *et al.*, 2007a). High levels of drug affects cardiac differentiation more compared to the differentiated cells of the MM system. Similar results were also observed with CBZ. This may mean VPA and CBZ use during cardiac cell differentiation is more lethal compared to later stages of heart cell development. It would be interesting to see how these drugs affect adult cardiomyocyte contractile activity in *in vitro* systems. During ES cell differentiation VPA and CBZ affect normal cardiogenesis by increased oxidative stress, which is not observed in the MM system. That is possibly due detoxifying enzyme levels which increase with developmental stage, while antioxidant supplements have shown a positive impact on detoxifying these intermediates (Zhang *et al.*, 2010). CBZ, at doses above serum therapeutic levels, arrested ES cell growth, which has connotations with earlier studies. The effects of the same drugs appear to be different on neurogenesis, since earlier stage neural tube development is inhibited by VPA and CBZ and results in NTDs, while late stage neuronal differentiation remained unaffected as observed with chick NSC. The

drug effects on NTD were found to be the result of decreased neuroectodermal cell proliferation, but this is reversed by the addition of FA. HDAC inhibition properties could be the other source of reduced cell proliferation at the earlier stage commitments (Gurvich *et al.*, 2004), while at later stages of neurogenesis HDAC inhibition was actually found to induce neuronal differentiation (Yu *et al.*, 2009).

In the *in vitro* systems ROS production takes place within the cells in the 24-well plates, without the need for cells to migrate to a target tissue. *In vivo* ROS produced in the mother cannot migrate to the embryo because the electrophiles have a very short half-life. There needs to be some mechanism to produce intermediates and reactive species either by placental metabolism or metabolism inside the embryo to produce toxic effects. Increased ROS production caused by VPA and CBZ may have effects on other proteins including Cx43, which shows reduced and disturbed dose dependent levels in both the systems. However, in the MM system the ROS levels were shown not to be significantly elevated after drug treatment, so effects might be attributed to some other mechanism, especially for VPA, such as PPAR δ, β-catenin, activator protein 1, myo-inositol, PkC and Erk1/2 (Gotfryd *et al.*, 2011; Gurvich *et al.*, 2004). The role of AA to counteract CBZ cardio-toxic effects, especially in ES cells, is mainly due to the cardiac induction properties through production of low levels of peroxide, along with its antioxidant effects. SOD failed to be protective in ES cells, possibly because these cells either cannot respond to SOD or it does not reach the target site.

In the MM and ESDC cardiomyocytes PHT showed a dose and stage dependent effect on cardiogenesis. PHT effects on contractile activity are mainly attributed to its inhibitory effects on potassium channels (Danielsson *et al.*, 2007), while increasing ROS production through epoxide intermediates (Ozolins *et al.*, 1995) might result in toxic effects which were more prominent in the ESDC system. The toxic effects on cell proliferation and to some extent on viability were only evident in the ESDC system. This shows the role of PHT in producing malformations before cardiac lineage commitment compared to the treatment of the differentiated cells of the MM system. These different effects with PHT were not only observed at stages of

development but also in different strains mainly due to enzyme levels that metabolize PHT or detoxify the highly reactive intermediates (Ozolins *et al.*, 1995).

The fourth anticonvulsant molecule primidone in the MM system showed dose and time dependent effects on contractile activity, while in ESDC increased dose resulted in decreased contractile activity at all intervals observed. The ESDC exhibited inhibitory effects on contractile activity at lower doses compared to the MM system. It may be that the effects of the drug on development are through some unknown mechanism compared to the contracting cardiomyocytes of the MM system. In vivo primidone is metabolized to PEMA and phenobarbital, and the phenobarbital is mainly responsible for its anticonvulsant effects (Pizzi *et al.*, 1996). Whether primidone is metabolised in this system is unknown, while its metabolite phenobarbital when tested in MM and ESDC was found to affect cardiomyocyte contractile activity (Ahir, 2009). Primidone itself is less toxic compared to its metabolite in the *in vitro* cardiomyocytes system, while in *in vivo* the drug itself and its metabolite synergistically may induce toxicity. If the drug is not metabolised it is comparatively less toxic, but its therapeutic activity is mainly due to phenobarbital production. In order to avoid toxic effects, it may be possible to increase the efficacy of the parent molecule itself without its metabolism or may be by drug metabolism to a phenobarbital analogue with reduced toxic effects.

In humans the use of FA and AA supplements at the start of pregnancy may eliminate the risk of producing NTDs and CVS defects with AEDs. But AA at high doses was found to act as pro-oxidant through the Fenton reaction in *in vitro* systems, so maintaining adequate levels of AA is also necessary to avoid any unwanted effects. Less teratogenic analogues like methyl valproate may provide an alternative with reduced drug induced toxic effects.

In ADP the toxic effects produced did not show any common outcome. BPN had effects on cell proliferation in all systems. The chick cardiomyocytes isolated from day five old embryos or the embryonic stem cells derived cardiomyocytes both exhibit a reduced cell proliferation after BPN treatment. When the drug was used to treat NSC derived neurons the effects on cell proliferation were not very different

from the cardiomyocyte system. Interestingly in the ES cells, the size of EBs was not affected as much as observed in CBZ treated groups, where reduced EBs size was seen from the start of the experiment. In MM the cultured cardiomyocytes ability to proliferate and form connections was seriously affected after drug treatment. BPN has been reported to affect cell proliferation without producing metabolites but by increased cell apoptosis (Jang *et al.*, 2011). This means the mechanism of decreased cell proliferation is independent of the cell type used and stage of development. BPN effects on cell viability seemed to be dependent on cell proliferation, but the increased LDH leakage into medium with increase in drug dose might have been the result of increased apoptosis. Estimating the caspase activity with BPN treatment in these systems might provide a better elucidation. Drug effects on cell proliferation also affect the establishment of the contractile unit, especially in the case of the MM system, where cultured cardiomyocytes proliferate and make contact and then establish gap junctions so they can share information. The connexin43 levels after BPN treatment were reduced in both systems and especially a disturbed localization was observed in the MM system. BPN affects the Ca^{2+} levels in the cells and Ca^{2+} is involved in the regulation of gap junction permeability (Lurtz & Louis, 2007). Reduced permeability might have affected Cx43 turnover and reduced gap junction levels in the cardiomyocytes, which in turn affects the contractile activity. Early cardiac and neuronal differentiation markers analysis will provide the better explanation that drug only inhibits cell proliferation or also its commitment to different progeny. BPN drug treatment with high doses of BPN during pregnancy carries the risk of producing convulsions and antiproliferative effects, which both may confound the pregnancy outcome. Maintaining the serum therapeutic levels during pregnancy or chemically modifying the Bupropion structure specially to prevent the effects on proliferation might provide solution to these side effects.

The effects of the second antidepressant molecule, lithium carbonate, on cardiomyocytes were observed through two different mechanisms. Firstly inositol synthesis - the drug affects inositol synthesis through inhibition of the IMPase enzyme which results in reduced recycling of free inositol at the cell membrane. This

eventually results in reduced production of IP_3 in the cells (Giles & Bannigan, 1999; Klug *et al.*, 1992; Segal, 2004), a molecule that has an important role in cell contraction and initiation of pace maker activity in the heart (Kockskämper *et al.*, 2008). When the effect of lithium on contractile activity was compared in MM and ESDC systems, the results were very different because cells at same the dose were contracting in the MM system while in ESDC no contraction was observed. Two things are important here: firstly MM cardiomyocytes have already differentiated and contract and exhibit GJIC at the time of isolation, while ESCs are unspecialized cells which can acquire any lineage. When lithium treatment was given to cells, the MM cardiomyocytes had already experienced contractility, so contractile activity re-establishment was not affected much compared to ESDC, where the early mechanism to start the first beat relies on IP_3, which was eliminated by lithium treatment. Myo-inositol supplementation compensated for the effect of Lithium, by providing the requisite inositol. The other important potential mechanism is lithium activation of Wnt pathways, which results in accumulation of β-catenin, and β-catenin has an important role in Cx43 regulation (Zhaowei *et al.*, 2000) and embryo hyperdorsalization (Eisenberg & Eisenberg, 2007; Martin *et al.*, 2011). When the results for Cx43 in MM are analysed, the cellular localization appeared disturbed after lithium treatment compared to ESDC, where protein content was increased. This means that it is likely that lithium acting by some other mechanism has disturbed Cx43 localization in MM, while in EDSC the activation of Wnt negatively regulates cardiogenesis and β-catenin accumulation, which induces CX43 expression. Wnt signalling has a role in producing hyperdorsalized embryos which might parallel the fact that in ESDC differentiated cells there was increased cell proliferation. In NSC lithium exerted a neuroprotective effect on neural differentiation, with no altered effects on cell viability, which is in accordance with the results observed earlier (Ishii *et al.*, 2008). Lithium activation of the Wnt pathway can affect the cell-lineage fate (Sommer, 2004), which was observed in both cardiomyocytes and the neuronal system. The lithium molecule exhibits cardiac inhibition and with no effects on neuronal differentiation. Altering the molecule's properties may not provide the

solution because it is thought that lithium acts by these mechanisms to produce antidepressant effects. Maintaining lithium therapeutic levels during pregnancy will reduce the risk of producing malformations.

St. John's wort active constituents, hypericin and hyperforin, were shown in some cases to affect normal development. When evaluated in the MM system the effects of hypericin, as observed in other studies, seemed to be light dependent, and initiate ROS formation that induced toxic effects (Theodossiou *et al.*, 2004; Wurglics & Schubert-Zsilavecz, 2006). The other constituent hyperforin, with caspase activation properties (Hostanska *et al.*, 2003), showed some inhibitory effects on contractile activity at levels above the normal serum concentration of the drug. Synergistically both drugs affected cardiomyocyte proliferation and contractile activity significantly but again above the drug's serum therapeutic levels. The MM system suggests that HP and HF use at low doses showed no significant toxic effects on cardiomyocytes, but the mixtures effects on development need to be proved before making any definite conclusion.

Most pregnancies are unplanned; thus when the mother finds out that she is pregnant she has already been exposed to teratogens. A drug's effects on development depend on the molecular mechanism with which they interfere, and this varies with lineage and the stage of development. The proper elucidation of the molecular mechanism of teratogenicity may help to counteract drug induced toxicity. Adding various markers and genetic exploration will make more clear the toxic profile of the drug, especially in the case of contractile activity by using some automatic scoring system or contractile protein markers to estimate the extent of effects on cardiac differentiation will make the EST more reliable. The chick NSC is a newly developed system and it needs more evaluation at the molecular level to become a better and more reliable screening method. Differentiation into different neuron types using growth factors and supplements and drug treatment during differentiation would provide an easy system for screening neuroteratogens. Using

different molecular markers and biochemical analyses in all three systems can provide a better explanation of toxic effects.

By increasing the molecular size of the drug molecule so that it cannot cross the placenta, without losing its efficacy would be another way to reduce the risk of drug exposure to the embryo, but on the other side it may affect passage of the drug through the blood brain barrier. Before the formation of the placenta drug transfer directly to the embryos poses another threat. Planned pregnancies and supplementing the mother with counteracting molecules or chemically attaching these molecules to the drugs, which on metabolism resulted in the availability of these detoxifying agents at the site of production, may be a more sophisticated solution to the problem.

The toxic effect of a drug during development depends on exposure conditions, time, dose, tissue and developmental stage. The complex effects produced by epilepsy, depression and their medications during development remained unresolved. Induction of oxidative stress by AEDs, antiproliferative effects of BPN or effects of Li_2CO_3 acting via IP3 /Wnt pathway interactions might have affected cell differentiation. These effects were more pronounced in differentiating cells (ESC) compared to primary culture of embryonic cardiomyocytes. The same molecule may produce different, even opposite, effects with different cell lineages. This totally depends on the molecular pathways with which it is interacting and their role in development. By counteracting or eliminating the exact toxic molecular mechanism, tissue injury and developmental defects as a whole can be prevented.

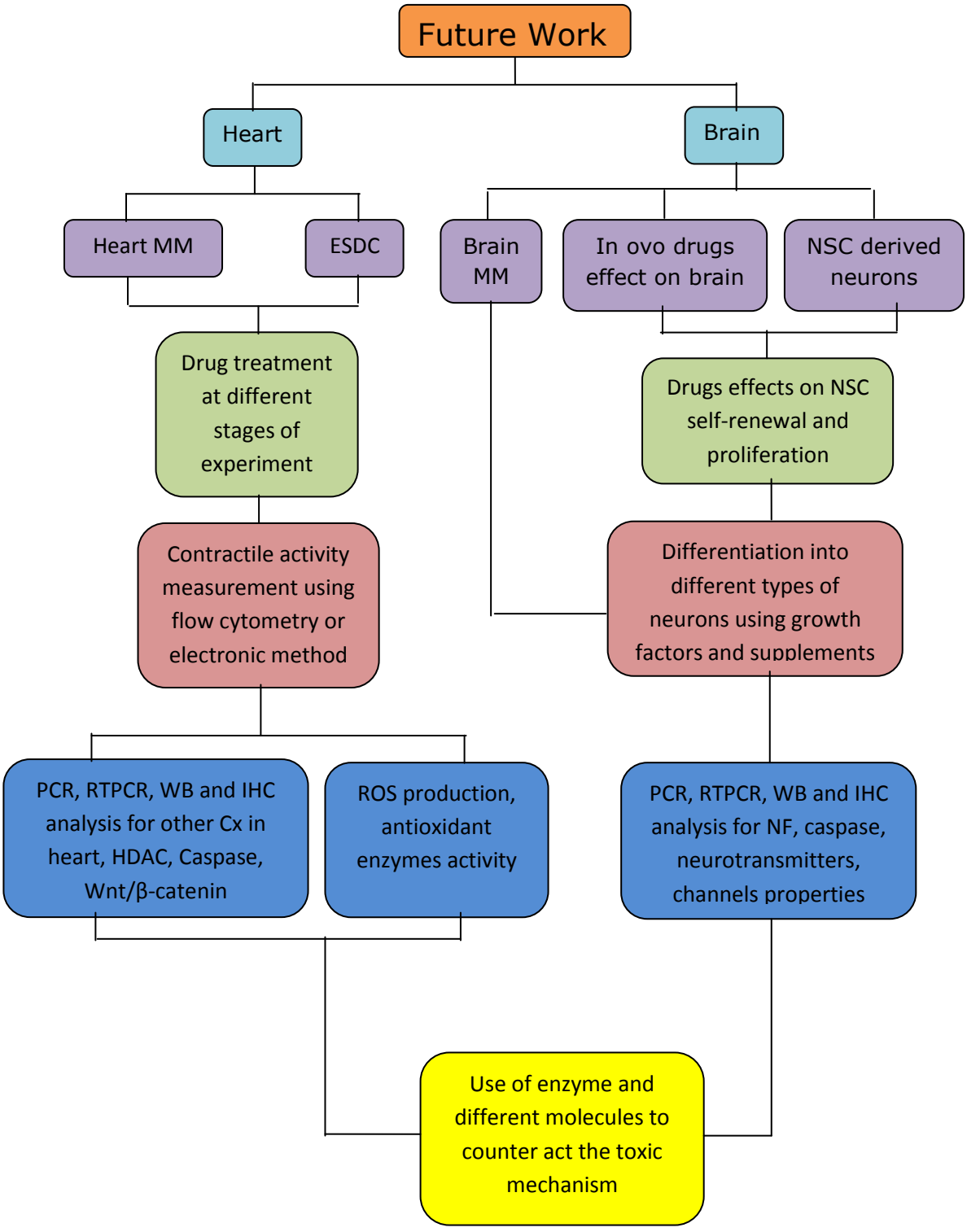


Fig 9.1 Flow chart for possible future research direction in these in vitro systems

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Appendix 1. Materials

All the chemicals were purchased from Sigma-Aldrich.UK unless otherwise stated.

1.1 Plastic and Glass wares

24 well plates	Nunclon, UK
25cm^2 tissue culture flasks	SLS, UK
90mm round Petri dishes	Nunclon, UK
96 black well plates (clear bottom)	Costar, UK
96 well plates	Nunclon, UK
Aero disc filter (0.2μM)	Sartorius, UK
Bijou bottles (7ml)	Sterilin, UK
Chamber slides (8 well)	Thermo scientific, UK
Cover slips	SLS, UK
Cryovials	Sarstedt, UK
Eppendorf tubes 0.5ml and 1.5ml	Sterilin, UK
Serological pipettes	Greiner bio-one, UK
Square Petri dishes	Sterilin, UK
Syringes 50ml, 10ml and 1ml	BD Biosciences, UK
Universal tubes 20ml	Sterilin, UK
Universal tube 15ml and 50ml	Greiner bio-one, UK

1.2 Equipment

Assorted dissecting tools	A. Dumont, Switzerland
ASYS HITEC Expert 96 plate reader	SLS, UK
Autoclave	Denley, UK
Automated egg turner	MFG, USA
Balance D40T	Stanton, UK
Calibrated pipettes	Gilson, France
Centrifuge (centaur 2)	Fisons, UK
Class I sterile laminar flow hood	Glossop, UK
Class II sterile laminar flow hood	Thermo scientific, UK

CO_2 incubator	Sanyo, UK
Confocal microscope TCS4D	Leica, Germany
Cooled egg incubator	Gallenkamp, UK
Dissecting microscope (Nikon SMZ-10a)	Nikon, Japan
Dymax 30 aspirator vacuum pump	Charles Austen Pumps
Eggs Incubator (38^{o}C)	LEEC, UK
Fluorescence microscope	Olmpus 1X70, Japan
FLUORStar Galaxy plate reader	BMG Cambridge, UK
Freezer (-80^{o}C)	New Brunswick, UK
Haemocytometer	Hawksley, UK
Inverted phase microscope	Nikon, Japan
Liquid nitrogen Dewar	Dilvac, UK
Mr Frosty	SLS, UK
Plate shaker	Luckham Ltd, UK
Stirrer hot plate	Gallenkamp, UK
Warming block	Camlab, UK
Water purifier	Egga, UK

1.3 Western blot materials

Cassettes	Millipore, Uk
Centrifuge Cold	Sigma, UK
Centrifuge (RT)	Sanyo, UK
Electrophoresis module	Bio-Rad laboratories, UK
Filter paper	Whatman's Ltd, UK
Fish skin gelatine (FSG)	Sigma-Aldrich, UK
Loading marker	Lonza, UK
Nitrocellulose membrane	Shleicher & Schuell, Germany
Odyssey scanner	Licor, UK
Plate reader	Spectra, Max, UK
Polytron homogenizer	Heildolph, UK

Precast gels (10% 1mm)	Lonza, UK
Transfer module	Bio-Rad laboratories, UK
Tanks	Bio-Rad laboratories, UK
Transfer cassettes	Bio-Rad laboratories, UK

1.4 Consumables

Autoclave bags	NHS supplies
Pipette tips (200µl and 1000µl)	Sarstedt, UK
Scalpels	SLS, UK
Universal graduated glass bottles	SLS, UK
Vinyl gloves	Ansell, UK
Yellow biohazard bags	NHS supplies

1.5 Software

EndNote X5	Thomson Reuters, USA
Odyssey V3.0	LI-COR Biosciences, UK
Prism statistical software, V5.0	Graph pad, USA
Volocity workstation	PerkinElmer, Inc, USA

Appendix 2. Preparation of cell culture media

2.1 Culture medium for chick embryonic cardiomyocytes (Micromass)

For chick embryonic cardiomyocytes culture medium Dulbecco's Modified Eagles Medium (DMEM) was supplemented with 10% heat inactivated fetal bovine serum (FBS), 2mM L-glutamine and 50unitsml^{-1} penicillin/50µgml^{-1} streptomycin. The medium was stored at 4^{o}C and used within two weeks.

2.2 D3 mouse embryonic stem cells culture medium (ESDC)

The D3 mouse ESCs culture medium was prepared by supplementing DMEM with 20% heat inactivated FBS, 2mM L-glutamine, 50unitsml^{-1} penicillin/50µgml^{-1} streptomycin, 0.1mM β-Mercaptoethanol and 1% X100 non-essential amino acids. Leukaemia inhibitory factor (LIF) (Chemicon international, UK), was aliquoted and stored at 4^{0}C. LIF 1000Uml^{-1} was directly added to the culture flask.

2.3 Preparation of NSC culture medium

For the preparation of NSC culture medium, equal amounts of DMEM high glucose and Ham's F12 were supplemented with B27×50 (2%) (Invitrogen, UK), N2×100 (1%) (Invitrogen, UK), EGF 20ngml^{-1} (BD, Bioscience, UK), bFGF 10ngml^{-1} (BD, Bioscience, UK), Heparin 100µgml^{-1}, 2mM L-glutamine and 50unitsml^{-1} Penicillin/50µgml^{-1} Streptomycin.

2.4 Preparation of NSC differentiation medium

For the preparation of NSC differentiation medium Neurobasal medium (Invitrogen, UK) was supplemented with B27×50 (2%), N2×100 (1%), bFGF 5ngml^{-1}, Heparin 100µgml^{-1}, NT3 5ngml^{-1} (Abcam, UK), NT4 5ngml^{-1} (Abcam, UK), BDNF 5ngml^{-1} (Abcam, UK), 2mM L-glutamine and 50unitsml^{-1} Penicillin/50µgml^{-1} Streptomycin.

Note: The culture media quality was checked by placing 5ml prepared medium in bijou bottle for 2-3 days at 37^{o}C, 5% CO_2 (British oxygen company) in an incubator. The medium was inspected microscopically for contamination. If contamination was present, the medium was discarded.

Appendix 3. Preparation of stock solutions

3.1 Immunohistochemistry Stock solutions

3.1.1 Bovine serum albumin (1% BSA w/v) solution

1g BSA was added to 100ml PBS and agitated until dissolved. The solution was stored at 4^{o}C.

3.1.2 Normal goat serum (5% NGS v/v)

5% v/v NGS solution was prepared by adding 500µl NGS in 9.5ml PBS immediately before use.

3.1.3 Paraformaldehyde (4% PFA) solution

4g of PFA was added to 100ml PBS inside the fume cupboard, the solution was heated at 60^{o}C with continuous agitation until completely dissolved. The solution was filtered and stored at -20^{o}C.

3.1.4 Triton X-100 Solution (1% v/v)

100µl Triton X-100 was added to 9.9ml PBS prior to use. The solution was vortexed and stored at 4^{o}C.

3.2 Preparation western blot Solutions

3.2.1 Lysis Buffer pH 7.6

20mM (12.1g) Tris (Invitrogen, UK), 1mM (19.1g) EGTA, 0.1% Triton X-100 (500µl), 1mM (0.021g) NaF, 10mM (1.08g) β-Glycerophosphate dissolved in 500ml Distilled water. One tablet of protease inhibitor (Roche, UK) was added to 10ml lysis buffer prior to use.

3.2.2 2X Solubilisation Buffer

0.5M (2.5ml) Tris (Invitrogen, UK), 2ml Glycerol, 10% (2ml) SDS (Fisher Scientific, UK), 1ml β-mercaptoethanol (Fisher Scientific, UK), 2.5% (40µl) Bromophenol Blue (Fisher Scientific, UK).

3.2.3 TBST (0.1% Tween-20)

25mM (30.29g) Tris and 125mM (73.12g) NaCl were dissolved in 1L distilled water, pH was adjusted to 7.6 using HCl (approximately 17ml conc HCl), total volume was made up to 10L and 10ml Tween-20 (0.1%) was added.

3.2.4 10X Electrophoresis buffer

Tris 30.3g, Glycine 144g and SDS 10g were dissolved in 1L distilled water. The solution was stored at 4^{o}C.

3.2.5 Transfer buffer

Tris 30.3g and Glycine 144g were dissolved in 8L distilled water and 2L methanol was added. The solution was stored at 4^{o}C.

3.2.6 Lowry A and B

500ml Lowry A solution was prepared by dissolving 2g NaOH (Fisher Scientific, UK), 1g SDS, 10g Na_2CO_3 (Fisher Scientific, UK) in 500ml distilled water. For Lowry B 1% $CuSO_4$, 2% NaK Tartrate (Fisher Scientific, UK) were dissolved in distilled water.

3.2.7 Folin's Reagent

Folin reagent solution was prepared by dissolving 1:1 of reagent in dH_2O.

Appendix 4. List of antibodies

4.1 Immunohistochemistry (IHC)

4.1.1 Primary antibodies for immunohistochemistry

Antigen Specificity	Host Species	Antibody Type	Reactivity	Dilution	Source
Connexin43	Mouse	Monoclonal	Bovine, chicken, mouse, human, hamster, rat	1:500	Sigma-Aldrich.UK
SOX-2	Rabbit	Polyclonal	Human, mouse, chicken	1:100	Sigma-Aldrich.UK
Myosin heavy chain	Mouse	Monoclonal	Chicken	1:100	DSHB, Iowa, USA
Troponin T	Mouse	Monoclonal	Broad species	1:100	DSHB, Iowa, USA
Neurofilament-L	Mouse	Monoclonal	Pig, rat, chicken, human	1:400	Sigma-Aldrich.UK

Table 4.1 The primary antibodies used for immunostaining.

4.1.2 Secondary antibodies for immunohistochemistry

Antigen specificity	Host	Type	Reactivity	Label	Dilution	Source
Rabbit IgG	Goat	Monoclonal	Rabbit	Alexa 488	1:1000	Invitrogen, UK
Mouse IgG	Goat	Monoclonal	Mouse	Alexa 488	1:500/1000	Invitrogen, UK

Table 4.2 The secondary antibodies used for immunostaining.

4.2 Western blot

4.2.1 Primary antibodies for western blot

Antigen Specificity	Host Species	Antibody Type	Reactivity	Dilution	Source
Connexin43	Mouse	Monoclonal	Bovine, chick, mouse, human, hamster, rat	1:3000	Sigma-Aldrich.UK
Phospho-Connexin43	Rabbit	Monoclonal	Chick, murine, human, bovine, zebra fish	1:750	Thermo scientific, UK
SOX2	Rabbit	Polyclonal	Human, mouse, chicken	1:1000	Sigma-Aldrich.UK
β-Tubulin III	Mouse	Monoclonal	Mouse, Pig, Rat, Chicken, Human	1:1000	Abcam, UK
GFAP	chicken	Polyclonal	Mouse, Rat, Chicken, Cow, Cat, Human	1:15000	Abcam, UK
GAPDH	Mouse	Monoclonal	Human, mouse, rat	1:10000	Sigma-Aldrich.UK
GAPDH	Rabbit	Monoclonal	Human, mouse, rat	1:20000	Sigma-Aldrich.UK

Table 4.3 Primary antibodies used in Western blot analysis.

4.2.2 Secondary antibodies for western blot

Antigen Specificity	Host Species	Antibody Type	Dilution	Source
IRDye800CW	Mouse	Polyclonal	1:10000	LICOR, UK
IRDye680CW	Mouse	Polyclonal	1:10000	LICOR, UK
IRDye800CW	Rabbit	Polyclonal	1:10000	LICOR, UK
IRDye680CW	Rabbit	Polyclonal	1:10000	LICOR, UK
IRDye800CW	Chicken	Polyclonal	1:10000	LICOR, UK

Table 4.4 Secondary antibodies for NSC Western blot.

4.3 In cell western blot

4.3.1 Primary antibodies for In cell western blot

Antigen Specificity	Host Species	Antibody Type	Reactivity	Dilution	Source
Neurofilament-L	Mouse	Monoclonal	Mouse	1:300	Sigma-Aldrich.Uk
GAPDH	Rabbit	Monoclonal	Human, mouse, rat	1:3000	Sigma-Aldrich.UK

Table 4.5 Primary antibodies for NSC In cell western blot.

4.3.2 Secondary antibodies for In cell Western blot

Antigen Specificity	Host Species	Antibody Type	Dilution	Source
IRDye800CW	Mouse	Polyclonal	1:3000	LICOR, UK
IRDye680CW	Rabbit	Polyclonal	1:3000	LICOR, UK

Table 4.6 Secondary antibodies for NSC In cell Western blot.

Appendix 5. Calculation of cell number

The cell number was determined using the Neubauer Haemocytometer. 20µl cell suspension was incubated with 20µl of Trypan Blue (0.4%w/v) (1:1 dilution) for few min at room temperature. The dead cells take up the dye and were stained blue. Cell viability is determined by counting the number of live and dead cells.

Total cells ml^{-1} = Average of squares counted × dilution factor × 10^4

For total percentage of viable cells:

$$\text{Percentage viability} = \frac{\text{Live cell count}}{\text{Total cell count}} \times 100$$

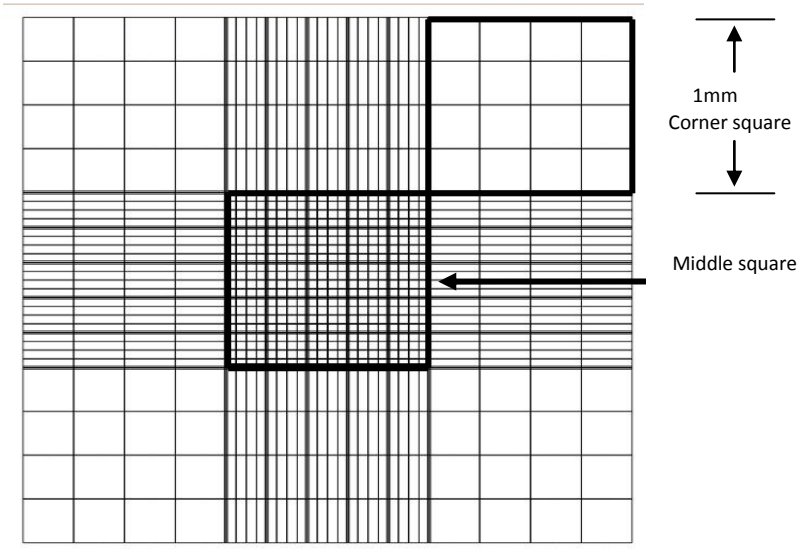

Fig 5.1 Appearance of the Haemocytometer grid visualized under the microscope.