Human Neural Stem Cell Culture and Other In vitro Models for Prediction of Embryotoxicity and Neurotoxicity

By

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Thesis submitted to The University of Nottingham for the degree of Doctor of Philosophy

2016

Abstract

Generally, most of the in vitro tests used in neurotoxicology are limited to transformed cell lines which are derived from rodent or human. For an in vitro test to have high rate of predictability of neurotoxicity and teratogenicity it should undergo the important processes of embryological development, such as cell proliferation, cell migration, and differentiation. Human neural stem cells have been proposed for this purpose, which have the ability to divide, differentiate, and migrate. In this study, it was found that double coating of laminin with either poly D lysine or poly L lysine was most suitable for growing human neural stem cells rather than coating with a single extracellular molecule. Several chemicals and drugs were then chosen to assess the utility of neural stem cells as an assay for neurotoxicity: methyl mercury and lead acetate; four anti-epileptics drugs (sodium valproate, phenytoin, carbamazepine, and phenobarbitone); anti-oxidants (folic acid and melatonin). These anti-oxidants were tested alone and when added to sodium valproate and to phenytoin (which are well known in their teratogenicity), and other drugs (lithium, diazepam, and amitriptyline), which are weak teratogens. To assess the effects of these molecules on human neural stem cells cell survival, total cellular protein, neuronal process length, neurosphere sizes, migration distance, Glial Fibrillary Acidic Protein, and tubulin III protein expression were measured.

The study shows that methyl mercury caused significant reduction in most of the end points from the dose of 1µM and it led to significant increase in Glial Fibrillary Acidic Protein expression (which is a sign of reactive gliosis). Lead acetate led to a significant reduction in cell migration 48hours after treatment with

10µM. In the case of the anti-epileptics, sodium valproate appeared to reduce neurosphere size significantly from the dose of 500µM and decrease migration distance significantly 48hours after treatment with 1000µM. Moreover, phenytoin treatment resulted in significant reduction in neurosphere sizes from the dose of 25µM and reduced cell migration significantly from the dose of 50µM. However, the other anti-epileptics (carbamazepine and phenobarbitone) revealed their effect only at high doses which are above their therapeutic range. On the other hand, adding the anti-oxidants (Folic acid or Melatonin) to sodium valproate or phenytoin had to some extent beneficial effects, by making their toxic effect appear at doses which were higher than when used alone. Regarding the other drugs (lithium, diazepam, and amitriptyline), it seems that their toxic effect appeared only at doses which are higher than the therapeutic range.

Therefore, it can be concluded that human neural stem cells are a sensitive model in detecting the neurotoxicity of methyl mercury and lead acetate at low doses and can predict the neurotoxicity of sodium valproate and phenytoin at their therapeutic doses.

To my wife, my sons (Humam and Jamal),

and my daughter (Dania)

Acknowledgement

The completion of my Ph.D study could not been possible without the help and support of so many people whose names may not all be mentioned. Their contributions are sincerely appreciated and gratefully acknowledged.

However, I would like to express my deep appreciation and indebtedness particularly to **Dr. Margaret Pratten** for her superb supervision, kind guidance and motivation throughout my study to do this interesting novel model in In vitro Neurotoxicology and for her endless support apart from my study.

I would like also to express my sincere thanks and gratitude to **Dr. Peter Wigmore** for his kind supervision and invaluable support in my study.

I would like to acknowledge and thank many people that I have met during my study in particular **Mr. Laique Latif** for his kind help and support, all **AMU staff**, and **Dr. Lisa Storer** for their valuable help.

Special thanks to all **my family** and **relatives** for giving me emotional support throughout my study.

I pay my deep sense of gratitude and indebtedness to **Iraqi Ministry of Higher Education and Scientific Research** (MOHESR) for funding my Ph.D scholarship.

Above all, I would like to thank **Allah, the Great Almighty**, the author of knowledge and wisdom, for giving me the strength and support to accomplish and to study.

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Chapter 1

Litrature Review of In vitro Neurotoxicology

1.1 Historical Background

The history of neuroscience goes back over a century but started in a meaningful way with the advent of in vitro systems. One of the earliest theories had been proposed by Cajal in 1890, which was based on microscopic analysis of histological sections, who stated that immature neuronal cells can send out neurites which elongate freely, having a growth cone at the tip. Competing theories proposed that growth of neurites did not occur, but the neurites formed from elements produced from other cells, or formed by stretching of a protoplasmic bridge between the central and peripheral cell bodies of a multinucleated cells (y Cajal, 1989). These theories could not be tested at that time because the histological methods were primitive and the growth of neurites of living neurons could not be observed, since the old histological techniques were not applied to live cells. Harrison was the first who developed a culture system for living neural tube tissue for the long term examination of neuronal differentiation (Harrison et al., 1907) and he proved that Ramon Cajal theory in 1890 concerning the development of nerve fibres was correct. His finding that the growth of neurites was from cell bodies has been considered as " one of the most revolutionary results in experimental biology" (Shephard, 1994).

50 years later, the discovery of nerve growth factor (NGF) by Levi-Montalcini, Hamberger, and Cohen provided a profound advance in tissue culture (Cohen et al., 1954). NGF became the basis for the discovery of other neuronal growth and differentiation factors. Those investigators used a chick ganglion bioassay technique to detect NGF in other sources. Neurons in the chick ganglion explants extended neurites toward NGF when they perceived its presence in extracts of snake venom, submaxillary gland of male mouse, and S-180 mouse sarcoma.

Levi-Montalcini won the Nobel Prize, which was published in the journal of the Tissue Culture Association with the consideration of its editor, Gordon Sato, that the award to Levi-Montalcini and Cohen was " an affirmation of the growing importance of cell culture in biological research" (Levi-Montalcini, 1987).

1.2 Brain Spheroid in In vitro Neurotoxicology

In 1950s Garber and Moscona showed that a single cell suspension taken from the fetus at a specific time from mammalian or avian sources can reaggregate on constant gyratory agitation to form a three dimensional structure called a spheroid (Moscona, 1952, Garber and Moscona, 1972). Close observation of the spheroids demonstrated that they could reproduce the complex cellular organization and development of the tissue from which the cells had been isolated. In monolayer cell culture, the cells lost their phenotype as they remain in the culture for a long time. However, the brain spheroid restricts cell division and stimulates morphological and biochemical differentiation similar to in vivo development (Seeds et al., 1980). The dissociated cells are avid to attach with their counterparts to form neurospheres, and the cells within the neurosphere are able to migrate and to interact with the neighbouring cells by direct cell- cell junctions and through the exchange of nutritional and signaling factors. This environment enables the cells to form tissue-specific structures such as synapses and myelin . Under certain environmental conditions, the cells within the neurosphere can migrate and reorganize themselves to form aggregates which histologically resemble the tissue from which the cells had been isolated (DeLong, 1970). The maturation process of the neurosphere makes it a suitable model in evaluating the effect of some chemicals on the developmental maturation of the neurosphere and whether these chemicals can produce structural or functional defects.

1.3 Cellular development in the CNS

Stem cells are the cells which have the ability first to proliferate and make identical copies of themselves, several times for several generations, and second to differentiate into the cell lineages of the tissue they are derived from (pluripotency and multipotency in case of embryonic stem cells and neural stem cells respectively), third they can regenerate the tissue and organ they resided in (Ahmed, 2009). Stem cells are present in all tissues to some extent, under strict growth control to maintain them, to prevent over growth and tumour formation and to mobilize them during injuries in a bid to compensate and replace the cell loss (Evans and Potten, 1991).

All the cells in the central nervous system are formed from the neuroectoderm, the neural plate, which located along the dorsal midline of the developing embryo. This neuroepithelium is a single layer of pseudostratified epithelium which grows and proliferates continuously to close the developing neural groove and form the hollow neural tube. The neural tube later on gives rise to the ventricular system of the CNS, while the epithelial layer will form the neuronal cells and glial cells (Clarke, 2003). The neuroepithelial stem cells differentiate into neural cells at first, then into glial cells. Neural differentiation is an early event in mammalian embryogenesis. It appears that the neural plate formed by suppression of signals that induce non-neural differentiation, examples for these signals are bone morphogenic proteins (BMPs) and transforming growth factors-β (TGF-β) superfamily molecules (Wilson and Edlund, 2001). Several molecules that enhance neural differentiation such as noggin, follistatin, and chordin are BMP- antagonists. BMPs suppress neural fate differentiation of neuroepithelium

and there are two receptors for BMPs: BMPR-1A and BMPR-1B. The expression of these two receptors is inhibited by sonic hedgehog (Panchision et al., 2001)

The differentiation of neuroepithelium proceeds in a temporal way, which is specific for each region of the developing embryo (Rao, 1999). This patterning of the neural tube is thought to start from the stage of neural plate development by inductive cellular interactions which form organizing centres in both poles (Altmann and Brivanlou, 2001), the ventral and the dorsal one. These specialized cells release signals, sonic hedgehog (Shh) and (BMPs) in variable concentrations, to form a gradient of signal concentration. This induces the expression of patterning genes in the adjacent cells of neuroepithelium in a concentration dependant manner. These genes encode homeodomain transcription factors, and their expression divides the cells into different domains (Kobayashi et al., 2002). These patterning genes may specify the identity of the neurons and might control the duration of neurogenesis during each developmental process.

The neural tube in the human is formed during the third and fourth weeks of gestation. Initially, the neuroepithelium consists of a single layer of neural stem cells. These cells proliferate symmetrically and asymmetrically to enrich the pool of neural stem cells and to provide more progenitor cells from which more mature neuronal and glial cells develop. The signals which determine the symmetry and asymmetry are still unknown (Jan and Jan, 1998), presumably, BMPs are involved. Retroviral labelling studies have shown that 48% of the dividing cells in Ventricular Zone (VZ) remain in colonies suggesting self- renewal in this area (Cai et al., 1997). The active division of these progenitor cells is controlled by apoptosis to maintain a constant population but the mechanisms are not clarified yet (Raoul et al., 2000).

The neural stem cells in the neuroepithelium locate at the luminal surface of the neural tube called the Ventricular Zone (VZ). These stem cells start to proliferate generating the neuroblasts which accumulate in this zone and activate a series of genes which are involved in neuronal differentiation. These genes also activate the expression of specific cascades of factors which control neuronal determination and differentiation in different areas in the neocortex. The newly matured neuroblasts migrate beyond the VZ of the neuroepithelium with the aid of the radial glial cells which provide the essential substrate for neuronal migration (Clarke, 2003). The radial glial cells extend from the ventricular surface to the outer pial surface of the neural tube, guiding neuronal migration away from the VZ and forming a second zone called the SubVentricular Zone (SVZ) as shown in the figure below.

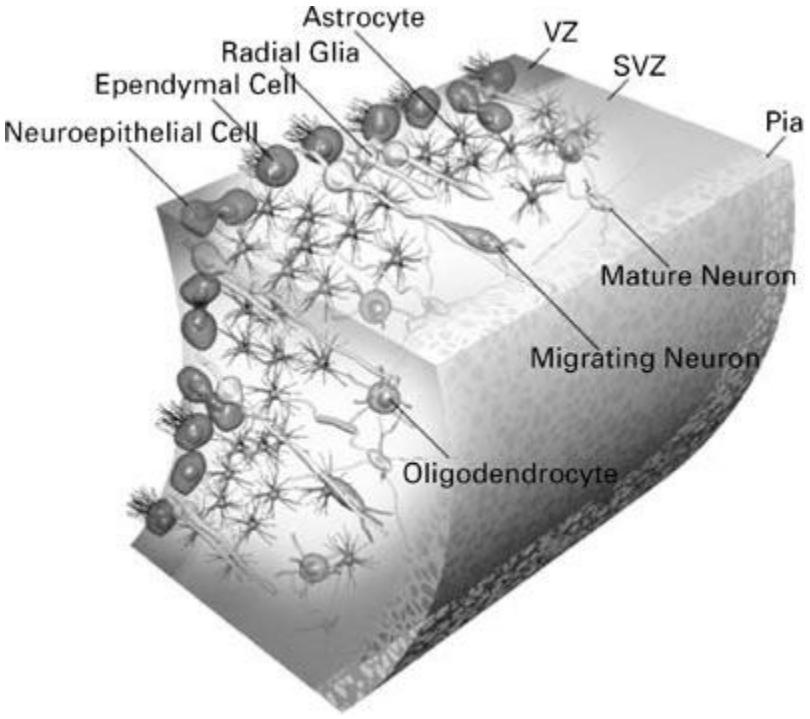


Figure 1: The neuroepithelium in the neocortex of the brain (Clarke, 2003)

When the formation of neuroblast has stopped, the remaining cells in the neuroepithelium will differentiate into glioblasts. Clonal studies pointed out that most of the glial cells originate from the stem cells of the neuroepithelium (Barres and Barde, 2000) and these cells migrate to the adjacent area, the SVZ, to reside there and proliferate and differentiate into astrocytes and oligodendrocytes. Lineage tracing studies showed that most of the progenitors in the germinal matrix are glial precursors that differentiate into either astrocytes or oligodendrocytes (Levison and Goldman, 1997). The glioblast formation ceases after birth shortly, then the VZ disappears throughout the neuroaxis and most of the remaining neuroepithelium differentiate into ependymal cells. Ependymal cells line the ventricular system of the brain and the central canal of the spinal cord throughout adulthood. These cells have multiple cilia at their apical surfaces which help the cerebrospinal fluid to pass effectively through these regions. The SVZ also decreases in size and persists adjacent to the ependymal cell layer throughout all ventricular region.

In CNS development, the temporal patterning leads to the generation of neuronal cells before oligodendrocytes. In spinal cord development, it appears that these two cells develop from the same precursor and the final step in cell differentiation relies on extrinsic signals and activation of specific transcriptional factors (Jessell, 2000). There are two main groups of transcriptional factors which determine cellular fate, which are the homeodomain factors such as NKx2.2 (Vetter, 2001) and the basic helix-loop-helix family of transcription factors such as Olig1 and Olig2 (Zhou et al., 2001). The expression of Olig transcription factors is controlled by extrinsic factors, such as Shh, and their expression leads to the formation of oligodendrocytes. With the progression of development, Olig1 and

Olig2 expression continues and starts to overlap with homeodomain transcriptional factor NKx2.2, these cells with double expression migrate from the midline and differentiate into oligodendrocytes. Olig1 and Olig2 mutant mice showed absence of oligodendrocytes, the stem cell progeny which normally differentiate into neuronal cells then into oligodendrocyte. Instead they developed into interneurons and then into astrocytes (Zhou and Anderson, 2002).

The expression of the pro-gliogenic transcription factor might be controlled through receptors on the surface of the cells such as notch. A notch ligand-jagged-1 which is signalled by neurons suppresses the oligodendrocyte phenotype (Rogister et al., 1999). When the number of neurons become enough, jagged-1 is diminished and pro-oligodendrocyte signals enhance oligodendrocyte formation and stimulate myelination. When the neural precursor has committed to the oligodendrocyte lineage, the final step to differentiate into myelin forming cells needs the presence of SOX10. Lacking this factor in the neural stem cells leads to failure in axon myelination (Stolt et al., 2002).

1.4 Neuronal Migration

Neuronal migration is an important process in the development of the nervous system that determines the final position of the neurons, and also provides the basis for the subsequent contact with other cells to complete the neural circuitry. This process is so complicated, started by instruction from extracellular compartment which cause activation of certain receptors with their downstream signalling pathways, and this enables the newborn neurons to migrate through the developing nervous system until they reach their final destination. The migratory cycle of the neuron is defined by leading process dynamics (see below), by which

process the migration is directed, and somal translocation involving the migration of the nucleus, perinuclear organelles and materials takes place (Valiente and Marín, 2010). This cycle is not applied to all the neurons, some classes of neurons may modify and change some of the basics of the cycle depending on the pathways by which the neurons migrate. For example, pyramidal cells in cerebral cortex, pass through three different phases during their migration towards the cortical plate (Kriegstein and Noctor, 2004) during which the pyramidal cells adopt different morphologies each of which require special molecules whose disruption might lead to neurological deficits (LoTurco and Bai, 2006).

1.4.1 The leading process dynamics

Several experiments have studied the behavioural dynamics of the leading process of the migrating neurons. Figure (2) shows that the leading process has a special morphology, reflecting their ability to adapt to different environment. The rear part of the nucleus and the proximal part of the leading process are enriched with actin and myosin filaments. The migration cycle starts with extension of the leading process, dilatation of the perinuclear area and forward movement of the nucleus and adjacent organelles, such as Golgi complex and the centrosomes.

This simple dynamic is applied to some cells such as pyramidal cells which migrate radially (Rakic, 1972), but in case of tangentially migrated neurons, such as the interneurons and immature enteric neurons, these have distinct leading process morphology, all of which have branched leading processes (Marín and Rubenstein, 2001). The branches continue to extend and retract until one of them has been selected. At this time, the nucleus and perinuclear organelles move forward until reaching the branching point as shown in figure (2), then the branch selected to be the leading process continues to grow and extend its branch while

the other branches start to retract, and the cycle is repeated (Martini et al., 2009). Thus, the guidance cues control the orientation and the frequency of branching to form a new leading process, to change the direction of migration without having to change the orientation of the preceding leading process. This migration guidance differs from that described for axon growth, in which the growth cone steering controls movement direction (Lin and Holt, 2007).

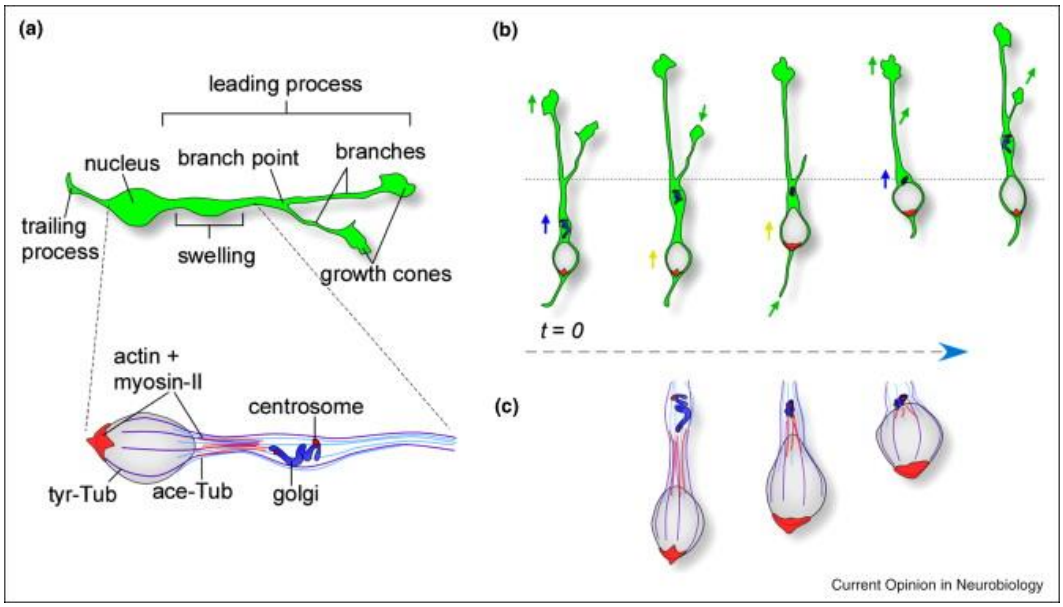


Figure 2 : The cell biology of neuronal migration (Valiente and Marín, 2010)

The branching of the leading process has been suggested to be the general strategy for neuronal migration, which may be in certain conditions suppressed, like that during the glial guided radial migration (Marín et al., 2006). The figure below (3) shows the steps of glial guided radial neuronal migration of pyramidal cells. The radial glial cells which are located at the VZ are the progenitors of cortical neurons. They send out a long branch that extends from the VZ to the pial surface over the marginal zone (MZ). The newly born neurons start to polarize and migrate close to the radial glial cells at VZ; when they actively interact with the radial glial cells (Kriegstein and Noctor, 2004), at the SubVentricular Zone

(SVZ), the migrating neurons stop their migration toward the cortical plate (CP) and begin to change their morphology to be multipolar neurons by preventing the interaction with the radial glial fibres (Elias et al., 2007). The cells at this stage form multiple processes around the cells and start to make tangential displacements. After that, the cells return back to be polarized and acquire bipolar morphology and migrate through the intermediate zone (IZ) toward the CP. One of the proteins which regulates this process is GTP binding protein-Rnd2. Knocking down this protein lead to disturbed transition of migrating neurons from multipolar to unipolar cells and the cells which manage to polarized appear to have multiple leading processes (Heng et al., 2008). The migrating neurons at this phase in the IZ are in close contact with the long process of the radial glial cell, the interaction between these cells is by connexin Cx26 and Cx43. Silencing these two proteins might result in the same abnormalities as knocking down Rnd2.

The stability of the leading process depends on a system of microtubules which are longitudinally arranged to link the leading edge of the cells with the soma. This arrangement provides structural support for the leading process and allows the flow of vesicles required for intracellular communication. This system is directly controlled by a novel microtubule associated protein (MAP) called p600 (also known as Ubr4) which interacts with the endoplasmic reticulum (ER). Knocking down this protein resulted in the absence of ER in the leading process and excessive reduction in acetylated tubulin which made the leading process to have a wavy appearance and affected migration (Shim et al., 2008) as shown in figure (3). These findings support the notion that the stability of microtubules and membrane trafficking are essential for the leading process.

The signalling mechanism that controls leading process branching still unknown, but work in cerebellar granular cells indicated that Ca^{+2} and cAMP might modulate this process. Increasing the intracellular concentration of Ca^{+2} or stimulating adenlyl cyclase increased leading process branching. On the other hand, reducing intracellular concentration of Ca^{+2} decreased the frequency of branching (Kumada et al., 2009).

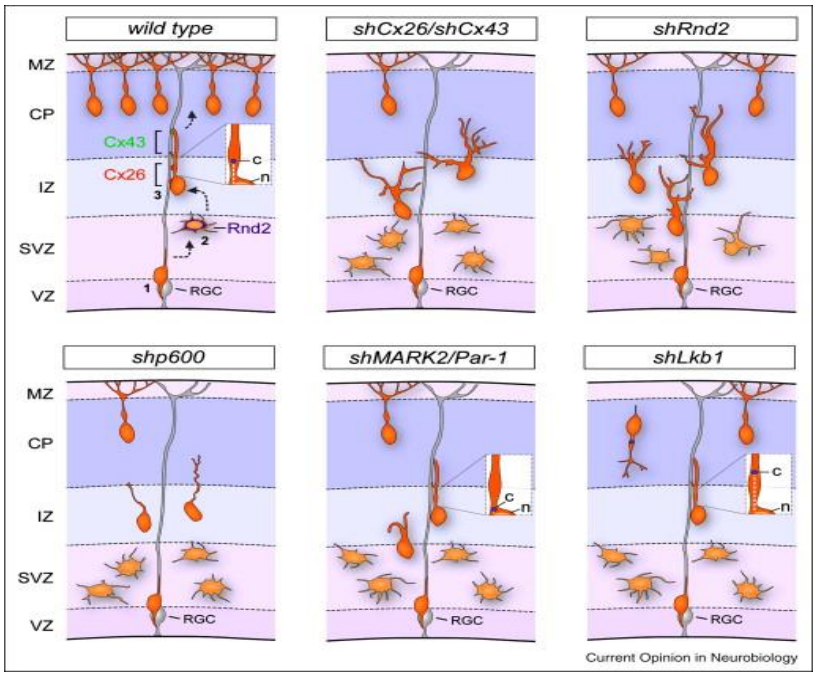


Figure 3: Glial guided radial migration. The steps in the wild type, RGC (radial glial cell) which reside in the ventricular Zone (VZ) send out a long process extending from VZ to the marginal zone (MZ). **1:** The newly born neuron becomes bipolar and interacts with RGC. **2:** The migrating neuron stops migration and becomes multipolar. **3:** The neuron returns back to bipolar type and interact with the RGC by Connexin 43and 26 (Cx43, 26). The other figures show the abnormalities in migrating cells when there is a deficiency in one factor which control cell migration.(Valiente and Marín, 2010).

1.4.2 Nucleokinesis

While the leading process determines the direction of migration, effective neuronal migration is performed only when this is followed by translocation of

cell soma, the small organelles and the nucleus. Translocation of soma occurs in two phases, one following the other. In the first step, there is a swelling or dilatation of the cytoplasm in the proximal part of the leading process, towards which the centrosomes and the Golgi complex move. This is followed by forward movement of the nucleus to invade the cytoplasmic swelling (Solecki et al., 2004), as shown in figure (2). The movement of the nucleus and the centrosomes is highly controlled and relies mainly on a rich microtubule array that extends between them. The microtubules surrounding the nucleus are tyrosinated, which mean that they are dynamic while the microtubules which located in the anterior pole of the nucleus, near the centrosomes, are acetylated which mean that they are more stable (Solecki et al., 2004).

It has been suggested that the microtubules surrounding the nucleus are anchored to the centrosome, which is the microtubule-organizing centre of the cell. Therefore, it was thought that the pulling force of the microtubules on the nucleus converge at the centrosome (Higginbotham and Gleeson, 2007). However; another study pointed out that the microtubules are not attached to the nucleus but instead they are extending distally toward the leading process, and this fact was confirmed by the finding that the nucleus moves forward passing the centrosomes (Umeshima et al., 2007). Therefore; the exact mechanism which explains nuclear movement is not fully revealed.

Microtubules Associated Proteins (MAPs) and other related proteins are crucial regulators for the movement of the organelles. Regardless of the exact function of MAPs and the other proteins in soma translocation, they are unlikely to be the only force which propels the nucleus and the organelles forward. It has been shown that there are Myosin II filaments behind the nucleus of the migrated

neurons, and the pharmacological blocking of its ATPase activity inhibits nuclear movement, suggesting that the contraction of actomyosin filaments might move the nucleus forward (Schaar and McConnell, 2005).

1.4.3 Cell adhesions in neuronal migration

Neural migration in the developing nervous system requires dynamic regulation interaction is integrin-mediated adhesion in many cell types, but the exact mechanism of this system in neuronal migration is unclear (Lauffenburger and Horwitz, 1996). Cell adhesion dynamics in the CNS has been well studied in the glial guided neuronal migration. The locomotion occurs in several steps, in which, there is a breakage and formation of new cell adhesions between the migrating neurons and the radial glia. Because of this unique property, the integrins, a family of cell adhesion receptors mediating cell- cell and cell- ECM interactions, have been speculated to have a role in this process (Huang, 2009). Integrin heterodimer α3β1 are expressed in migrating neurons and α3 integrin gene knocking down resulted in abnormal cytoskeleton dynamics and slow radial migration (Schmid et al., 2004).

Other studies showed that gap junction proteins Connexin 26 and 43 are found concentrated at the points of contact between the migrating neurons and the radial glia and their downregulation can result in abnormal radial neural migration (Elias et al., 2007). These junctions are well known in their capability in electrical and chemical cellular coupling, or releasing of some materials from the cells to the extracellular space. The dynamic adhesions between the migrating neurons and the radial glia, which is provided by the gap junctions, help in stabilizing the leading process of the migrating neurons along the radial glia (Elias et al., 2007). Limited information is available about the mechanism of controlling the dynamics

of gap adhesion, but it was thought that the C-terminal tail of connexin is essential for this process. It has been shown that this domain interacts with several types of cytoskeleton proteins and it has been thought that it can regulate mobility in several cell types (Cina et al., 2009).

1.5 Dendrite Growth

The nervous system is composed of a huge number of neurons with different characteristic afferent and efferent projections and dendritic morphologies. In general, the neurons in various parts of the nervous system show different patterns of dendritic arbors with highly specialized membrane properties. As the dendrites represent the site of synaptic contact, dendritic development determines the number and pattern of synapses in each neuron. Consequently, any defect in dendrite growth might result in neurodevelopmental disorders.

In the first stage of nervous system development, the neurons are formed and migrate to their final destination. The neurons then elaborate their axons and dendrites in a pattern which is cell specific. Finally, the neurons make special contacts with other cells - the synapses. In many cases, early synaptic connections are remodelled by neuronal activity to result in the mature pattern of neuronal connectivity (Katz 1996).

More than a century ago, Ramon y Cajal proposed that the dendrites (he described them as protoplasmic processes) are special morphological structure which receive neuronal input (Cajal, 1995). Before elaboration of dendrites, the neurons undergo polarization in which axon and dendrites are specified with their distinct functions and morphologies. In most neurons, the development of the axon occurs before the development and arborisation of dendrites (Cajal, 1995), although

some neuronal types adopt their specific programs of dendritic development at an earlier stage. In general, dendrite morphogenesis, can be defined in four steps (Puram and Bonni, 2013), as shown in the figure (4):

First, the dendrites extend from the cell body into their target by using guidance cues to steer toward them. During that time, the dendrites grow to attain length and width. Whereas the dendrites and axons extend with actin based structure, dendritic shafts are rich in microtubules and have dendrite specific microtubule associated proteins (MAP), which play an important role in regulating dendritic size and stability (Harada et al., 2002). Second, as the dendrites grow and extend further, branching is essential to cover the target field. Dendrites can ramify several times, with extensive secondary and tertiary branches. Primarily, dendrite branching occurs through interstitial branching in which the branches appear from the side of a pre-existing dendritic shaft. Branches appear at first as filopodia then become cone like structures and extend to become stable branches (Dailey and Smith, 1996). Third, as the dendritic arbor reaches the defined area, dendritic growth would be restrained, giving rise to the mature shape of the dendritic tree (Gao et al., 1999). Fourth, dendrites differentiate into a special structure to house the synapses, as in formation of dendritic spines in hippocampal pyramidal neurons or formation of dendritic claws in cerebellar granular neurons (Puram and Bonni, 2013). Finally, pruning, is an important step in establishing the mature dendritic tree, refers to modification of arbors through retraction and elimination of unwanted dendrites. For example, in rodent cerebellum, the exuberant dendrites are pruned to establish their mature shape and undergo postsynaptic differentiation (Cajal, 1995). The process of pruning exempts the properly innervated dendrites, leaving them for full maturation (Ramos et al., 2007).

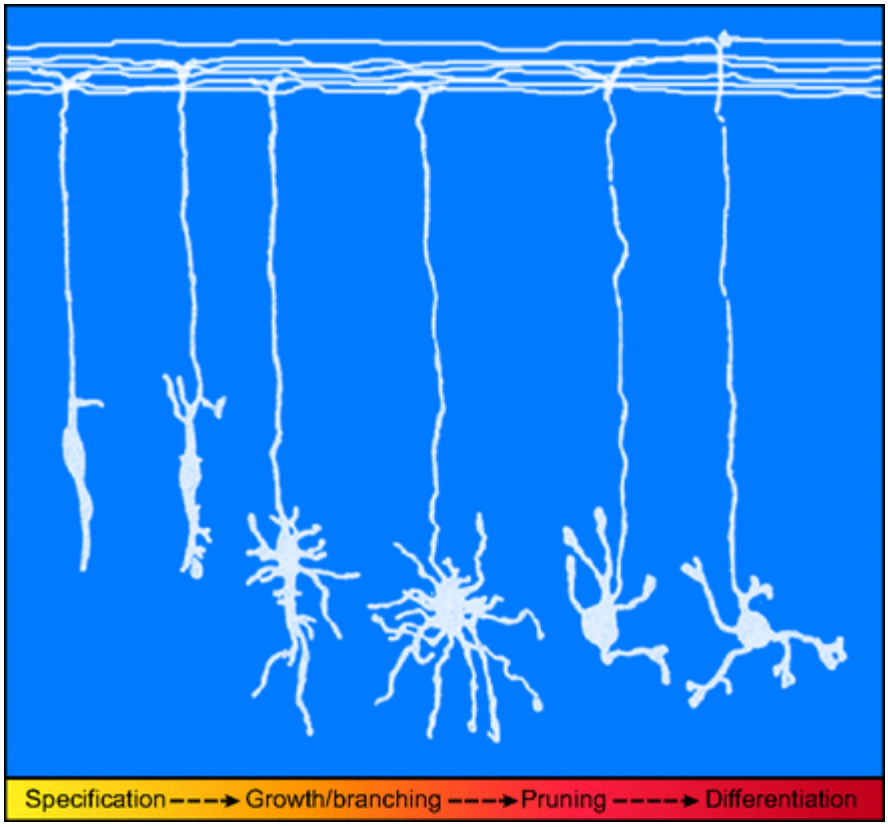


Figure 4: The steps of dendritic growth and arborization (Puram and Bonni, 2013)

Once the newly formed neuron is polarized, early dendrites sprout and start to branch to form the dendritic tree. At the same time, filopodia formed from the new dendritic shaft, some of these filopodia will be stabilized and form the dendritic spines, depending on whether or not they participate in synapse formation (Togashi et al., 2002). The shape and size of the dendritic tree reflects the function of that neuron (Jan YN, 2001). For example, when hamlet, a transcription factor in Drosophilia, is expressed in a multidendritic neuron, it will change it into a simple dendritic neuron. This shows that the intrinsic program of the neurons is controlled genetically. In general, the extent of dendritic arborisation is regulated by the intrinsic program and the extrinsic environmental factors:

1.5.1 Extrinsic cues regulate dendrite formation

Developing neurons are surrounded by a complex environment containing other neurons, glia. Afferent input of axons from other neurons, which arrive before or at the start of dendritic growth have a potential effect on dendritic development, since early exposure of the dendrites to neurotransmitters and action potential regulate the growth of the dendritic tree (Wong and Ghosh, 2002). The neurons also encounter several growth factors such as neurotrophins during development which strongly stimulate dendritic growth.

1.5.1.1 Neurotrophins

Neurotrophins are one of the molecular signals which control the growth of dendrites. These include four groups of growth factors which are: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (Arevalo and Chao, 2005). These factors play an important role in nervous system development. They interact with two types of receptors, Trk tyrosine kinase receptors (TrkA, TrkB and TrkC) and p75 neurotrophin receptors ($p75^{NTR}$). These two receptors signal independently or together to regulate neuronal survival, cell differentiation and synaptic plasticity. The Trk receptor can interact with ion channels and p75 can associate with membrane–associated Nogo receptors, which are involved in axonal regeneration (Chao, 2003). The neurotrophins and their receptors are highly expressed in the developing nervous system when there is active neuronal growth and cell differentiation. Also, these factors demonstrated a dramatic effect on axonal growth of the peripheral and central nervous system (Snider, 1994). Adding neurotrophins to slices of cortex caused an increase in the length and complexity of dendrites of cortical pyramidal neurons (McAllister et al., 1995). In addition to

neurotrophins, there are many other factors which are involve in dendrite growth and maturation, such as Semaphorins (Sema3A), Cpg15 gene expression, Epherin, Osteogenic proteins, Notch protein, and cell adhesion molecules (McAllister et al., 1995).

A general hypothesis stated that the growth of the axon is supported by a group of trophic factors and matrix molecules while dendrite growth is supported by another set of signals (Goldberg, 2004), as shown in the figure (5) . For example, sympathetic and cortical neurons show dendritic growth in response to bone morphogenetic proteins (Withers et al., 2000). Similarly, other adhesion and matrix molecules may stimulate axon growth but not dendrites, for example, chick retinal ganglion cells (RGCs) cultured on top of glial endfeet showed growth of axons, while RGCs cultured on top of glial somata showed growth of dendrites only (Bauch et al., 1998).

1.5.2 Intrinsic factors regulating dendrite formation

Different neuronal types encounter the same environment during development. However, an intrinsic program within the neurons regulates their interpretation of the external cues and forms distinct pattern of dendritic trees. For example, neurotrophin-4 induces dendrite growth and increases its complexity in cortical pyramidal neurons layer V, but has no effect on layer IV neurons. Brain derived neurotrophic factor (BDNF) highly stimulates dendrite arborisation in layer IV and moderately affects the neurons in layer V (McAllister et al., 1995). Differential expression of neurotrophin receptors may explain the different response of each cortical layer to different neurotrophins, explaining that cell-intrinsic mechanisms control the cell response to the external cues (Puram and Bonni, 2013).

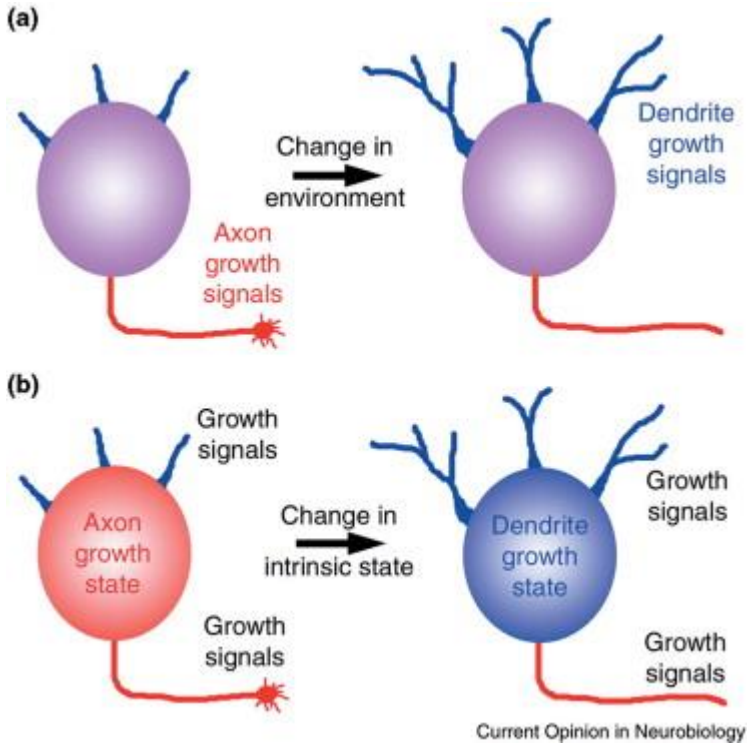


Figure 5: Extrinsic factors control hypothesis (a) and intrinsic factors control hypothesis (b) on axonal and dendritic growth (Goldberg, 2004)

Intrinsic refers to a phenotype expressed independently from the environment. Many intrinsic phenotypes are programmed at the progenitor stage and maintained throughout part or all the life of the neurons. The expression of the complement of receptors of survival, growth and initial axonal guidance may be intrinsic to the neurons. For example, BDNF is able to enhance axonal growth from retinal ganglion cells (RGCs). If BDNF is removed for few days and added again, RGCs are still able to respond to this growth factor and axonal growth is stimulated. This demonstrates that the responsiveness to BDNF is intrinsic to the cells (Goldberg et al., 2002). The differences in the intrinsic phenotypes of the adjacent neurons make them respond differently when they are exposed to the same environment, and this lead to appearance of variable neuronal patterns.

Thus, the same growth factor has a different axon or dendritic effect on different neurons, this explain the diversity in their final phenotype (Goldberg, 2004).

Regarding the intrinsic control of axon and dendrite growth, in the CNS, most of projection neurons extend axons at first, which are then followed by dendrite arbor growth. This suggests that axon and dendritic growth in vivo are separated by time. Another fact about the intrinsic control has come from in vivo studies showing that embryonic spinal projection neurons are able to regenerate their axons after injury, whereas this ability is lost completely after birth (Saunders et al., 1992). Other study using explant slice cultures showed that young explants illustrated better axon regeneration than the older explants (Chen et al., 1995). Therefore, it appears that there is a developmental modification in neuronal ability to regenerate their axon, but these studies are confounded by the simultaneous changes which occur in the extrinsic glial environment during development which inhibit axon growth and regeneration (Schwab and Bartholdi, 1996). However, the lack of successful regeneration of axons in studies designed to overcome the CNS glial cues confirm the intrinsic regulation for axon growth, For example, axon regeneration in adult RGCs take 2 months through a peripheral neuronal graft (Bray et al., 1987). These experiments also were confounded by the finding that reactive astrocytosis, may contribute to developmental changes through residual axon growth inhibitors. Therefore, it is difficult to explain developmental modification in axonal growth whether due to intrinsic or due to environmental changes.

1.6 The Time of Brain Development

Brain development in humans occurs from sixth week of gestation to several years after birth as shown in the figure (6). In the CNS, the organs build by cell

proliferation, migration and differentiation. The neurogenesis of specific neuronal populations has been shown in rodent brain and the results were extrapolated to human brain (Bayer et al., 1992). This study showed that different brain areas develop at different times, and even in a single brain area, groups of neurons develop at different times and at a variable rate. For example, cerebellar Purkinje cells develop during embryonic days 13-15 of gestation in rat which corresponds to 5-7 week of gestation in humans, on the other hand, granular cells are formed later, about 4-19 postnatal days, which correspond to 24-40 week of gestation in human (Bayer et al., 1992).

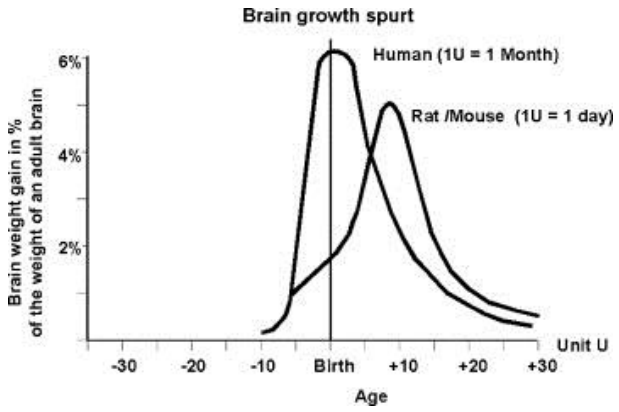


Figure 6: Illustrate the brain growth spurt in humans and rats, the units of time for humans are months and for rats days (Ikonomidou and Turski, 2010)

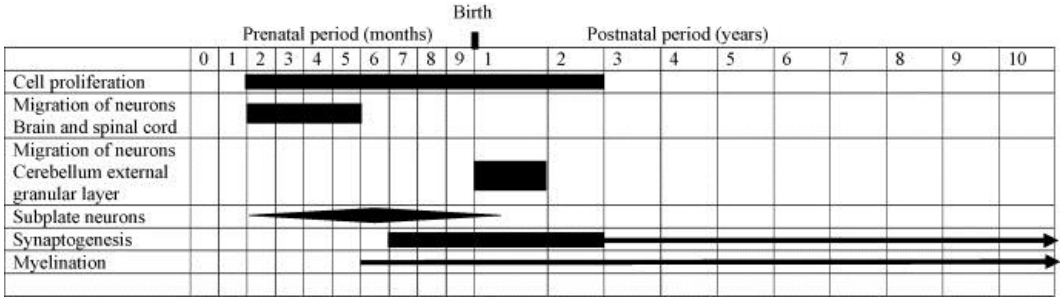


Figure 7: Illustrate brain development adopted from (Rice and Barone Jr, 2000)

Chapter 2

Micromass System

2.1 Teratogenesis and Malformation

A congenital anomaly is an anatomical, metabolic or functional defect in the newborn due to several factors, such as maternal exposure to infectious agents, drugs or due to genetic or environmental factors. It is the cause of high mortality and long lasting disability for surviving children and has a significant impact on families, social communities and health care. 5% of all the newborns have a birth defect, 3.2 million birth defects are registered annually, 270,000 newborns died by 28 days of age in 193 countries in 2010 due to congenital anomalies (WHO, 2012). 90% of these birth defects are of unknown cause and the confirmed causes account for only 10 %. Drugs as a cause for these congenital malformations represent only 1% or less of all congenital defects (De Santis M., 2001).

Teratology is the science that deals with developmental abnormalities which result from maternal exposure to physical, chemical or environmental factors during pregnancy which affect normal development, differentiation and/or behaviour. A teratogen is that factor which causes an irreversible effect on the development of the embryo anatomically and/or functionally. Therefore, a teratogen may be a xenobiotic which includes a wide range of chemicals that are used during pregnancy or to which a pregnant women is exposed such as pharmacological materials, cosmetics, food additives, heavy metals, cigarette smoking. Also, it can be other factors, for instance, stress, nutritional deficiency or infection with some viruses (cytomegalovirus and rubella) which can cause developmental abnormalities to the foetus (Stegmann and Carey, 2002).

This science was brought to light in the 1920s when piglets were born from mothers which ate a diet deficient in vitamin A; those piglets suffered from several congenital anomalies including absence of eyes (Hale, 1933). In the

human, the first confirmed case reported to have congenital malformation was due to pelvic X irradiation of the pregnant mother (Goldstein L, 1921). After several decades, Gregg (1991) was the first to report a case of congenital cataract due to infection of the mother with Rubella virus in Austria, but the evidence which proved that chemicals can cause foetal defects occurred after administration of high doses of a folic acid antagonist (aminopterin) which was used to induce abortion; the aborted foetuses and the living children showed multiple malformations (Tiersch, 1952). About 10 years later, the thalidomide disaster happened, in which thalidomide was used as an antiemetic drug during pregnancy from 1957-1961, more than 10000 children from mothers who had taken the drug exhibited variable anomalies ranging from Amelia (absence of the limb), phocomelia (shortening of the limb), absence of the ear and deafness and others. These birth defects happened especially when the drug was taken during day 35-50 of gestation (Branch, 2004). Thalidomide had minimal effect on the adults but it was very toxic to the embryos (McBride, 1961). From that time, the regulatory agencies especially the Food and Drug Administration (FDA) in the USA instigated the requirement to study the drugs thoroughly on animals before marketing them and they issued guide lines for perfect evaluation of drugs for human use (Goldenthal, 1966), identification of teratogenic agents is crucial in preventing birth defects, since 10% of all the congenital malformations are due to teratogens (Brent, 1995) which affect the quality of life for millions of individuals in the world and also can cost billions of dollars in health care every year. Many studies were undertaken to identify the teratogencity and embryotoxicity of these compounds.

Wilson (1959) summarized 6 principles for teratology and these are as follows:

1- The susceptibility of the conceptus to the teratogen depends on the genotype and how it interacts with the environment:

The differences in the genetic composition, environmental factors, metabolic pathways and the placental features explain inter and intra-species variability in response to teratogens (Schardein, 1993).

2- The susceptibility of the conceptus depends on the gestational age at which it is exposed to the teratogens

This principle brings to light the concept of the critical period of organogenesis which is day 22-55 of human gestation, and is the most sensitive period to drugs. Before that time the embryo either reacts with the drug and grows normally, or dies, so it follows the role either all or none. When the exposure occurred in the early part of the critical period, this leads to damage to the CNS, and when there is late exposure, this leads to urogenital malformation and growth retardation. Lastly, after organogenesis the foetus will be less vulnerable to teratogenesis, rather it causes a functional deficit (Bailey et al., 2005), figure (1).

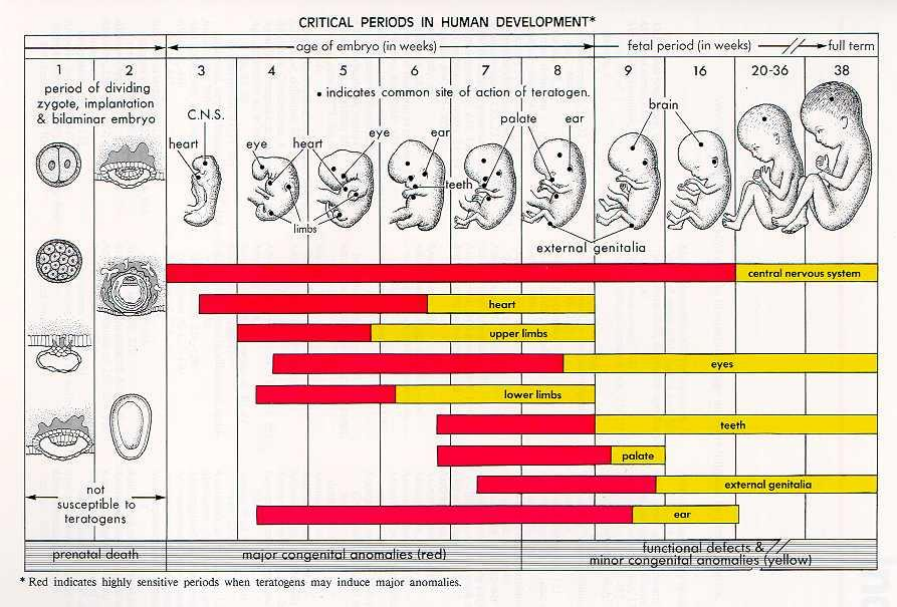


Figure 8: Demonstrates the critical periods in human development (Keith L. Moore, 2016)

3- Teratogens act in different ways to produce developmental abnormalities, such as modification in the cell matrix, cell membrane, changes in the transport processes and cell migration, alteration in RNA and protein synthesis or interference with energy resources. Also each teratogen can act in different ways and it is not necessary that a specific cellular change is related to specific teratogen (Bailey et al., 2005)

4- The final manifestations of a developmental abnormality are malformation, growth retardation, fetal death or functional impairment

5- Several factors affect the ability of any agent to influence the development of the conceptus which are the nature of the agent itself, the dose and the duration of exposure, the rate of placental transfer, and maternal metabolism of that agent in a bid to eliminate it (Polifka and Friedman, 1999).

6- The final manifestation of developmental abnormality depends on the dose of the teratogen. The teratogenicity appears when the dose exceeds the lower limit -

the threshold (Brent, 1995). Each teratogen has its own threshold; if the conceptus is exposed to a dose lower than that of threshold there will be no effect but once it is exposed to a higher dose, this will affect its development in a dose dependent way

Also Branch (2004) realized that when a teratogen is described, three principles of it must be considered:

1- The teratogen may be organ specific.
2- The teratogen could be species specific.
3- It can be dose specific.

FDA categorized drugs according to their pregnancy risk in to five categories (Briggs et al., 2012):

Category A: Controlled studies in women showed no evidence of risk to the fetus (in the first and other trimester)

Category B: Animal studies showed no foetal risk but there are no controlled studies on pregnant women.

Category C: The drug has foetal risk, but there are no controlled studies in the human, the drug can be administered when a benefit outweighs the risk.

Category D: The studies confirmed that the drug showed a positive fetal risk, the uses of these drugs is restricted to life threatening condition when safe drugs are not effective.

Category X: The drug is contraindicated in pregnant women, the risk outweighs the benefit, the risk has been demonstrated in both animal and human.

2.2 Mechanism of teratogenesis

Some of the mechanisms which are involved in teratogenesis are folate antagonism, neural crest disruption, endocrine disruption, oxidative stress, specific receptor or enzyme mediated teratogenesis (Van Gelder et al., 2010). In general, the teratogenicity and embryotoxicity of any drug is often not due to the parent compound (which is then called the proteratogen), but due to toxicity of the intermediate metabolites that are produced through bioactivation by cytochrome P450, prostaglandin H synthase and lipoxygenase. The metabolites are usually into electrophilic materials and/ or free radical reactive intermediates that oxidize or covalently bind to the macromolecules of the cells (protein, lipid and DNA) which may lead to cell death (Juchau et al., 1992).

The teratogenic effect of any drug depends on the balance between the teratogenic activity of the intermediates and the protective mechanisms, such as maternal proteratogen elimination, embryonic detoxification, cytoprotection against oxygen reactive species and repairing of the damaged cells (Wells et al., 1997).

2.3 Teratogenic testing:

Before the thalidomide tragedy, chemicals were tested by what is called the Litter test, in which the animals, usually male and female rodents, were exposed to the chemicals during the reproductive cycle and monitored for two successive pregnancies. Foetal survival was the only parameter for toxicity (Ujházy et al., 2005). The thalidomide disaster in the early 1960s instigated governments and scientific communities to test chemicals more thoroughly. Since that time, drug teratogenicity testing has been a subject of great interest, to discover harmful drugs and to save the developing embryos. The Food and Drug Administration (FDA) established guidelines which made the tests more comprehensive than

before. Since then, in 1994, new testing protocols were issued with acceptance by the International Conference of Harmonization (ICH) (Branch, 2004). Given the complexity of the reproductive system and the large number of tissue targets where birth defects or postnatal effects could be induced, there was reason for the standardization of the tests and there followed international acceptance (Tandon and Jyoti, 2012).

Embryo foetal development is a very sophisticated process; it involves many events such as gene expression, cellular proliferation, interaction, migration, differentiation, organ formation and the achievement of the physiological functions of the embryo. The ideal test is one which can provide full information about the whole process of embryonic development.

In general, there are two types of teratogenesis testing:

1- In vivo tests

The main advantage of using animals for product safety tests is that it is an inclusive model of all the factors that are involved in human exposure. Dosing can be by the oral or parenteral route, and the chemical is distributed and submitted to physiological and biochemical mechanisms that determine the concentration of the chemical or its active metabolite at the affected organs. Orally administered chemicals are first distributed in the liver to be modified into a water soluble compound so that the chemical can be eliminated from the body with the waste products. Sometimes, the liver metabolizes chemicals into long lasting active metabolites, which can be toxic in their own right, such as cyclophosphamide to acrolein and phosphoramide mustard (Garattini, 1985a), or they can be converted to toxic reactive metabolites in a remote organs such as the

nephrotoxicity of S-cysteine conjugates of haloalkenes (Chen et al., 1990). These interactions are not available in the in vitro models.

Using animals for product safety testing does not necessarily predict human toxicity. An example for that is the anti-viral drug fialuridine which caused hepatic failure in the human, but this was undetected in preclinical animal studies (Colacino, 1996). Another example is the anti- allergy drug 6,8- diethyl-5-hydroxy-4-oxo-4H-1- benzobyran-2-carboxylic acid (DHBC), which induced mild hepatotoxicity in the human, which was not predicted by laboratory animal tests (Clarke et al., 1985). These interspecies differences are due to differences in pharmacokinetics, drug metabolism, and organ perfusion rates (Garattini, 1985b). Therefore, it is impossible to rely completely on animal data until humans are exposed, and it is necessary to attempt to evaluate the human risk of these chemicals by any of those scientifically valid models.

In vivo tests can give a wide range of information about development, while in vitro tests reflect only one aspect of the process. Therefore, a combination of more than one test can provide a good view (Piersma, 2006). In vivo teratogenic testing is impractical for the huge number of chemicals that are produced annually because it could be highly expensive, time consuming, require large amount of chemicals to be used in the experiments and needs a large number of animals to be sacrificed. The advantages of In vitro methods are their being cheaper, quicker and they can serve as a pre-screen test for all the compounds to rank which need further studies by in vivo methods.

2. Alternative methods:

The drive to develop alternatives to animal studies in developmental toxicity has been increased in the last decades. The increased interest in this field stemmed from Russell (1959) who proposed the principle of 3 R,s (Reduction, Refinement, Replacement). The European Union white paper suggested the harmonization of testing requirements for chemicals marketed before September 2001 (Registration, Evaluation, Authorization of Chemicals- REACH) which involves about 30,000 chemical (Hartung et al., 2003). If these substances were tested by animal based methods, this would need 12.8 million animals and cost about 11 billion dollars for a period extending to up to 45 years (Hartung et al., 2003). The estimated number of chemicals that humans are exposed to exceeds 50,000 and there are more than 400 new chemicals introduced in to the market annually (Bournias-Vardiabasis, 1994). The alternative test methods in developmental toxicity include cell culture, organ culture and whole embryo culture. Cell culture can be the simplest and easiest and sometimes does not require animals. These tests reflect information about only one mechanism and can detect the effect of a drug on only one aspect of development, such as cell proliferation (Pratt and Willis, 1985) or cell adhesion (Braun et al., 1979). Flint and Orton (1984) used a high density of embryonic limb bud cells and brain cells to see the effects of xenobiotics on the differentiation of these cultured cells. However, this method is unable to provide enough information about the whole integrated system and its regulatory mechanisms.

While organ culture can present some parts of organogenesis in vitro, this method requires animal materials which make it laborious. The animal organs may involve lung, intestine, reproductive organs, limb bud etc (Faustman, 1988). In

the case of whole embryo culture, this provides an example of a complete in vitro alternative test. It presents embryogenesis in its full complexity from cell division and proliferation to pattern formation (Piersma, 2006).

Wilson (1978) summarized the main criteria for the ideal in vitro test:

1- Simple, easy and give interpretable results.
2- Rapid, use large number of samples.
3- Yield less false positive results.
4- Relevant to teratogenic mechanism.
5- Have some aspects of progressive development.
6- Can use different kinds of agents.
7- The used organism should be capable to absorb, circulate and excrete chemicals.

More than 30 in vitro tests using vertebrates and invertebrates have been proposed for assessing embryotoxicity (Tandon and Jyoti, 2012). Three of them have been validated, which are limb bud micromass-MM (Flint and Orton, 1984), rat post-implantation whole embryo culture- WEC (New et al., 1976), and the embryonic stem cells EST (Spielmann, 1997).

2.4 Chick Micromass System

Micromass culture is a good example of using cell culture. This test was devised by Umansky (1966) when he used undifferentiated mesenchymal cells of the chick embryo limb and cultured them in small volume in high concentration, when they formed numerous small foci of differentiated chondrocytes in a background of undifferentiated cells. The Micromass system involves some of the main mechanisms in development which are cell division, movement, cell-cell

communication, cell-matrix interaction and differentiation (Flint, 1983, Umansky, 1966). Therefore it has been proposed as a good screening test for detecting toxicity (Spielmann et al., 2001). This system is based on culturing cells from mesencephalon or limb of different species such as the rat (Flint and Orton, 1984), mouse (Tsuchiya et al., 1991) and chick (Wiger et al., 1988). In the micromass system, Formation of chondrocytes and neural cells is used as an end point, therefore, the ability of chemicals to disrupt the normal differentiation of primary cells can be identified with the aid of other cytotoxicity tests.

Chick can be considered as an attractive species in screening purposes because of its rapid development, and the avoidance of mother sacrifaction. Also, chick micromass system has been proven to be efficient in teratogen detection (Atterwill et al., 1991). It has been observed that there is no significant differences between rat and chick micromass systems in detecting teratogens (Brown and Methods, 1995, Brown and Wiger, 1992). For this purpose, sodium valproate and lithium carbonate have been used to detect their embryotoxicity on both cardiac and neural chick micromass systems.

2.4.1 Materials and Methods

2.4.1.1 The source of cells

Chick cells: Chick embryonic cardiomyocyte and neural cells were obtained from 5 day old embryos of white fertile Leghorn chicken eggs. These eggs were purchased from (Henry Stewart Company. UK)

2.4.1.2 Eggs storage and Incubation

White fertile Leghorn chicken eggs were stored in a cooled incubator at 12°C to suspend the embryonic development (SLACK, 2006). These eggs should be used

within 2 weeks from the first day being placed in the incubator. For each experiment, at least 2 dozen eggs were kept (for 5 days) in the warm incubator on an automatic egg turner with the broader ends faced up. The temperature was 37° C and the relative humidity was 100%. The day of incubation was regarded as day 0.

2.4.1.3 Cardiomyocytes and Neural cell culture

In the day of explantation, the eggs were swabbed by 70% alcohol then transferred to a class I laminar flow hood which was cleaned by Trigene 10% and sterilized by 70% ethanol. By gentle striking of eggs with the broader end of sterilized forceps the egg shell was broken and peeled off; the vitelline and chorioallantoic membranes were removed to reveal the embryo which was lifted up and placed in a 100ml Petri dish containing Hank's Balanced Salt Solution (HBSS). Under the dissecting microscope, the heart and midbrain were removed and placed in Bijou tubes containing 50% Horse Serum/HBSS. When all the eggs had been explanted and 24 hearts and midbrains were collected, the hearts and brains were washed twice with HBSS to remove the excess of horse serum. 2 ml 0.05% Trypsin and 0.02% EDTA was added to both these tubes and kept inside the incubator at 37° C, 5% CO2 and 100% humidity for 20 minutes with frequent shaking to enhance cellular disintegration, after that 8 ml of full culture medium (Dulbecco Modified Eagle Medium-DMEM, 10% Foetal Bovine Serum, Glutamine, penicillin and streptomycin) (see appendix 1) was added to stop the action of Trypsin and centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated and 1 ml medium was added to the pellet with frequent resuspension by pipette to produce a cellular suspension. A cell count was performed using a haemocytometer and the cell number was fixed on $3*10^6$/ml. 20µl cell

suspensions were placed at the bottom of each well of the 24 well plate, first left column was left without cells, with only media, then the cells were left for 2 hours to allow cellular attachment followed by adding 500µl medium. The drug was added 24 hours after cell seeding in a concentration double the required dose in a further 500µl medium. The treatment was for 6 days. The media changed every 3 days.

2.4.1.4 Cardiomyocytes Contractility Scoring

In the Cardiac Micromass System, the contractile activity of the cardiomyocytes was recorded every 24, 48 and 144 hours after cell seeding by using an inverted microscope and scoring depending on the number of contracting foci in the well. If the whole well shows contracting foci a score 3 is given, and the number of contracting foci decreasing the score decrease accordingly and becomes zero when there are no contracting foci.

The score	
0	No contractile activity
1	Few contracting foci in the well
2	Numerous contracting foci in the well
3	All the well is contracting

2.4.1.5 Neuronal processes number

$1*10^5$ cells from the trypsinized midbrain were seeded on glass coverslips which were coated with Poly D-Lysine and Laminin and left for 6 days, after that, 3 photos were taken blindly (for each coverslip) for the neurospheres that formed over these coverslips which start to attach and send neurites between them, as

shown in the figure (17). Neurite number was determined by using ImageJ software.

2.4.1.6 Resazurin Assay

Resazurin, a 7 –hydroxy-3H- phenoxazin-3-one 10 oxide redox dye (Bueno et al., 2002) has been used to detect bacterial and yeast contamination in milk and to detect semen quality for 50 years (Erb and Ehlers, 1950). It is also called the Alamar Blue test. The Resazurin test is easy, simple, rapid, performed by a one-step procedure, sensitive (it can detect viability even for 80 cells), efficient and cost effective (O'Brien et al., 2000). Non fluorescent blue resazurin is reduced by the action of mitochondrial enzymes (O'Brien et al., 2000); also cytosolic, microsomal enzymes can contribute to this reaction (Gonzalez and Tarloff, 2001), into fluorescent pink material called resorufin, then the fluorescence can be detected by optical density and the amount of fluorescent produced is directly related to the number of viable cells. This test is ideal for cells in primary cell culture, since the resazurin is not toxic to the cells and the test can be repeated several times per day. In addition to that, the cells can be reserved for other tests. Further reduction of resorufin produces colourless, non-fluorescent product which can be the cause of under estimation of large numbers of viable cells

The cell viability test was performed on the 6^{th} day of the cell culture by aspiration of the media and adding pre-warmed resazurin solution (see appendix 1) to each well and placing in the incubator at 37°C, 100% humidity, 5% CO2 for one hour. Optical density was measured by using FLUOR star plate reader with an excitation filter wavelength of 530±10nm and emission filter wavelength of 590±12.5 nm, and a gain of 60. The obtained data of optical density indicates the amount of resorufin produced by the cultured cells which was plotted from the

resorufin standard curve to know the exact amount of resorufin produced. After that, 300µl of Kenacid blue fixative (see below) was added to each well overnight for the Kenacid blue assay.

2.4.1.7 Resazurin Standard Curve

Different concentration of resorufin solution: 2000ng/ml, 1000ng/ml, 500ng/ml, 250ng/ml, and 125ng/ml were prepared in HBSS. 500µl of each concentration was added to each well leaving the first column of the 24 well-plate as a blank. The optical density was measured with the excitation filter wavelength 0f 530±10nm and emission filter wavelength of 590±12.5 nm, with a gain of 60. The data were plotted on a graph and the values were linked by the best fit straight line.

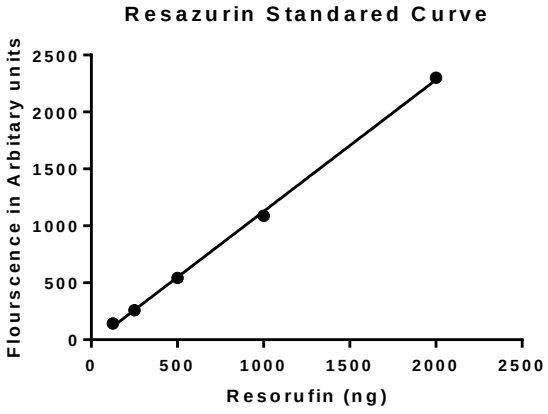


Figure 9: Resazurin assay standard curve

2.4.1.8 Kenacid Blue

Kenacid blue dye binds to cellular protein which is proportional to the total protein in the whole cell number in culture media (Clothier et al., 2006). This dye combines with $NH3^{+}$ terminal ends of the protein molecules (de St Groth et al., 1963). This assay is based on the idea that if a chemical or drug has an effect on

protein production, or membrane integrity, this will affect the rate of cell growth and cell number.

Kenacid Blue protein assay

After the resazurin assay, kenacid blue fixative (300µl) (see appendix 1) was added to each well and left to evaporate overnight, 500µl of kenacid blue working solution (see appendix 1) was added to the wells and kept on the plate shaker for 2 hours. At this time the kenacid blue dye will react with cell protein. Excess dye was removed by rinsing the well with kenacid blue washing solution (see appendix 1) then agitating the plate on the plate shaker for 20 minutes. The washing solution was replaced with 500µl kenacid blue desorb (see appendix 1), and left on the shaker for 1 hour to take the dye from the surface. The solution was transferred into a 96 well plate, and the optical density was determined by using ASYS HITEC Expert plate reader with reading filter 570nm and reference filter 405nm.

Kenacid Blue Standard Curve

In order to measure the amount of protein, a Kenacid blue standard curve was plotted by using bovine serum albumin (BSA). 2.4 mg BSA dissolved in 3 ml distilled water followed by adding 7 ml 100% ethanol to obtain 70% ethanol, 240µg/ml BSA. By adding 6ml 70% ethanol to 3 ml 240µg/ml solution, 80µg/ml BSA was obtained, then a serial dilution of this solution was done to obtain 70, 60, 50, 40, 30, 20, 10µg/ml. The 24-well plate was left in the refrigerator overnight to allow the ethanol to be evaporated, 300µl of kenacid blue fixative was added to each well overnight and the same steps were performed as described in the kenacid blue protein assay. The data were plotted on a graph and the values were linked by the best fit straight line.

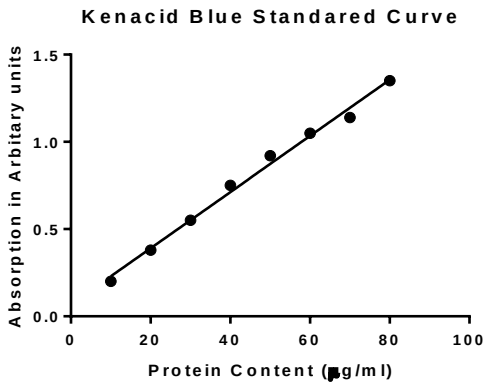


Figure 10: Kenacid blue assay standard curve

2.4.1.9 Drug preparation

Sodium Valproate and Lithium Carbonate stock solutions were prepared by dissolving them in Hank's Balanced Salt Solution (HBSS) and distilled water respectively. Different stock solutions were prepared at high concentration, the final required concentrations are: 500, 750, 1000, 1500, and 2000µM for each. The drug was added on the next day of the culture system. The first left column was without cells or drug, we only put media. The second column of the 24 well-plate was left without drug as control, as shown below. The stock solutions were kept in the fridge.

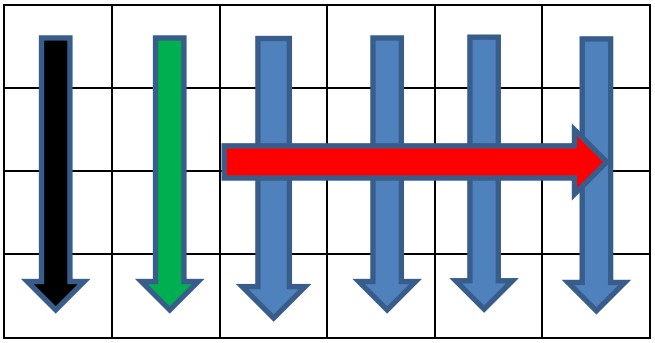


Figure 11: 24 well- plate showing the way of adding drugs, the left **black** column left as blank (only media), **Green** column as control (No drugs were added, only the solvent), the other **blue** columns are the treated groups, and the **red** arrow represent the direction of increasing the doses.

2.4.1.9 Statistical analysis

The raw data of 3 experimental repeats was analysed by using one way ANOVA test, because these parametric data belong to more than 2 groups and is normally distributed, then Dunnet's multiple comparison test to check the significance of difference. When the P value was >0.05 it was considered to be not significant. All statistical analysis was done by using Prism software version 6.

2.4.2 The results

1. Cardiomyocyte Micromass System

Different doses of Sodium Valproate (SV) were used to test the effect of these drugs on beating activity of the differentiating cardiomyocytes. It appears that with increasing the dose, ($F(5,36)=40.01$, $p<0.0001$), and with increasing the duration, ($F(2,36)=52.49$, $p<0.0001$), the inhibitory effect of this drug became more significant. Statistical analysis shows that 750µM causes significant reduction in the beating score of the cardiomyocyte after 6 days when compared with that of control, (concentration versus time: $F(10,36)=4.595$, $p<0.0003$, post-hoc: 750µM vs control: $p<0.01$). While the doses 1000 and 1500µM show their

inhibitory effect 48 hours and 6 days after drug treatment, and the highest dose, 2000µM inhibit beating activity of differentiated cardiomyocytes from the first day (24 hours), (2000µM vs control: p<0.0001) .

SV effect on cell viability appeared to be significantly affected at 1500 and 2000µM when compared with the control, (F (5,12)=91.82, p<0.0001, post-hoc: 1500µM vs control: p<0.0001), however, its significant effect on total protein amount revealed only at 2000µM, (F (5,12)=7.803, p=.0018, post-hoc: 2000µM vs control: p<0.01), figure (12).

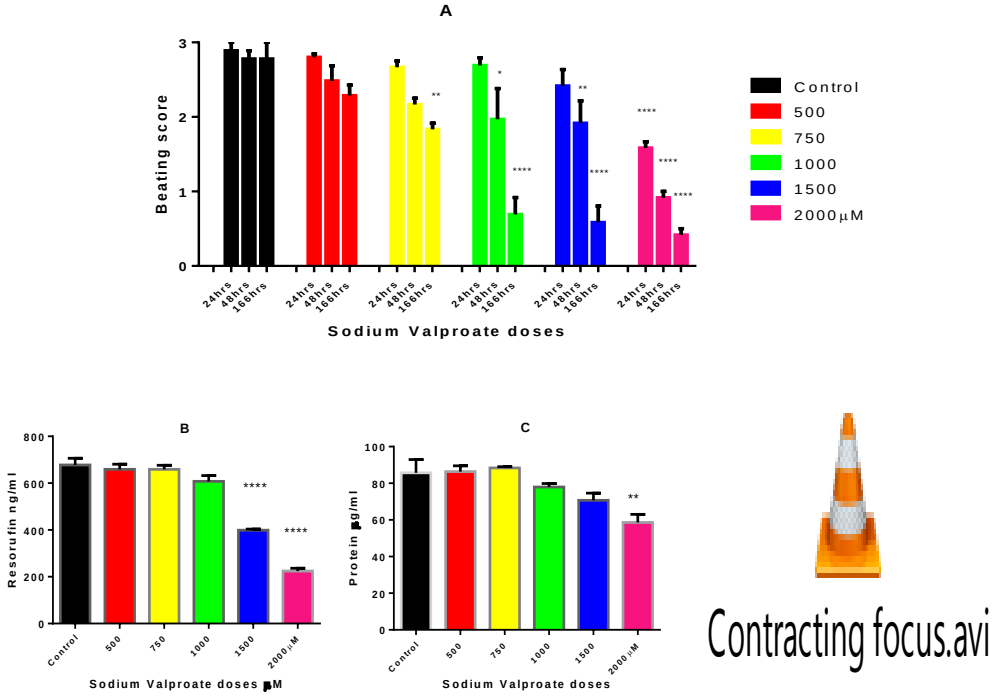


Figure 12: **Sodium valproate** effect on cardiac micromass. A- beating score 24, 48, and 144 hrs after drug treatment. B- Resazurin assay. C- Kenacid blue assay, and a video showing a contracting focus. The data here is represented by mean ± S.E, n=3 (average 4 wells from each experiment). *sign of significance when the P<0.05, ** when P<0.01, *** when P<0.001, **** when P<0.0001.The data was analysed by using two way ANOVA for beating score analysis, and One way ANOVA for resazurin and Kenacid blue assays, then Dunnett's multiple test was performed to compare the mean of control group with that of treated groups.

On the other hand, Lithium Carbonate has significant effect on the beating score of differentiating cardiomyocytes with increasing the dose, ($F(5,36)=43.72$, $p<0.0001$), and with increasing the duration, ($F(2,36)=35.96$, $p<0.0001$). Only at 1500µM, a significant reduction in the beating score appeared 48hrs after treatment, (concentration versus time: $F(10,36)=8.217$, $p<0.0001$, post-hoc: 1500µM vs control: $p<0.05$), but, at 2000µM, the inhibitory activity of Lithium appeared earlier after 24 hours of treatment, (2000µM vs control: $p<0.05$). The viability of differentiating cardiomyocytes, ($F(5,12)=4.644$, $p=0.0137$, post-hoc: 2000µM vs control: $p<0.05$), and total protein amount, ($F(5,12)=5.782$, $p=0.0061$, post-hoc: 2000µm vs control: $p<0.01$) were reduced significantly only at highest dose which seems to be significantly reduced when compared with control group, figure (13).

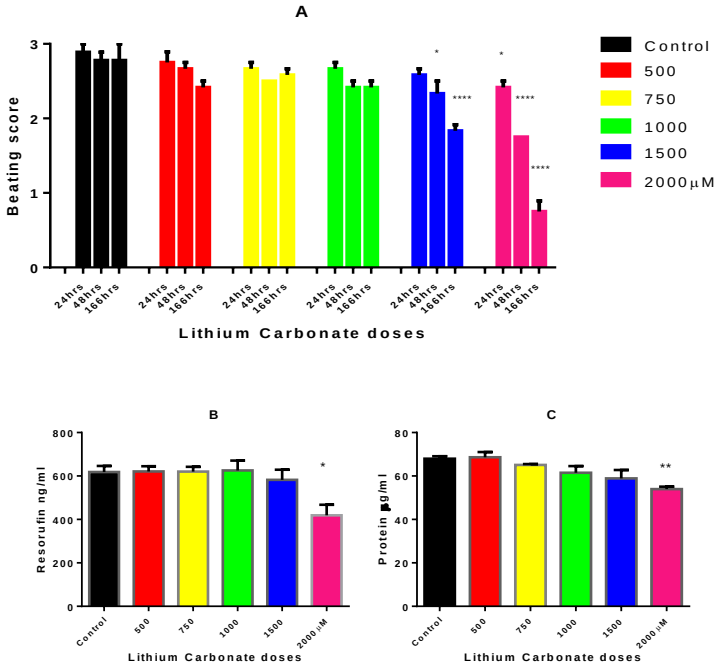


Figure 13: **Lithium carbonate** effect on cardiac micromass. A- beating score 24, 48, and 144 hrs after drug treatment. B- Resazurin assay. C- Kenacid blue assay, and a vedio showing a contracting focus. The data here is represented by mean ± S.E, n=3 (average 4 wells from each experiment). *sign of significance when the P<0.05, ** when P<0.01, *** when P<0.001, **** when P<0.0001. The data was analysed by using two way ANOVA for beating score, and one way ANOVA for resazurin and Kenacid blue assays, then Dunnett's multiple test was performed to compare the mean of control group with that of treated groups.

2. Brain Micromass System

Sodium Valproate in brain micromass culture reduce cell viability, ($F(5,12)=9.130$, $p=0.0009$, post-hoc: 2000µM vs control: $p<0.01$), and total protein amount, ($F(5,12)=8.280$, $p<0.0014$, post-hoc: 2000µm vs control: $p<0.01$), significantly only at the highest dose, while its effect on neuronal process number appeared to be at lower doses, 1000µM, ($F(5,12)=136.9$, $p<0.0001$, post-hoc: 1000µM vs control: $p<0.05$), figure (14).

Similarly, the same effect was observed after lithium treatment on cell viability, ($F(5,12)=7.092$, $p=0.0027$, post-hoc: 1500µM vs control: $p<0.05$), and total

protein amount, (F (5,12)=5.397, p=0.0079, post-hoc: 2000µM vs control: p<0.01), figure (15).

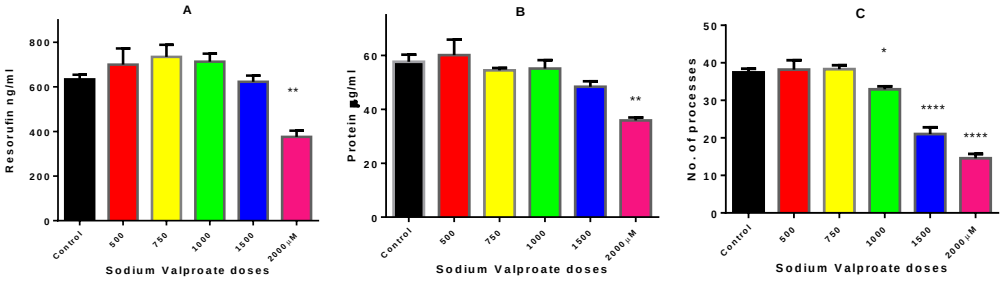


Figure 14: **Sodium valproate** effect on brain micromass. A- Resazurin assay. B- Kenacid blue assay. C- Neuronal process number. The data here is represented by mean ± S.E, n=3 (average 4 wells from each experiment). *sign of significance when the P<0.05, ** when P<0.01, *** when P<0.001, **** when P<0.0001. The data was analysed by using one way ANOVA, then, Dunnett's multiple test was performed to compare the mean of control group with that of treated groups.

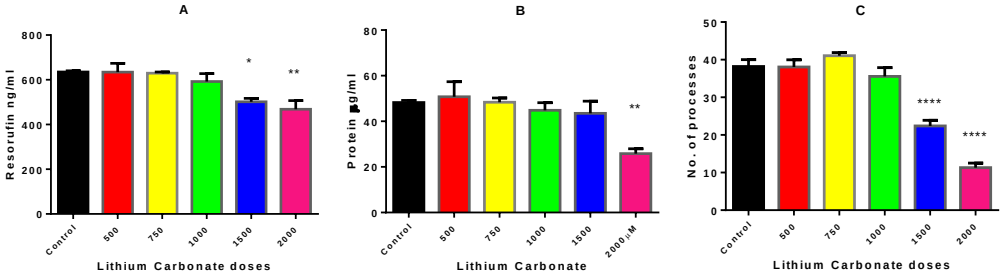


Figure 15: **Lithium carbonate** effect on brain micromass. A- Resazurin assay. B- Kenacid blue assay. C- Neuronal process number. The data here is represented by mean ± S.E, n=3 (average 4 wells from each experiment).

*sign of significance when the P<0.05, ** when P<0.01, *** when P<0.001, **** when P<0.0001. The data was analysed by using one way ANOVA, then, Dunnett's multiple test was performed to compare the mean of control group with that of treated groups.

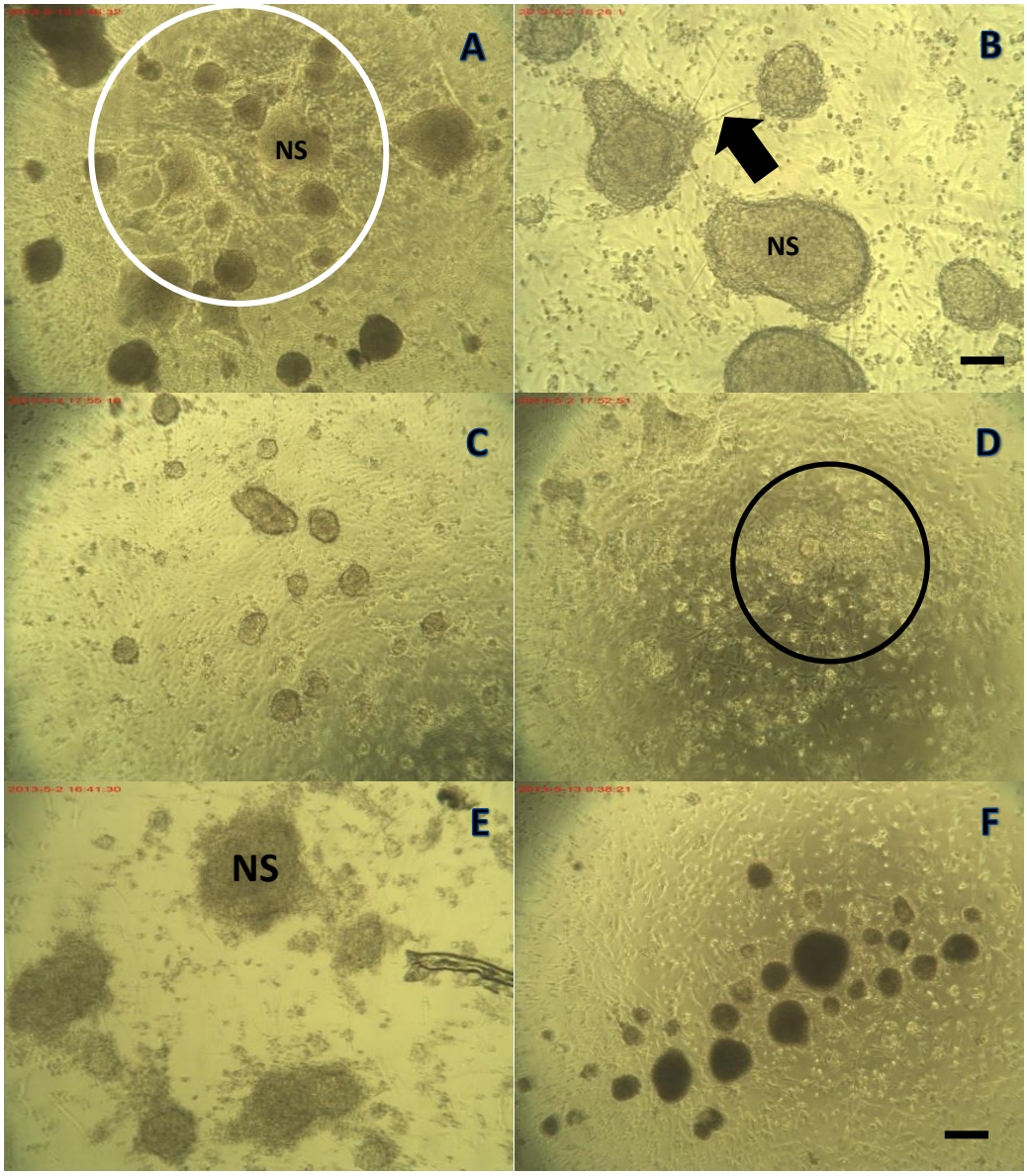


Figure 16: Morphological changes on brain micromass culture after treatment with **Sodium Valproate**. A, B represent the control group which show the neurosphere (NS) and how these NSs are connected to each other (black arrow) and notice the large population of neural cells as shown inside the white circle, C represents the treated group with 1000µM shows the disappearance of neuronal processes between the NSs. D represents the treated group with 1500µM showing floated (dead) cells inside the black circle. E&F treated group with 2000µM showing disintegrated NSs and small floated neurospheres. The scale bar is 100µm for all photos but for B it represents 250µm.

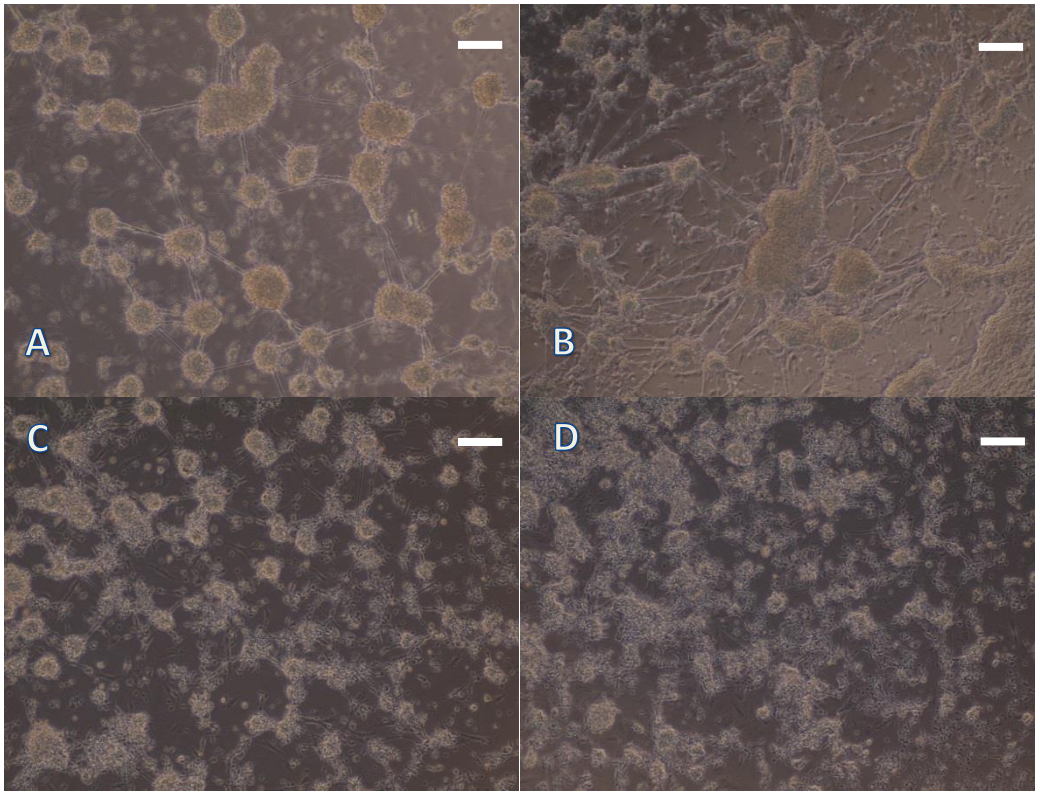


Figure 17: Chick Brain Micromass, $1*10^5$ cells from trypsinized midbrain seeded on coated glass coverslips illustrates the effect of **Lithium treatment**. A & B represent the control group, which show the normal spherical appearance of neurospheres, and how they attached with each others by neuronal processes, C: represents the treated group with 1500µM, and D represents the treated group with 2000µM lithium, these figures show the reduction in the number of neuronal processes between the neurospheres. The scale bar is 100µm.

2.4.3 Discussion

2.4.3.1 Sodium Valproate

SV appears to have a significant effect on the beating score of differentiating cardiomyocytes, this reduction is time and dose dependent, and appeared to be significant within the therapeutic range, 400-800µM (Chiu et al., 2013). There was significant reduction in beating score at the dose of 750µM, this indicate that cardiac micromass culture is a sensitive in-vitro method to test the embryotoxicity of the drugs. Also, its effect on cell viability and protein content was not

significant at lower doses, but those end points started to be reduced significantly at high doses. SV is well known in its teratogenic potential, SV administration during pregnancy associated with broad spectrum of congenital diseases, including cardiac and neural tube defects (Nau, 1994). SV is well known as Histone Deacetylase inhibitor (HDAC) (Gurvich et al., 2005), SV has been reported to cause chromatin condensation, changes in gene expression and cell death by apoptosis (Phiel et al., 2001) and leads to DNA methylation which result in change in gene expression which can associate with several developmental anomalies (Detich et al., 2003). SV has been recorded to inhibit mesodermal differentiation of embryonic stem cells (Murabe et al., 2007a) and resulted in oxidative stress and release of free radicals (Na et al., 2003) which may explain the reduction in cell viability and total protein in both cardiac and brain micromass culture systems.

2.4.3.2 Lithium Carbonate

In general, lithium carbonate can be considered as a weak teratogen, however, it has been reported that lithium treatment may associated with developmental abnormalities in the heart (Giles and Bannigan, 2006) and the nervous system (Jurand, 1988). These experiments reveal that Lithium Carbonate has no significant effect on the beating score of differentiating cardiomyocytes in the doses which are close to therapeutic doses, which is 600-1200µM (Su et al., 2007b), it inhibitory effect on beating score of differentiated cardiomyocytes appeared only at first at 1500µM, 6 days after lithium treatment and at 2000µM appeared after the first day of treatment.

Lithium acts by several mechanisms; it inhibits glycogen synthase kinase-3 (GSK-3) thereby affecting gene transcription leading to anti-apoptotic activity and

by which cell structure integrity can be improved (Chin et al., 2005) and might be a cause of teratogenesis (Giles and Bannigan, 2006). Also lithium prevents the conversion of Inositol 1,4,5 triphosphate (IP_3) into myoinositol by blocking the Inositol monophosphatase (IMPase), this leads to depletion of inositol from the cells. Dampening of the phosphoinositol (PI) cycle causes downregulation in MARCKS which is actin binding protein, its down regulation is essential in stabilizing the cell membrane (Machado-Vieira et al., 2009). It has been postulated that pretreatment supplement with myoinositol can prevent the teratogenic effect of lithium (Giles and Bannigan, 2006) and (Giles and Bannigan, 1999). IP_3 in developing heart regulates pace maker activity, enhance cardiogenesis, and controlling myocardial contraction by enhancing calcium release and influx, therefore, inhibition on PI cycle can disturb early developmental events of the heart and great vessels (Kockskämper et al., 2008).

Additionally, lithium is well known drug as neuroprotective agent, it induces BDNF production (Hashimoto et al., 2002), and control the release of GDNF in vivo and in vitro (Paratcha and Ledda, 2008) which are involve in maintaining cell survival and many other cellular activities. Moreover, lithium has an anti-apoptotic activity in several mechanisms, like enhancing Bcl2 protein, downregulation of P53 (pro-apoptotic protein) (Chen and Chuang, 1999). Also, it can activate PI3K/Akt, MEK/ERK pathways which stimulate the release of anti-apoptotic factors (Chiu et al., 2013), these may explain our finding that lithium has no significant effect on cell survival and total proteins throughout all the doses except in the highest doses in both cardiac and brain micromass system.

Finally, microsystem system has been used to detect the toxicity of herbicides, insecticides and mycotoxins (Daniels et al., 1996). To overcome species

difference in chick micromass system, and to rely on cells which have the capability to undergo most of the important processes in embryonic development, human neural stem cells have been suggested which are also reflect to some extent the normal cellular status, and not derived from tumours. The rest of this thesis will aim to establish a system using human neural stem cells to test drugs for neurotoxicity.

Chapter 3

Methods of Developing of Human Neural Stem Cell Culture as a Model in In vitro Neurotoxicology

3.1 The Neural stem cell niche

Embryonic Neural Stem Cells (NSCs) in vivo undergo time dependant changes in their microenvironment (which can also be called their niche) to enable them to fulfil their roles in the development of the CNS. NSCs have the capability to proliferate (self- renew) and to differentiate into the three major CNS lineages; astrocytes, oligodendrocytes, and neurons. NSCs have been the subject of intensive investigation because of their potential use in therapy of neurodegenerative disorders such as Parkinson disease and multiple sclerosis (Hall et al., 2008). Because of the scarcity of primary human tissue from which NSCs can be isolated, the need to develop a protocol to culture them in vitro has been escalated. As a first step toward accomplishing this goal, it is essential to know the composition of their niche in developing CNS and to identify the factors that regulate their growth and differentiation. The observation that the NSCs are not randomly distributed throughout the brain is one of the important clues for the nature of these factors. They are located in Ventricular and Subventricular zones in the developing CNS and in the Subependymal zone of the adult CNS (Temple, 2001). Studies on niche in different organisms and various tissues have shown that there are common signalling molecules which are involved in maintenance of stem cell populations and enhance their growth in cell culture. These molecules include growth factors, cell-cell signalling molecules, adhesion molecules and extracellular matrix molecules (ECM) (Li and Xie, 2005).

The evidence that laminins have a direct contact with adult NSC through the basal lamina of the blood vessel in SVZ (Kerever et al., 2007) and with the embryonic NSC in VZ, highlights the likelihood of involvement of this ECM in NSC regulation (Lathia et al., 2007). Laminin acts through the interaction with the

integrin family of receptors, which are the key regulators of all aspects of cellular processes such as cell proliferation, survival, migration and differentiation (Qin et al., 2004). Integrins are transmembrane αβ heterodimers which make conformational changes with ligand binding that lead to downstream effects, like actin polymerization and growth factor signalling (Qin et al., 2004). The elevated expression of integrin, especially the laminin-binding α6β1 heterodimer, is used for the isolation of stem cells from more differentiated cells (Fujimoto et al., 2002). Integrin β1 has been shown to maintain prostatic stem cells (Heer et al., 2006). Integrin-laminin interaction has been shown to regulate migration of human NSC in an integrin α6-dependant way (Flanagan et al., 2006).

Isolation of NSCs from CNS was first described by Reynolds through a neurosphere formation assay (NFA) (Reynolds and Weiss, 1992). The idea behind NFA is that the cells which have the ability to form the neurosphere (NS) are stem cells since they have the ability to proliferate (self-renew) and to differentiate into the main cell types in the CNS. These two criteria confirm cell 'stemness'. NS are spheroid 3D structures consisting of cells with a rich extracellular matrix. These NS produce their own ECM molecules (laminin, fibronectin, chondroitin sulphate proteoglycans) and growth factors (Fibroblast growth factor- FGF and Epidermal growth factor-EGF) (Lobo et al., 2003). Initial cell-cell contacts are retained by the proliferating cells, and the NS can create a niche which is more relevant physiologically than 2D culture models, and also can allow dynamic changes in the environment such as changes in growth factors or nutrient materials (Ahmed, 2009). The important features of NFA are; the identification of NSC, the simplicity, as a starting point in studying the neurodevelopment, and it can offer a model to screen chemicals and drugs for brain tumours and CND diseases.

3.1.1 Role of Growth factors

1- Basic Fibroblast Growth factor (bFGF) and Epidermal Growth factor (EGF)

Progenitors from developing brain of different species can grow in vitro in the presence of several growth factors, by which, the cells can preserve their capability to proliferate and differentiate into neurons and glial cells (Armstrong and Svendsen, 1999). The factors which stimulate these cells to proliferate are complex, and they are likely to involve contact and diffusible molecules (Svendsen et al., 1998), although it is well known that Epidermal growth factor (EGF) and Fibroblast growth factor (FGF) are important mitogens in stimulating these cells to proliferate. The receptors of these two factors are expressed in the highly proliferating NSCs of the adult brain (Doetsch et al., 2002, Frinchi et al., 2008). Supplementation of these mitogens to aged mouse brain stimulates proliferation of NSCs (Jin et al., 2003). Deletion of them from the genome results in reduction of proliferation of these cells (Zheng et al., 2004).

During early stages of embryogenesis, the neural tube is composed of a homogenous population of cells, the neuroepithelial stem cells, which reside in Ventricular zone (VZ) and lack the markers for any differentiated cells, such as neurons, or glial cells. These cells can be maintained and proliferate in culture with the presence of basic FGF (bFGF) (Qian et al., 1997). There is undetectable or low expression of EGF receptors in the ventricular zone neuroepithelial stem cells, therefore, it has been difficult to generate neurospheres from these stem cells by EGF alone (Pevny and Rao, 2003). As development progresses, by proliferation of neuroepithelial cells and migration of differentiated cells, the

subventricular zone (SVZ) will be demarcated, and a new population of stem cells can be isolated and maintained in culture (Reynolds and Weiss, 1992, Reynolds and Weiss, 1996), this type of stem cell has been called EGF-responsive stem cells, since there is high expression of EGF and EGF receptors in this area. Both EGF and FGF are able to stimulate their growth to form neurospheres (Vescovi et al., 1993).

Both FGF and EGF can be used to drive the stem cells to form neurospheres, but, a combination of these factors is able to generate more neurospheres than either factor alone (Lobo et al., 2003). It seems that there is a synergistic effect of both EGF and FGF on NSCs proliferation (Kelly et al., 2005). It has been proposed that EGF responsiveness of the cells could be primed by exposure to FGF (Ciccolini and Svendsen, 1998).

2- Role of Heparin

Heparin is also one of the important factors for the growth of NSCs in vitro. It has been shown that heparin is required for the growth of progenitor cells of primary tissue of the mesencephalon, combined with FGF-2 (Caldwell and Svendsen, 1998). Heparin appears to potentiate the action of EGF and FGF on rat embryonic neural progenitor expansion when these factors used individually or in combination. However, its potential effect was higher when these factors were combined (Kelly et al., 2005). FGF's are typically found bound to heparin sulphate components in the extracellular matrix (Gospodarowicz and Cheng, 1986). The importance of this interaction is thought to be due to activation or protection of FGF-2 molecules (Mason, 1994). Previous studies failed to generate free floating neurospheres from mouse by using FGF-2 alone in the absence of

heparin (Reynolds and Weiss, 1992). However, other studies succeeded in propagating them from adult murine brain after adding heparin to the growth media (Gritti et al., 1996, Caldwell and Svendsen, 1998).

The following experiment is to demonstrate cell behaviour in the media and to describe the neurospheres.

3.2 Neural stem cell growth and behaviour in media

3.2.1 Materials and methods

The foetal material was provided by the Joint MRC)/Wellcome Trust (grant # 099175/Z/12/Z) Human Developmental Biology Resource (www.hdbr.org). Foetal brain tissue was washed, dissociated mechanically into single cell suspension (Uchida et al., 2000). Single cell suspension of multipotent NSCs were grown in NSC maintenance medium (Dulbecco Modified Eagle Medium-DMEM and Ham's nutrient F12 in 1:1 ratio, bFGF, EGF, Glutamine, Heparin, N2, and B27 supplements) in 25 cm^2 non treated flasks and left in the incubator at 5% CO_2, 100% humidity, 37C° for several days.

3.2.2 Result and discussion

Our findings demonstrate that NSCs aggregate to form spherical structures which are called neurospheres, the progeny of neurosphere are variable in size, as shown in the figure (18) below. Neurospheres can grow more to become darker in the centre as they enlarge, the dark core suggesting necrotic event which may occur due to reduction in the amount of nutrition from the external medium (Bez et al., 2003). The cytoarchitecture of the neurosphere depend on the access of neurosphere forming cells to the nutrients in the medium and to the oxygen and also to the possibility to eliminate the waste products to the outside of the

neurosphere. Smaller neurospheres appear translucent and appear healthier. The cells in the external layer of the neurosphere may show small cytoplasmic processes similar to cilia. The behaviour of the neurosphere described above can be explained as an adaptation of NSCs to the in vitro culture environment. The NSCs may aggregate to form clusters to survive in the non-physiological conditions of the in vitro environment. The cells adapt themselves and optimize their interaction to acquire the most advantageous shape, the sphere, from the thermodynamic point of view (Bez et al., 2003). The neurosphere itself can be considered as a microsystem, in which the cells can survive, grow, and proliferate to reach a critical stage by which there is no self-restoration mechanism (Bez et al., 2003). Therefore, the neurosphere can be considered as an example of environmental adaptability.

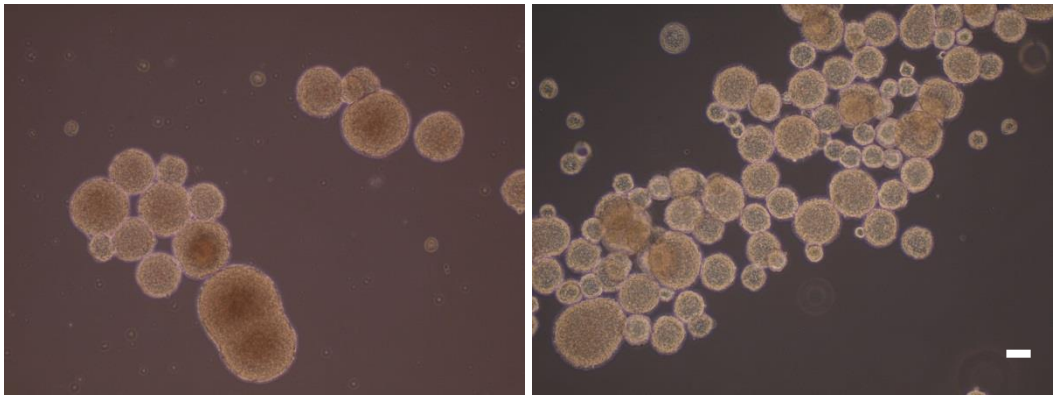


Figure 18: Variable sizes of neurospheres appear a few days after keeping NSCs in the medium, the larger spheres showing darker zones in the centre which denote cell necrosis, while the smaller spheres are transparent. The scale bar is 100µm.

3.3 Neural stem cell Identification:

Neural stem cells have the capability to divide, self-renew, and differentiate into their main three lineages. The ability of the cells to form neurospheres in vitro indicates that these cells are stem cells, because they fulfil the criteria of neural

stem cells in dividing and differentiation (Ahmed, 2009). Intermediate filaments, nestin, can be considered as an indicator for these stem cells (Miyagi et al., 2006). Nestin expression starts from the early embryonic period in the neural stem cells, and is used for stem cells identification, isolation, and cultivation as a marker protein. This protein can be re-expressed when there is brain injury; therefore, it has been used as a quick, early responding marker of brain impairment (Jin-ping, 2009). The expression of these intermediate filaments is down regulated when there is a transition from proliferating neural stem cells into post-mitotic neuronal cells (Zimmerman et al., 1994). There are several transcription factors which are used as a marker for neural stem cell, such as SOXB1 and three other related genes which are SOX1, SOX2, and SOX3. This family universally mark neural progenitor and stem cells throughout the vertebrate CNS. SOX2 is highly expressed in neuroepithelial stem cells and persists in neural stem cells until adulthood. This factor is well known to stimulate neuroectoderm development and inhibit mesendodermal development (Thomson et al., 2011). SOX2 expression has been shown to inhibit cellular differentiation and maintain the stem cell characteristics, while inhibiting SOX2 signaling leads to delamination of neural progenitor cells from the ventricular zone, which is accompanied by the loss of stem cell markers and the starting of expression of neuronal differentiation markers (Graham et al., 2003). SOX2 knock down in mouse leads to death around the implantation stage (Miyagi et al., 2006).

Other transcriptional factors used in addition to SOX2 to identify NSCs are OCT4, Klf4, and c-Myc. These four transcriptional factors are used to reprogram human astrocytes (Ruiz et al., 2010) and mouse and human fibroblasts (Kim et al., 2011) into induced pluripotent stem cells (iPSCs) with similar efficiency to NSCs.

Well controlled OCT4 expression leads to generation of tripotent neural stem cells which have extensive self-renewing capacity (Kim et al., 2011). Over-expression of OCT4 alone in adult NSCs can induce pluripotent stem cell (iPS) formation, which are similar to embryonic stem cells in vivo and in vitro which are able to differentiate into NSCs, cardiomyocytes and germ cells in vitro, but are also capable of teratoma formation in vivo (Kim et al., 2009). NANOG is another transcription factor which acts, at physiological state, in concert with SOX2 and OCT4 to maintain embryonic stem cell identity (Hanna et al., 2010).

There are some molecular mechanisms which serve to maintain the general cellular characteristics of stem cells, such as the ability to divide and to differentiate into their lineage. For example, one well-known molecular mechanism which is shared by neural progenitor cells is the Notch signalling pathway. This pathway seems to play a critical role in preserving the stem/progenitor cell pool. During development and in adulthood, Notch1 expression, or its downstream regulators, like HES-1, prevents neuronal differentiation and leads to preservation of the progenitor pool (Graham et al., 2003). The exact mechanism in which the Notch signalling pathway controls cell fate is not well determined. Another study suggested that Notch signalling, rather than inhibiting neuronal differentiation and maintaining the stem cell pool, may enhance glial differentiation (Gaiano and Fishell, 2002).

In order to establish the phenotype of cells within our neurospheres, we carried out immunohistochemistry (below).

3.3.1 Immunohistochemical staining of neurosphere sections

Neurospheres were collected from the flasks by centrifugation of the culture medium and they were fixed using 4% Paraformaldehyde (PFA) for 20 minutes. PFA was aspirated after centrifugation. The neurospheres were placed in 2% Agarose for a few minutes with gentle stirring of the solution to ensure even distribution of the neurospheres. Then the samples were transferred into an automatic tissue processor for dehydration, xylene, wax impregnation and wax embedding. Using an automatic microtome, 10µm thickness slices were taken from these samples and placed on gelatinized slides.

To prepare the slides for ICC, the slides were submitted into:

-Xylene 5 min*2 to dissolve wax from the slide, followed by keeping the slides in alcohol in different decreasing concentrations to rehydrate the samples as follows:

-Alcohol 100% 5 min*2

-Alcohol 90% 5 min

-Alcohol 70% 5 min

-Alcohol 50% 5 min

-Tap water 5 min

Then the sections were washed for 5 minutes 3 times with 0.01 PBS and permeabilized with a solution containing 0.01 PBS, 0.3% triton and 1 % Goat serum for 1 hour. The sections were washed again 3 times for 5 minutes. Anti-nestin primary antibody (Mouse monoclonal –BD Biosciences pharmingen) and was added to the sections in 1:1000 dilution and Anti-SOX2 primary antibody (Rabbit polyclonal-Abcam) in 1:2000 dilution was added to other sections, then the sections were incubated overnight at 4° C. On the next day, the primary

antibody was washed with 0.01 PBS for 5 min 3 times, and the secondary antibody in 1:500 dilution was added in a light protected area for 1 hour followed by washing by 0.01 PBS for 5 minutes 3 times, then mounting was performed in fluorescent mounting medium. Examination of slides was performed using Fluorescent microscope.

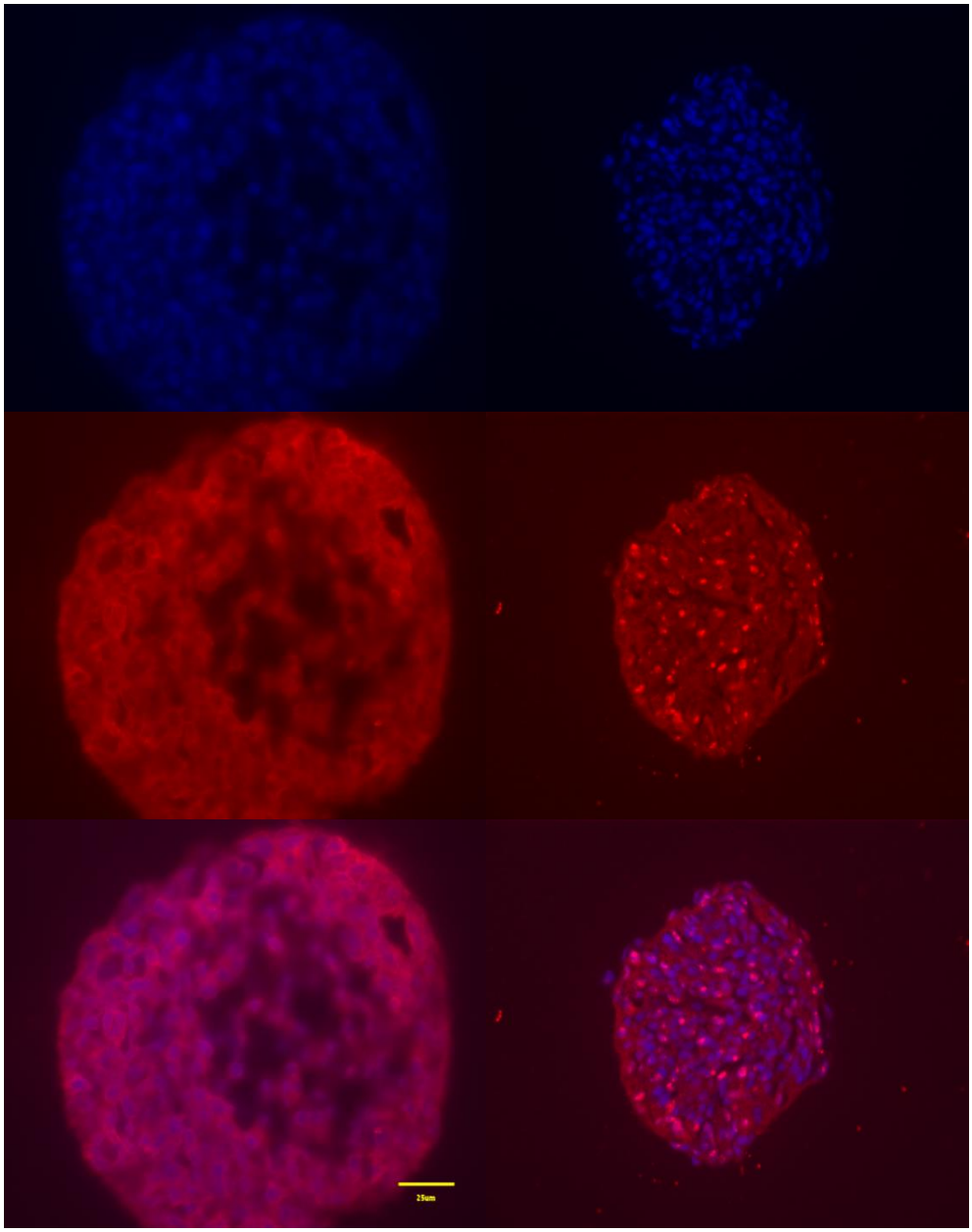


Figure 19: Immunohistochemistry staining for Nestin and SOX2 in Neurospheres of HNCs, the blue colour represent the nuclei stained with **DAPI**. The images at the bottom are the merged images. Left column shows **Nestin** positive cells in the neurosphere while right column illustrating **SOX2** positive cells.

3.3.2 Immunocytochemistry of seeded slides

In order to compare the phenotype of floating neurospheres with the same cells seeded onto slides, immunohistochemistry was carried out. $2*10^4$ were seeded over glass coverslips which were coated with PDL and laminin, then NSC

maintenance media was added to the wells in which the coverslips were placed and left for 6 days, after that the media was aspirated and 4% PFA was added for 15 minutes to fix the cells. Blocking and permiabilization was performed by solution containing PBS, 0.3% triton and 1% Goat serum, then, we followed the same steps as in immunohistochemistry.

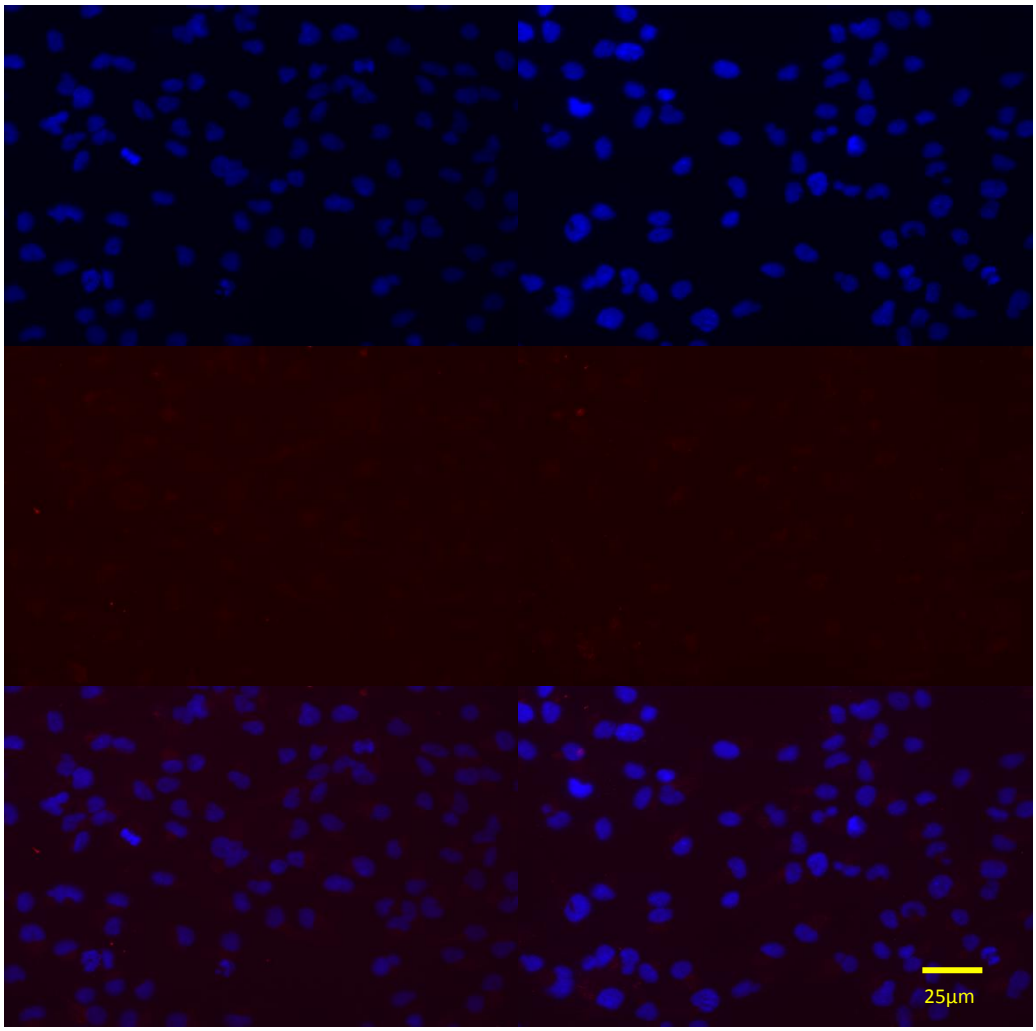


Figure 20: Immunocytochemical staining for NSCs after plating them on glass coverslips which was coated with PDL and Laminin. This figure illustrates the loss of cell stemness after attachment since the cells have become **Nestin** and **SOX2** negative.

3.4 Neuronal cell Markers

Neurons are diverse from a functional and morphological point of view, albeit they are structurally unique, once they have completed their migration to their final destination in the nervous system. The distinct morphology of neurons depends on their ability to elaborate elongated cell processes which are known as neurites. The growth, maintenance, stability and functionality of these neurites depend on subcellular structural organization, the cytoskeleton system, which is formed by three main filaments microtubules (neurotubules), intermediate filaments (neurofilaments) and microfilaments (actin filaments) (Pannese, 1994). The microtubules are 25nm hollow cylindrical structures, serving a variety of functional and structural roles, such as formation and control of cell morphology, mitosis, cell migration (especially during embryonic development), and cytosolic transport of cellular organelles and secretory vesicles (Ludueña, 1997). The structural constituent of microtubules is the globular protein tubulin, which interacts with a host of accessory microtubule associated proteins (MAPs), histones, chaperonins, G proteins and protein kinases. Tubulin is composed of two subunits, α and β, to form heterodimers. Six α- and seven β-tubulin isotopes have been identified in mammals and birds (Ludueña, 1997). In mammalian and avian development, β-tubulin III is considered to be among the earliest neuronal-associated cytoskeletal marker proteins. Its expression, before or during terminal mitosis in the CNS, is coincident with the lack of expression of this protein in other embryonic or adult cells (Katsetos et al., 2003), which indicates that this tubulin is controlled by transcriptional factors which are concerned with neuronal lineage commitment and morphological differentiation (Dennis et al., 2002). B-tubulin III is abundant in the central and peripheral nervous system and in the

brain it is prominently expressed during fetal and postnatal development (Katsetos et al., 2003). It is widely used to identify neurons in developmental studies as a neuronal marker (Svendsen et al., 2001).

3.5 Astroglial cells

Astroglial lineage consists of protoplasmic and fibrous astrocytes, together with specialized astrocytes which have specific development, function, and biochemistry, such as radial glial cells. They are related by the presence of glial fibrillary acidic protein (GFAP) and their developmental characteristics (Eng et al., 2000).

3.5.1 Radial glial cells

Radial glial cells are the first glial form in the developing CNS. These cells develop earlier and transiently in prenatal and postnatal neurogenesis in vertebrates. In general, they are bipolar cells, one of their processes extending to the ventricular surface and the other to the pial surface. These cells provide a framework during development for migrating neurons (Pentreath, 1999). Thus, these cells are essential for the normal construction of the CNS. The radial cell processes may provide information between the surface of the ventricles and centre of proliferation. Once the neuronal migration is finished, the cells transform into protoplasmic or fibrous astrocytes or other type of specialized astrocytes (Rakic, 1995).

3.5.2 Astrocytes

Astrocytes make up about half of the volume of grey matter (Pentreath, 1999). The overall functions of astrocytes in physiological condition are supportive and

protective for the neurons, which can be summarized as follows (Pentreath, 1999):

1- Taking up, inactivating, reusing neurotransmitters, and having receptors for these neurotransmitters and they may release some neurotransmitters. This function is critical for controlling synaptic activity.

2- Releasing growth factors, cytokines, secreting some of the extracellular matrix components and some molecules for cell adhesion. This is related to growth, and survival of the neurons.

3- Blood brain barrier formation by their interaction with the endothelial cells of brain capillaries.

4- Potassium siphoning, distributing potassium away from active neurons.

5- Detoxification of some exogenous and endogenous toxins.

Injury to the CNS in the form of trauma, degenerative diseases, or chemical exposure makes the astrocytes proliferate and hypertrophy. This form of reaction is called reactive astrogliosis, which is characterized by increased synthesis and expression of GFAP intermediate filaments (Zurich et al., 2004). Astrogliosis can be detected before the occurrence of the toxic effect on neurons, therefore it can be regarded that GFAP is an early indicator in neurotoxicity (O'callaghan, 1991). These reactive astrocytes secrete several molecules which can modulate the neurotoxicity of xenobiotics and may have neuroprotective and neurodegenerative effects (Aschner et al., 2002).

It is well established that GFAP is the principle intermediate filaments (8-9nm) of mature astrocytes and it has been proven to be a reliable marker for normal and

neoplastic astrocytes. As a member of the cytoskeleton system, GFAP is thought to play a role in regulating astrocyte motility and morphology by stabilizing the extension of the astrocytes (Eng et al., 2000).

For the purpose to identify neuronal cells and astrocytes, the same protocol of immunocytochemistry in 3.3.2 was performed on cells seeded on a slide, but with different antibodies, mouse monoclonal anti-tubulin III antibody (1:500), and rabbit polyclonal anti-GFAP antibody (1:800) were added, then goat polyclonal secondary antibodies to mouse (1:500) and rabbit (1:500) respectively were added (appendix 3).

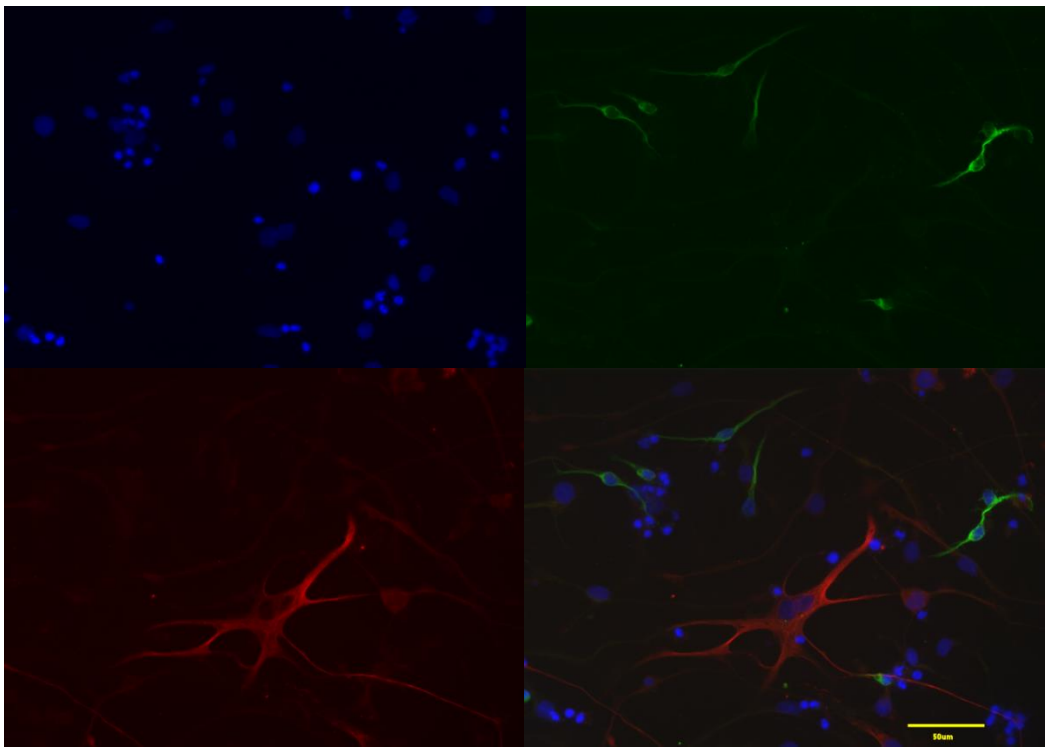


Figure 21: Immunocytochemistry staining of human neural stem cells, the nuclei stained blue with DAPI, Tubulin positive cells are green, and GFAP positive cells are red. The scale bar is 50µm.

3.6 Evaluation of different Extracellular molecules for coating:

Different ECMs have been used in our study for coating the plates and the slides to find out which one is best for the neurospheres and the cells to be attached on the surface. Normally NSCs are in the floating state in the medium, otherwise, once the cells attach, they would differentiate into their three main lineages; astrocytes, neurons, and oligodendrocytes. These ECMs are; Poly D-Lysine (PDL), Poly L-Lysine (PLL), Collagen I, and Laminin.

3.6.1 Materials and methods:

1- PDD, PLL, and collagen I alone protocol:

PDL and PLL were used independently at 5µg/cm^2 and collagen I was at 10µg/ cm^2. They were dissolved in sterile distilled water, PDL, PLL, and collagen I were added to separate plates and left overnight in the hood at room temperature. On the next day, the plates were washed with sterile distilled water three times, then, the plates were ready for use. Single cell suspensions were added to the wells of the plates and left in the incubator at 37C°, 5% CO_2, and at 100% humidity for several days to see whether the neurospheres were attaching, or not, to the bottom of the wells.

2- PDL and Laminin or PLL and Laminin

In this protocol, the first coating was with PDL or PLL which followed the same steps mentioned above. On the next day, after washing the plates from PDL or PLL, Laminin at 2µg/cm^2 was added to the wells and left in the incubator at 37C°, 5% CO_2, and 100% humidity for two hours, then the plate was washed by sterile distilled water three times, and when it dried, the plate would be ready for use.

3.6.2 The results:

NSCs in the wells which are already coated with PDL, PLL, or collagen I alone, aggregated to form floating neurospheres which were not attaching to the bottom of the wells as shown in the figure below. In contrast, the cells that were seeded in wells which were coated with PDL and laminin or PLL and laminin form a monolayer sheet of cells that attached rapidly to the bottom of the wells. The figure also demonstrates the morphology of the differentiated cells and how they elaborate their processes.

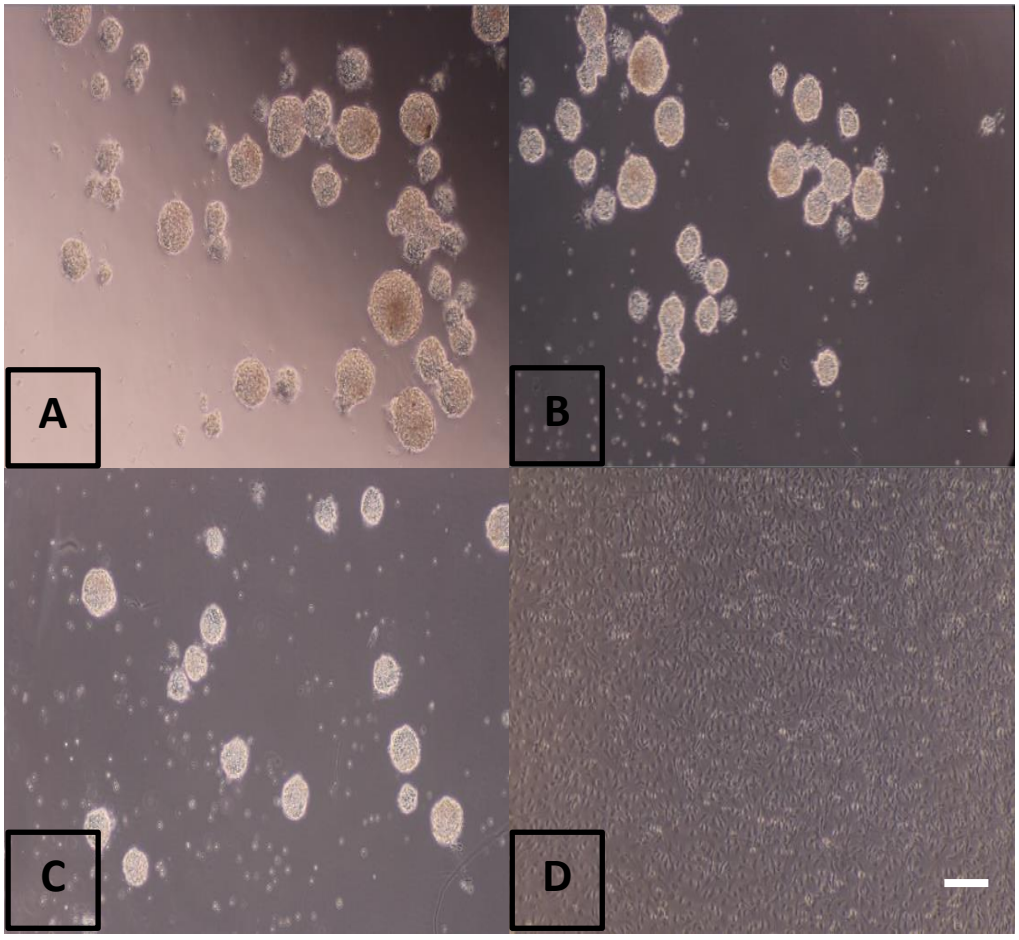


Figure 22: NSCs after seeding them on coated wells, **A: PDL, B: PLL, C: Collagen I, D: PDL+ Laminin.** The scale bar is 100µm.

3.6.3 Discussion

PDL, PLL, and collagen I alone was not enough to enhance cell attachment. It seems that the addition of laminin to PDL or PLL is the best to enhance attachment to the base of the wells. It was mentioned above that laminin is one of the important molecules which are present in the NSCs niche during development (Kerever et al., 2007). This molecule also can make interaction with the integrin family molecules of the NSCs, which are the key regulators for cell proliferation, migration and differentiation (Qin et al., 2004).

Extracellular Molecules (ECMs), when used in in vitro culture, can enhance cellular behaviour. Cell response depends on the cell phenotype and on the type of ECMs that have been used. The cells in vitro usually behave better when the ECMs used are similar to ECMs which are found in vivo. Cells in this case survive for a longer duration with these ECMs, and the existence of such ECMs can make the cells grow in the absence of serum and other growth factors (Kleinman et al., 1987).

The ECM is secreted by the cells located within it. It is composed of proteins, proteoglycans and hyaluronan. These components interact with each other and with the proteins on the outer surfaces of the cells. It has been considered that ECM plays a structural role, and it has been shown that ECM is critically important in determining the functional responses of the cells toward the environment. Thus, in the CNS, ECM plays an important role in regulating several functions during development and adulthood. ECMs send signals for cell growth, maturation, differentiation, cellular migration, tissue homeostasis and tumour cell invasion. These activities are very important in CNS development and organization and any disruption of ECM might lead to severe developmental

abnormalities (Novak and Kaye, 2000). Several molecules in ECM have been reported to control rodent NSC proliferation, migration, and differentiation (Kearns et al., 2003). Cells express some receptors on their surfaces for ECM components to mediate their response.

ECM is a component of the NSC niche which regulates their behaviour. Permissive substances may underlie the pathway of migration for neurosphere differentiation, while the non-permissive substances mark the more sedentary cellular zone. Laminin is one of the potent permissive substances for different types of cells in vitro. It is mainly present in the basal lamina but it is not present in high amounts in adult animal and humans (Novak and Kaye, 2000). It has been shown that laminin is present in developing cerebellum and it acts to facilitate migration of granular cell precursors from the external granule cell layer into the internal granule cells layer (Pons et al., 2001).

Laminins are large flexible proteins of three polypeptide chains which are connected by disulfide bonds. This protein has several domains which facilitate its interaction with type IV collagen, heparin sulfate, entactin, and laminin receptors of the integrins (Ryan and Christiano, 1996). It is a multifunctional protein and it plays roles in development, migration, and differentiation, as it interacts with cell surface receptors. Several genes encode the three different types of polypeptide chains and mutation or deletion of these genes produces a range of congenital abnormalities such as a form of muscular dystrophy and epidermolysis bollusa (Novak and Kaye, 2000).

3.7 Migration study:

3.7.1 Materials and methods

We tried to find out which method is suitable to study cell migration of NSCs, two methods were investigated: The first method was by making a scratch with the tip of a pipette through a monolayer of cells, which was formed by using a single cell suspension placed on a surface coated with PDL and laminin, and the second method was by placing the neurospheres on a surface which was already coated with the same coating materials (PDL and laminin). This was followed by monitoring cell migration from the edge of the scratched monolayer and from the edge of the attached neurospheres.

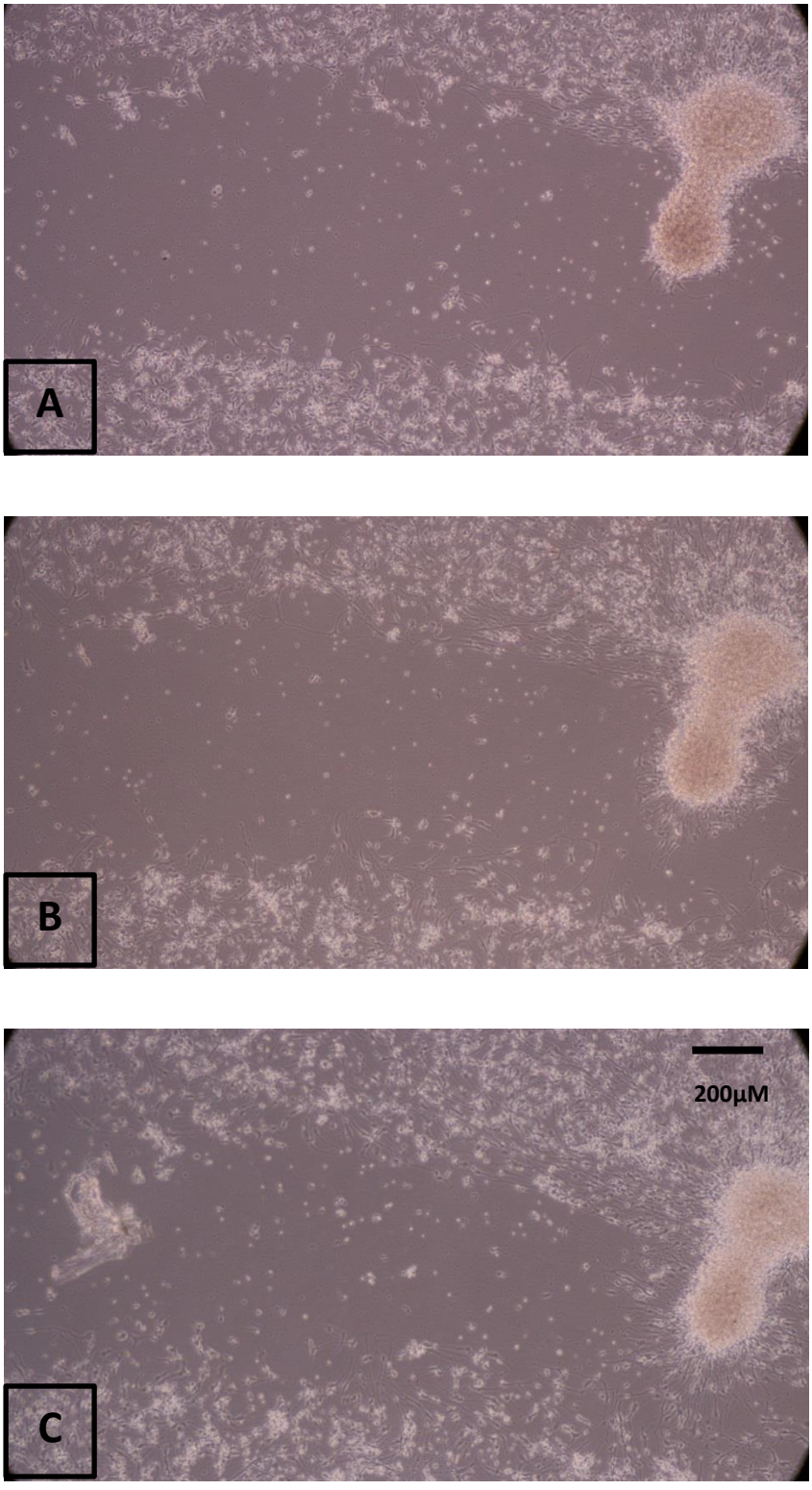


Figure 23: Illustrate cell migration in scratch method, the gap between these cells represent the scratch. On the right side, there is two merged small neurospheres showing their cell migration. **A: 2days, B: 4 days, C: 6 days** after cell seeding. The scale bar is 200µm

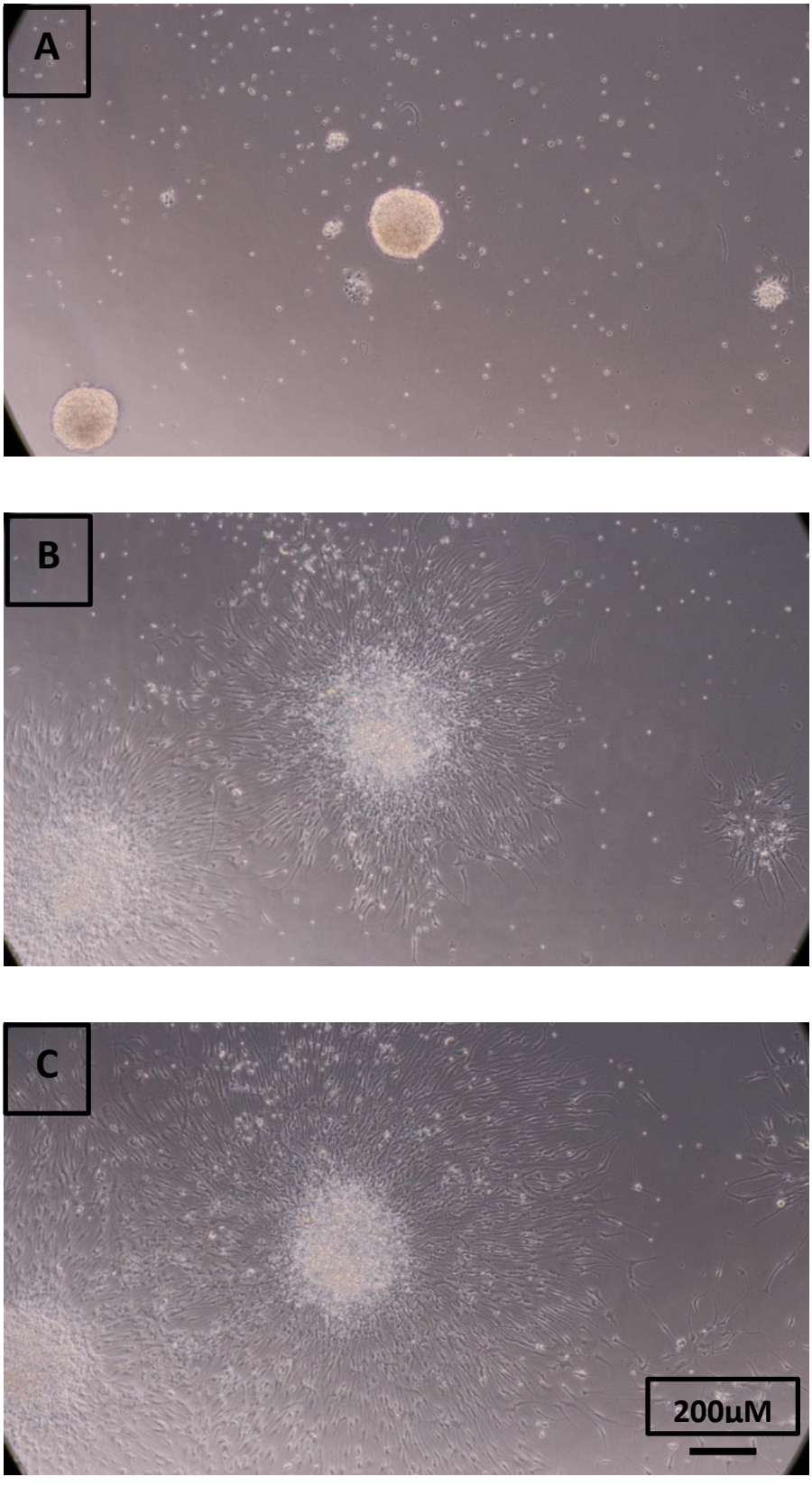
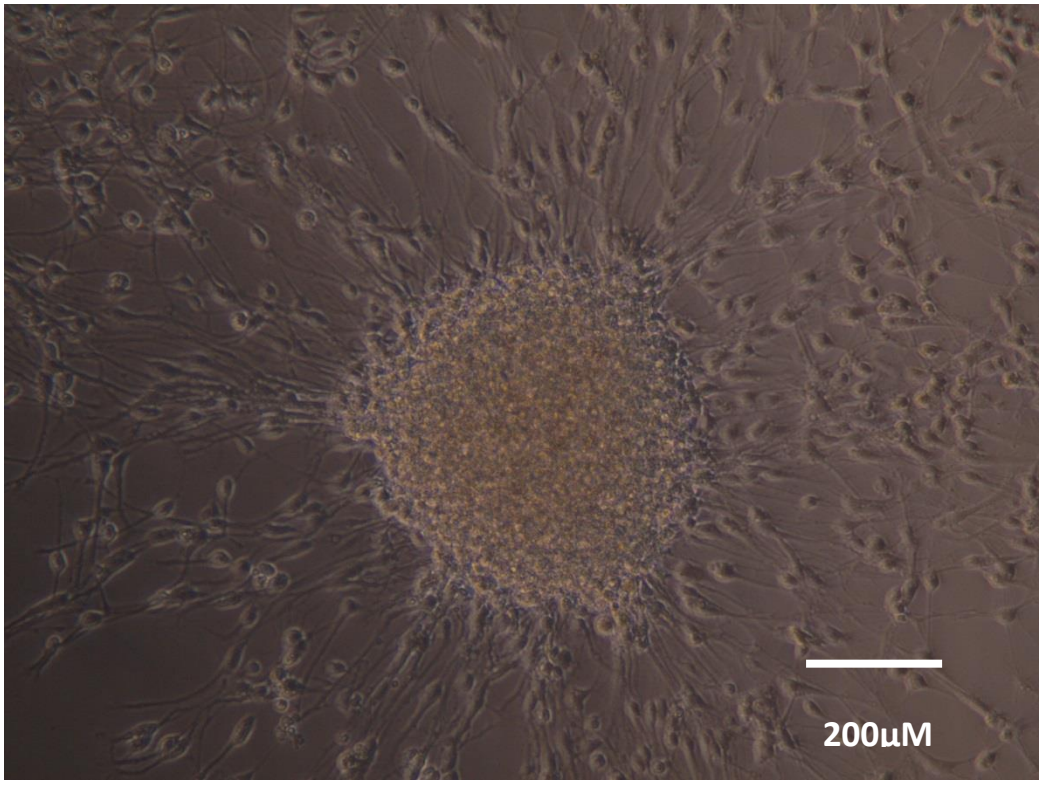


Figure 24: Two large and one small neurospheres attaching to the surface migrate away from their edges. **A: 0 hour, B: after 24 hours, C: after 48 hours.** The scale bar is 200µm.



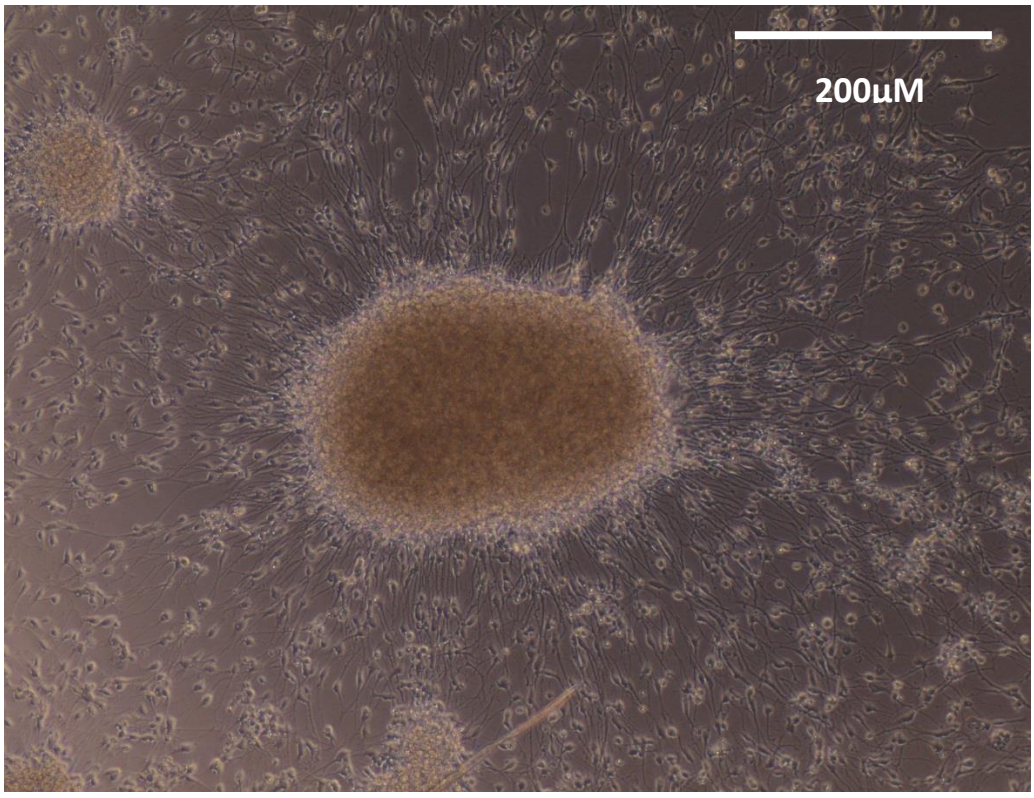


Figure 25: Migrating neurosphere after its attachment, showing radial cellular migration 10X, 20X

- Two videos showing neurosphere migration and merging with each other.

3.7.2 Discussion

It appears that the first method was less reliable since all the cells were differentiated at the time of cell attachment with no more cell proliferation. It showed migration and cellular processes for only the cells which resided at or near the edge of the scratch, therefore, migration distance was short and did not represent the real migration of all cells. On the other hand, using neurosheres, as shown in the videos, the cells still had the capability to proliferate, not all the cells were differentiated, and they continued to migrate for a long distance away from the edge of neurospheres, therefore the second method can be recommended to study cellular migration.

Chapter 4

Materials and Methods

4.1 Human Neural Stem Cell source and Cryopreservation

The human embryonic and foetal material was provided by the Joint MRC)/Wellcome Trust (grant # 099175/Z/12/Z) Human Developmental Biology Resource (www.hdbr.org). It was stored in a special medium composed of 70% Neural Stem Cell maintenance medium (DMEM, F12 Ham's Nutrient, Glutamine, B27 supplement, N2 supplement, Basic Fibroblast Growth factor, Epidermal Growth factor, and Heparin), 20% Foetal Calf Serum and 10% DMSO. The number of cells in each cryovial ranged from 1 to $3*10^6$. Cryovials were labelled with the passage number and the date of storage. The vials were kept at first in -80° C freezer for one day; then, the cryovials were transferred into liquid nitrogen for long term storage. The passage number used was 20-25.

4.2 Resuscitation of cryopreserved Human Neural Stem Cells

The stored cryovials of hNSCs were removed from liquid nitrogen and thawed rapidly in the water bath at 37°C for 2-3 minutes. 3ml NSC maintenance media was added then centrifugation was performed at 1200 rpm for 5 minutes, the supernatant was aspirated and 1ml NSC maintenance medium was added and through re-suspension by pipette, a homogenous cell suspension was produced. Cell counting was carried out using haemocytometer. After that, the cell suspension was transferred to a non-treated tissue culture flask and more NSC medium was added to it. The flasks were kept in the incubator at 37° C, 5% CO2 and 100% humidity.

4.3 Subculturing of Human Neural Stem Cells

The single cell suspension of human neural stem cells was grown in stem cell maintenance media (1:1 DMEM and F12 Ham's nutrient, N2 (1ml of 100X in

100ml media) and B27 supplements (2ml of 50X in 100ml media), Glutamine 2mM, Heparin 5µg/ml, EGF 20ng/ml, and bFGF 10ng/ml), and kept in 25 cm^2 non-treated flasks. The flasks were placed in the incubator at 37°C with 100% humidity, 5% CO_2 for several days. The cells proliferate to form neurospheres which start to increase in size with time. When the diameter of the neurospheres became more than 500µm, the culture medium was collected in 15 ml conical centrifugation tubes. Centrifugation was performed at 800rpm for 3 minutes. The supernatant was aspirated and 1 ml of Accutase was added to the pellet at the bottom of the tube to enhance cellular dissociation. The tube was placed in a water bath at 37°C for 5 minutes with shaking to prevent the neurospheres settling down. Cellular dissociation was completed by frequent resuspensions by pipetting. 2ml medium were added to the cell suspension to stop the action of Accutase followed by centrifugation at 1200 rpm for 5 minutes. The supernatant was removed and 1ml medium was added to the pellet and re-suspended to obtain a single cell suspension. Cell number counting was performed using a haemocytometer, then the cell suspension was transferred into 25 cm^2 tissue culture non treated flasks, 4ml medium was added to the flasks, passage number, cell number and the date of splitting were recorded on the flasks.

4.4 Drug preparation

A stock solution was prepared for each dose of each drug at high concentration, 300-500 times the final required concentration (depending on the drug), by dissolving the drug in its specific solvent. At the time of the experiment, these stock solutions were diluted by the media to achieve the final required concentrations to make the volume of the solvent fixed in all concentrations (0.2-0.3%). Also, the same volume of the solvent was added to the control group.

The drug	The solvent
Methyl mercury	Distilled water
Lead acetate	DMSO-Dimethylsulfoxide
5,5-Diphenylhydantoin (phenytoin)	DMSO
Sodium valproate	HBSS- Hank's Balanced Salt Solution
Carbamazepine	DMSO
Phenobarbitone	DMSO
Folic acid	NaOH
Melatonin	Ethanol
Lithium carbonate	Distilled water
Amitriptyline	DMEM
Diazepam	Ethanol

4.5 Cell seeding

48 well plates were used and were coated first with Poly D-Lysine (PDL) and laminin as described before. After splitting the neurospheres using Accutase, as described previously, the cells were seeded into the wells at 30,000 cells per well to form a monolayer, then NSC differentiation medium: 1:1 DMEM and F12 Ham's nutrient, N2 (1ml of 100X in 100ml media) and B27 supplements (2ml of 50X in 100ml media), and Glutamine 2mM containing the drug was added, 500µl in each well for 6 days. The media was changed every 3 days. Then, resazurin test was performed.

4.6 Resazurin Assay

Has been described in chapter 1, 1.2.6

4.7 Kenacid Blue

Has been described in chapter1, 1.2.7

4.8 Immunocytochemistry

8 well chamber slides were coated with Poly D-Lysine and laminin as described before. In order to perform double immunostaining for Tubulin III and GFAP proteins, 20,000 cells were seeded in each well of the slide and 500µl NSC differentiation media with the drug in different concentrations were added for 6 days, with changing media every 3 days. At the end of 6 days, the medium was aspirated, and paraformaldehyde (4%) was added for 15 min to fix the cells. Permeabilization and blocking was done by Triton (0.25%) and BSA (1%) for 30 minutes followed by washing with Phosphate Buffered Saline- PBS three times, 5min for each wash. Primary antibodies, anti-tubulin III (Mouse monoclonal) in 1:500 dilution and anti-GFAP (Rabbit polyclonal) in 1:800 dilution were added together for double staining. The cells were incubated with the antibodies at 4° C for 24h in the cold room. On the next day, three washes with PBS (5 minutes each) was followed by adding the secondary antibodies in 1:500 dilution for 1h. Again the same washing with PBS three times, after that, DAPI in 1:1000 dilution was added for 2 minutes then washed with PBS. Non fluorescent mounting media was used to cover the slides with suitable coverslips; nail varnish was used to seal the coverslips. Lastly the slides were examined by Leica microscope DMIRE2.

4.9 Neuronal processes length

Several random photos were taken at 40X magnification by the same microscope blindly from each sample for three repeats. By using Volocity software version 6.3.1 neuronal processes were measured to examine the effect of different doses of drug on the length of these processes.

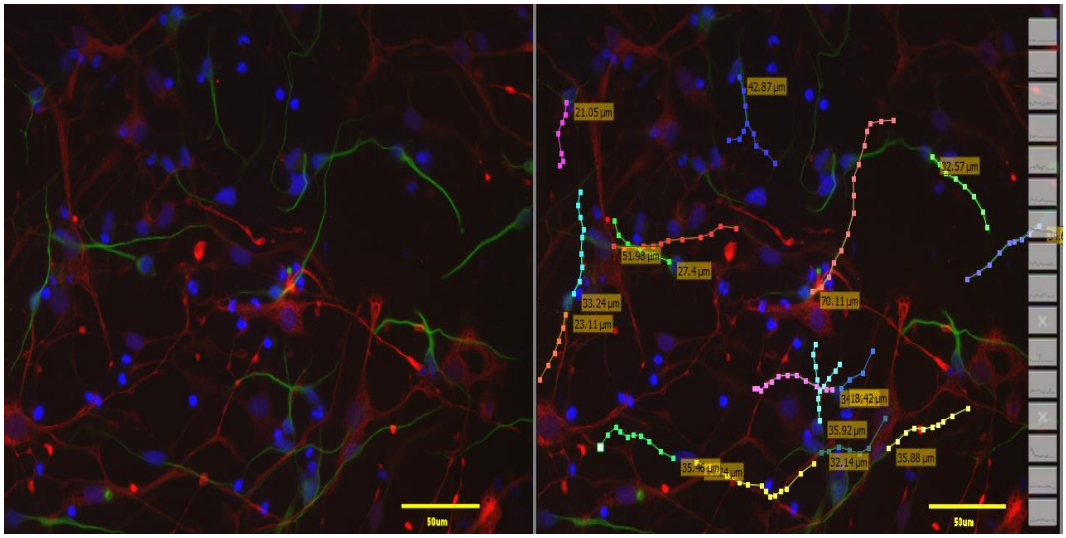


Figure 26: The method of measuring the length of neuronal processes by using Volocity software version 6.3.1, the length of each neurite appears inside the box which appears beside each neurite.

4.10 Neuronal proliferation study

An ultra-low attachment 96 well plate was used. $1x10^4$ cells/100µl stem cell maintenance media were seeded in each well, then the plate was centrifuged at 300 RCF/3 minutes and left for 3 days to form only one neurosphere, after that the drug was added to the wells. 10 days later, during which the media was changed every three days, the neurosphere sizes were determined by measuring the diameter and comparing the mean for each group with that of the control group.

4.11 Cell migration study

The method mentioned above promoted formation of only one neurosphere in each well and with similar sizes. When the neurospheres reached a diameter of 350-400µm, they were transferred into uncoated 48 well plates to be incubated for 1 day with stem cell differentiation media and the drugs, 500µl in each well. Then, they were transferred into another 48 well plate which was coated with Poly D- Lysine and laminin. Normally, the neurospheres attached to the bottom of

the wells within few hours and started migration. Photographs were taken at 0, 24 and 48 hours after incubation. To study migration distance, all the migrated cells were outlined, 2 diameters were taken, one perpendicular to the other, and the mean of these 2 readings was taken, neurosphere diameter was deducted, then, the result was divided by 2 to get the net migration distance, as shown in the figure below.

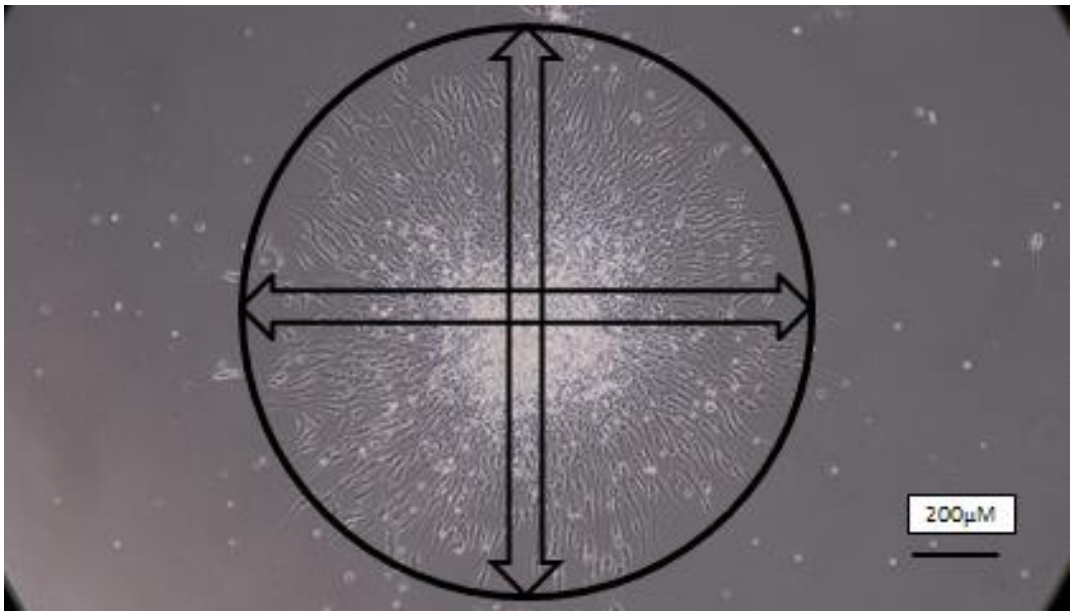


Figure 27: Illustrates the way of measuring the migration distance of the migrating cells from the neurosphere.

4.12 Western Blot Analysis:

4.11.1 Lowry test:

For western blots, 48 well plates were used with $10*10^4$ cells seeded in each well, 6 wells for each group. The drug and media were added on the same day, 500µl in each well. On the next day, the cells started to proliferate and form the neurospheres. The media and the drug were changed every 3 days. After 6 days, the media and the neurospheres were aspirated and placed in the Eppendorf tubes

and centrifuged at 13000 RPM for 1 minute. The supernatant was removed and the pellets were washed twice with ice cold PBS. A freshly prepared lysis buffer and protease inhibitor 1:1 (see appendix 1) was added and mixed with the pellet by pipetting up and down to produce a homogenous solution of cell lysate. Some of the cell lysate was used for a Lowry protein test and the rest was mixed with 6X solubilisation buffer (see appendix 1), vortexed and kept in the freezer at -20 C° to be ready for Western Blotting.

A Bovine Serum Albumin (BSA) standard curve was prepared by using 1mg/ml BSA. 1 ml Lowry solutions (A&B) (see appendix 1) were added for both the cell lysate of the samples and the standards. 10 minutes later, 100µl of Folin solution and water 1:1 was added and the tubes were incubated at room temperature for 45 minutes. 200µl of each sample and BSA standard were pipetted in triplicate in a 96 well plate, as shown the figure. The plate was read at 750nm.

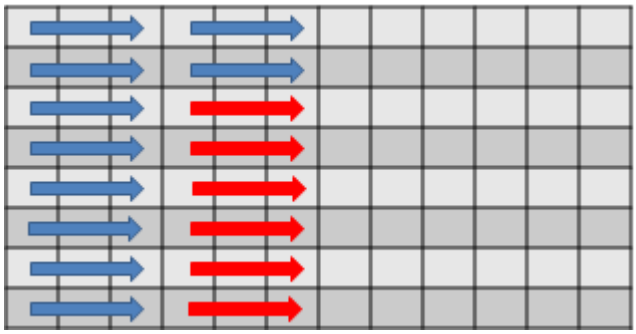


Figure 28: Illustrate the method of Lowry test, the **blue arrows** represent the standard samples and the **reds** represent the experimental samples

4.11.2 Western Blotting:

For western blot analysis for tubulin III and GFAP proteins, the cell lysate was heated at 95°C for 5 minutes followed by vortex mixing and centrifugation at 13000 RPM for 5 minutes. 6µg protein, were loaded in each well in the precast

polyacrylamide gel (4-20%, 12 well comb). The gel was run in 1X electrophoresis buffer (see appendix 1) at 175 V for 40 minutes. After that the gel bands were transferred to a nitrocellulose membrane by running in cold transfer buffer (see appendix 1) at 100 V for 60 minutes. The membrane was collected after 60 minutes, and a few drops of Ponceau stain were added to reveal the protein bands. The membrane was divided into 2 pieces, one for Tubulin III and GFAP and the other was for GAPDH. The membrane was washed quickly with TBST (see appendix 1) to remove the Ponceau stain. 5% milk solution in TBST was used as a blocking solution to incubate the membrane for 1 hour on a plate shaker at room temperature. The primary antibodies (see appendix 3) were diluted in 5% milk solution and the dilution was as follow: Anti- tubulin III (mouse monoclonal antibody-Abcam) 1:2000, Anti-GFAP (Rabbit polyclonal antibody-Abcam) 1:20000, Anti-GAPDH (mouse monoclonal antibody-Sigma) 1: 5000. Appropriate amounts of these antibodies were used in sealed nylon bags to incubate the blots overnight in the refrigerator at 4°C on a plate shaker. On the next day, the blots were washed by TBST, 3X quick washes, 3X- 5 minutes washes and 3X- 15 minutes washes to remove the non-specific binding. The secondary antibodies (see appendix 3) (Goat anti-mouse, and Goat anti-Rabbit-Li-Cor Odyssey) were diluted in 5% milk solution in TBST and the dilution was 1:30000. The blots were incubated with the secondary antibodies for 1 hour on a plate shaker at room temperature, after that, the blots were washed with TBST, 3X quickly, 3X- 5 minutes, and 3X-15 minutes. At the end, the blots were scanned by using an Odyssey scanner at resolution of 84μm.

4.12 Statistical analysis

The parametric data which represent three experimental repeats was analysed by using Prism version 6. Since we need to make comparison between more than two groups, one way ANOVA was performed for the all the data except for cell migration, two-way ANOVA was performed - two sets of data were used (1^{st} set - after 24 hours, 2^{nd} set - after 48 hours); then Dunnett's multiple comparison test was undertaken to check the significance of difference by comparing the mean of the control group with that of experimental group.

Chapter 5

Evaluation of This Model with Neurotoxic Chemicals

5.1 Neurotoxic Chemicals

5.1.1 Mercury

Mercury is present in the environment because of either natural processes or due to human activities (anthropogenic). It can be found in three different chemical forms: mercury vapour, inorganic mercury salt and organic mercury (Clarkson, 2002). Methyl mercury (MeHg) has been studied thoroughly because it can reach the CNS and result in a neurotoxic effect (Aschner et al., 2007). MeHg is an environmental contaminant that has neurotoxic effects on both humans and animals (Farina et al., 2010). As a result of methylation of inorganic mercury, which is released from local industrial discharge into the aquatic environment, MeHg- containing fish represents the major source of human exposure. Communities which rely greatly on fish for food are liable to be exposed to highly toxic levels of MeHg (Clarkson et al., 2003). For more than 150 years human poisoning by MeHg has been reported in the laboratory by accidental exposure (Edwards, 1865). Most of the information about the toxic effect of MeHg has come from the catastrophic episodes of poisoning which occurred in the 1950s and 1960s in Japan (Harada, 1978) by consumption of fish from water which was heavily polluted with MeHg daily for long periods. Another major health disaster occurred in Iraq in the 1970s, where hundreds of people died and thousands became clinically ill when they ate bread made from grain which was treated with an organo-mercury fungicide (Bakir et al., 1973).

Adult intoxication with MeHg is characterized by damage to focal anatomical areas in the brain such as the visual cortex, and the granular layer of the cerebellum. Its effect on the peripheral nervous system manifest themselves by axonal degeneration with myelin disruption of the sensory branches of peripheral

nerve, excluding the motor part (Hunter and Russell, 1954, Takeuchi, 1981). The signs and symptoms of adult intoxication (Minamata disease) may take several weeks to be manifested. These include constriction of the visual field, paraesthesia to the extremities (glove and stock type) and in perioral area, cerebellar ataxia, hearing loss, muscle weakness, tremor and mental retardation (Castoldi et al., 2001). On the other hand, the immature CNS is extremely sensitive to MeHg and the fetal brain may be affected even if the mother was asymptomatic. The effects of MeHg poisoning on the developing brain is diffuse unlike that in adult intoxication, with a high dose of MeHg resulting in cerebral palsy, blindness, hearing loss, and severe mental retardation (Castoldi et al., 2001).

5.1.2 Lead

Lead (Pb) is a heavy metal that is normally found in the earth's crust and is considered a ubiquitous pollutant in the ecosystem (Nava-Ruiz et al., 2012). Environmental levels of Pb have increased 1000 times in the last 3 centuries; the maximum increase was in 1950-2000 (ATSDR, 2007). The general population is exposed to Pb through air and food, while occupational exposure to Pb occurs in workers in Pb refining, battery plants, mines, welding of lead painted metals. Pb emissions in developed countries have decreased significantly in the last few decades due to the use of unleaded fuel (Jarup, 2003). Blood Pb levels were revised in the general population because the results showed the negative effect of Pb on child neurodevelopment, therefore, Pb levels which were considered hazardous were reduced to 40μg/dl in 1971, 30μg/dl in 1975, 10μg/dl in 1991 (Bellinger and Bellinger, 2006). In spite of efforts to reduce exposure through regulation, excessive exposure still exists and Pb still is a public health concern.

The targets of Pb toxicity are cardiovascular, kidney, bone, the haematological system, whilst the CNS is the most susceptible system to its effect (ATSDR, 2007). Children are the most sensitive to the effect of Pb. Pb exposure in utero, infancy, and early childhood produces dramatic effects manifested by behavioural disturbances, difficulties in learning, reduction in IQ scores even at low doses. This is because the developing CNS is more sensitive to PB than the adult CNS (Sharma et al., 2015). The high vulnerability of children to Pb toxicity is due to the different exposure pathway and due to variable toxicokinetics (Bellinger and Bellinger, 2006). Childhood lead exposure contributes to about 600,000 new cases of intellectual disabilities yearly, 99% of them are living in developing countries (Sharma et al., 2015). In humans, about half of Pb enters the human body through the inhalation route, while only 10-15% is absorbed by the oral route. In both these cases, 90% of the internalised Pb is retained in the body and distributed in the bones (Links et al., 2001). In the human, acute intoxication is less common than chronic intoxication and is manifested by central abdominal pain, headache, neurological signs; chronic poisoning is characterized by sleeplessness, poor attention span, convulsion, vomiting and coma (Bellinger et al., 1992). In children, Pb encephalopathy is characterized by lethargy, anorexia, vomiting, irritability, mental dullness; in severe cases, exposure for a long duration can decrease cognitive function and increase behavioural disorders like aggression and hyperactivity (Bellinger et al., 1992, Jarup, 2003, ATSDR, 2007).

In summary, methyl mercury and lead acetate have been chosen because they are well known in their neurotoxicity, as mentioned above. They have been used to evaluate human neural stem cell model in predicting their neurotoxicity, and to know at which level it can detect their toxicity.

5.2 The results

5.2.1 Mercury

Our study shows that Methyl mercury MeHg affects cell viability and total protein content of cultured cells. It appears that cell viability significantly reduced from the dose of 2.5µM, ($F(7,16)=26.99$, $p<0.0001$, post-hoc: 2.5µM vs control: $p<0.001$), also, total protein amount was reduced significantly from the same dose, ($F(7,16)=98.06$, $p<0.0001$, post-hoc: 2.5µM vs control: $p<0.0001$) as shown in the figure 29A and B. However, MeHg reduced neurite length significantly at lower doses (1µM) than that of cell viability and protein, ($F(5,12)=69.55$, $p<0.0001$, post-hoc: 1µM vs control: $p<0.05$). Similarly, the neurosphere sizes were reduced significantly from the that dose, ($F(5,12)=85.25$, $p<0.0001$, post-hoc: 1µM vs control: $p<0.05$). This means that neurite length and neurosphere sizes are more sensitive measures than cell viability and total protein amount. Neurite length and neurosphere reduced more with increasing the doses as shown in figure 29C and D and figure 30.

In the case of the effect on GFAP and tubulin III proteins in the western blot, it appears that MeHg significantly increases GFAP from the dose of 1µM, ($F(5,12)= 64.02$, $p<0.0001$, post-hoc: 1µM vs control: $p<0.05$), while in the effect of MeHg on tubulin III protein, it seems that this protein is reduced significantly at the same dose, ($F(5,12)= 6.574$, $p<0.0036$, post-hoc: 1µM vs control: $p<0.05$), as shown in figure 29E and F and figure 31. Regarding the effect on cell migration, it seems that MeHg at low dose does not have any significant effect on cell migration at either 24 or 48 hours of treatment. Cell migration appears to be significantly reduced at the dose of 1µM, (concentration vs time: $F(5,24)= 1.568$, $p=0.2071$, post hoc: 1µM vs control: $p <0.05$) (24hrs after treatment), and $P<0.01$

(48hrs after treatment), then migration distance reduced more with increasing the doses (F (5,24)=35.66, p<0.0001) and with increase the duration, (F (1,24)=228.4, p<0.0001).

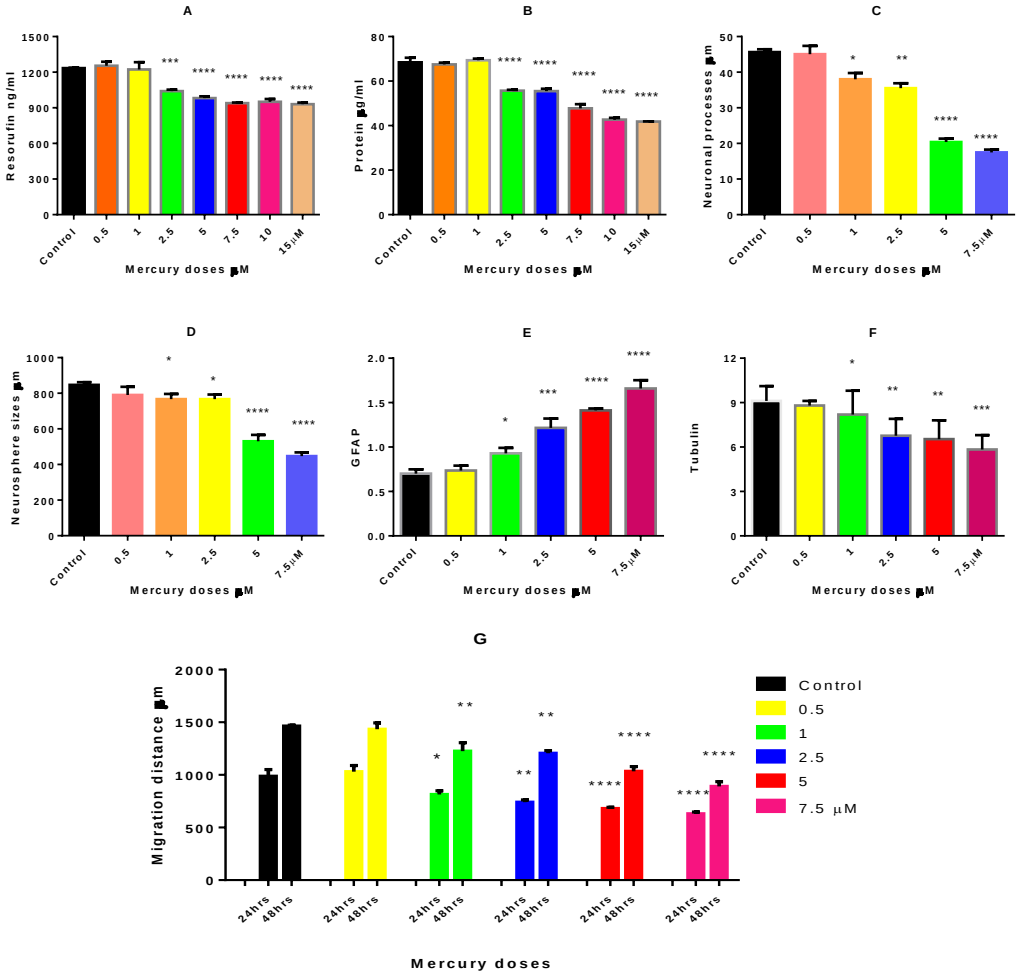


Figure 29: Effect of **Methyl Mercury** at different doses on A: Resorufin production, B: Total protein. Mean± SE., n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

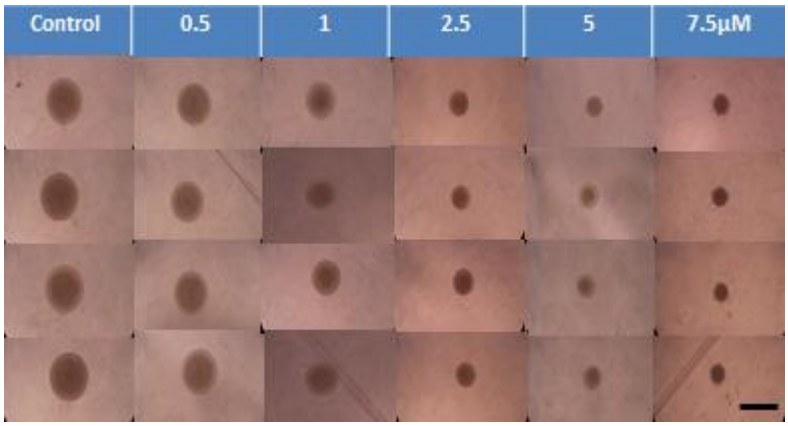


Figure 30: The effect of different doses of **Methyl Mercury** on the sizes of neurospheres. This figure illustrates photos of 24 wells of the same plate at the same magnification. The scale bar is 700µm**.**

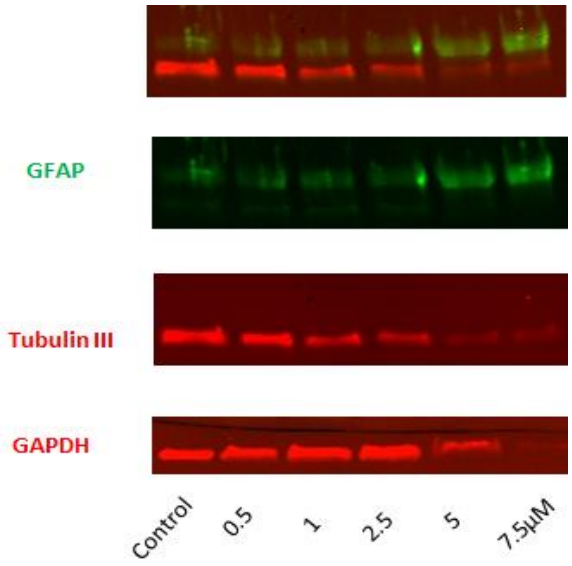


Figure 31: Western blot analysis for hNSC cultured cells illustrating GFAP, Tubulin III, and GAPDH proteins after adding **methyl mercury.**

5.2.2 Lead

Our study found that Lead (Pb) caused significant reduction in cell viability at a dose of 75µM, ($F(7,16)=3.587$, $p=0.0163$, post-hoc: 75µM vs control: $p<0.05$). It appears that cell viability was severely affected with increasing the doses. However, total protein amount was reduced significantly at 50µM, ($F(7,16)=12.12$, $p<0.0001$, post-hoc: 50µM vs control: $p<0.05$), as shown in figure 32A and B. Pb treatment results in significant reduction in neurite length and neurosphere sizes from the dose of 25µM, ($F(5,12)=23.07$, $p<0.0001$, $F(5,12)=43.88$, $p<0.0001$ respectively, post-hoc: 25µM vs control: $p<0.05$), figure C and D. Regarding its effect on GFAP and tubulin III proteins, it seems that this chemical has no significant effect on tubulin III protein, but it increase GFAP significantly at 25µM, ($F(5,12)=5.144$, $p<0.0094$, post-hoc: 25µM vs control: $p<0.05$), and the GFAP expression increased significantly with increasing the doses, figure 32E and F and 33. In case of its effect on cell migration, it appears that Pb has no significant effect at the dose of 5µM, however, it reduce migration distance with increasing the dose, ($F(5,24)=26.14$, $p<0.0001$), and with increasing the duration, ($F(1,24)=199.4$, $p<0.0001$). Lead reduces migration distance 48 hours after treatment at the dose of 10µM, (concentration vs time: $F(5,24)=1.944$, $p=0.1$, post-hoc: 10µM vs control: $p<0.05$) and has no significant effect 24 hours after treatment, but at 25, 50 and 75µM, Pb causes highly significant reduction in cell migration 24, and 48 hours after treatment, figure G.

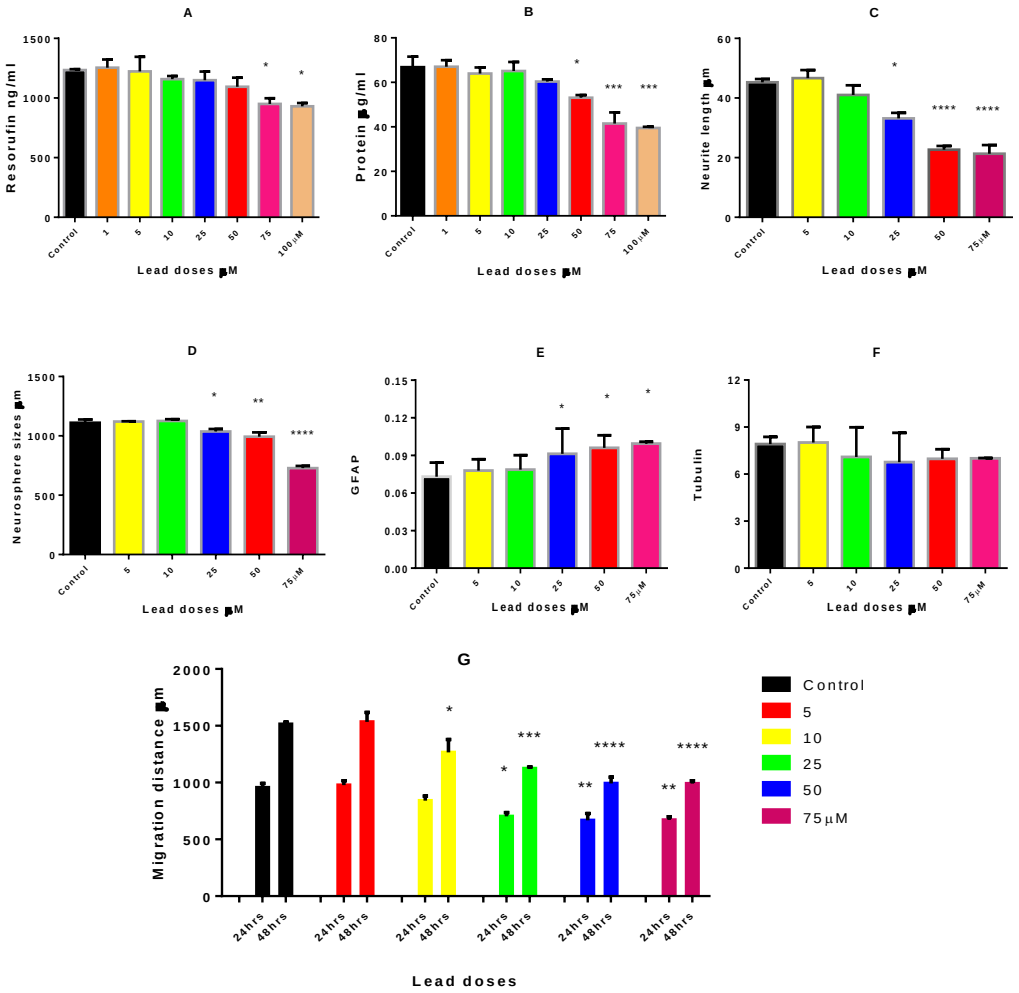


Figure 32: Effect of **lead acetate** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

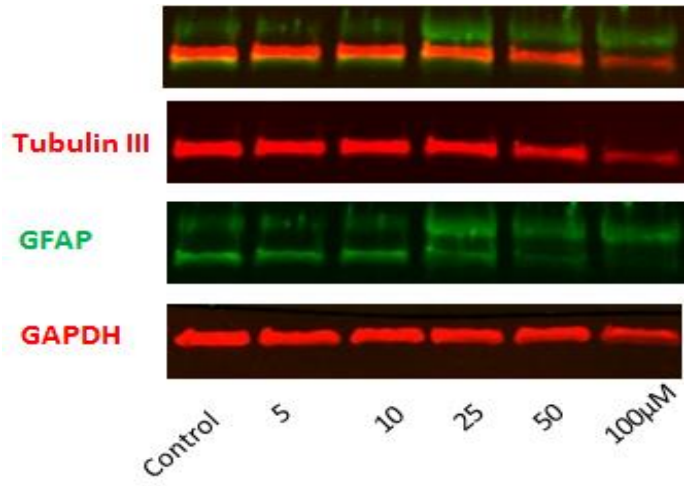


Figure **33**: Western blot analysis for hNSC cultured cells illustrating GFAP, Tubulin III, and GAPDH proteins after adding **Lead acetate.**

5.3 Discussion

5.3.1 Mercury

Methyl mercury (MeHg) is well known as environmental neurotoxicant for both humans and animals. Transplacental passage of MeHg readily occurs in humans and the developing CNS is more vulnerable to its neurotoxicity (Costa et al., 2004, Grandjean and Landrigan, 2006). High affinity of MeHg for sulfhydryl (thiol) groups makes proteins and peptides containing cysteines vulnerable to structural and functional changes. Combination of MeHg with sulfhydryl group in enzymes leads to inhibition of these enzymes (Rocha et al., 1993). Also, combination of MeHg to thiol groups in non- protein compounds such as glutathione GSH disturbs the antioxidant GSH system of the cells. Therefore, these facts represent the keys to the mechanism of MeHg neurotoxicity (Aschner and Syversen, 2005).

In this study, it appears that MeHg affects cell viability and total protein in a dose dependant way. MeHg can cause cell death in two ways, either by apoptosis

(programmed cell death) (Nagashima, 1997, Bulleit and Cui, 1998, Ceccatelli et al., 2010) or by necrosis (Miura et al., 1987, Nakada and Imura, 1983). The dose of MeHg determines the type of cell death; cell death at lower doses is by apoptosis, while at higher doses, is by necrosis (Castoldi et al., 2000). It seems that high doses of MeHg >5μM cause rapid impairment in mitochondrial activity and also causes plasma membrane lysis which results in massive cell necrosis. At low doses, the cell death seems to be by apoptosis (Castoldi et al., 2001) which appears to be caspase dependant (Chang et al., 2013).

Regarding the effect of MeHg on neurite length and cell migration, microtubule integrity is important in the development of the CNS such as cell proliferation, migration to form cortical layers of the cerebrum and cerebellum, neurite formation, extension and stabilization. MeHg appears to affect the cytoskeleton especially microtubules. MeHg has been found to have a high affinity for sulfhydryl groups, to depolymerize microtubules in vitro, and inhibit their assembly (Sager et al., 1981). Cytoskeleton fragmentation and neural network disruption have been shown in cultured cerebellar granular cells when exposed to MeHg, and it seems that these effects appear earlier than cell death (Castoldi et al., 2000). MeHg interference with microtubules is clear by the findings in the autoptic brains of full term infants from the Iraq outbreak in 1970s, who were exposed in utero to MeHg in early pregnancy, these include, brain size reduction, abnormal neuronal arrangement and alteration in cerebral cortex alignment (CHOI et al., 1978). MeHg caused a dose dependant reduction in cell migration, which is consistent with the finding of Kunimoto when he used cerebellar granule neurons and explained the reduction in cell migration due to extensive cell death (Kunimoto and Suzuki, 1997).

Calcium ions play an important role in cell death in the CNS. Ca^{+2} overload has been shown to trigger cell death either by apoptosis or by necrosis. The mechanism by which Ca^{+2} causes this problem is unknown, but raised calcium ions may lead to activation of degradative enzymes like proteases, endonuclease, phospholipase, mitochondrial dysfunction, and disruption in the cellular cytoskeleton (Orrenius and Nicotera, 1993). MeHg has been reported to affect Ca^{+2} homeostasis and elevate Ca^{+2} intracellularly in cerebellar neuron cultures (Okazaki et al., 1997, Oyama et al., 1994), as shown in figure 33. Calcium channel blockers, such as nifedipine and MVIIC, delay MeHg induced elevation of Ca^{+2} significantly. Also, the Ca^{+2} chelating agent BAPTA protects granule cells from cell death induced by MeHg. Additionally, in agreement with these findings, voltage gated Ca^{+2} channel blockers prevent the appearance of neurological disorders in rats which received MeHg in vivo (Sakamoto et al., 1996).

The results show that MeHg reduced neurons (tubulin III protein) in a dose dependant way; this can be explained by MeHg accumulating in astrocytes to induce cell swelling and inhibit excitatory amino acid uptake (Aschner et al., 2000). Glutamate represents the major excitatory neurotransmitter in the mammalian CNS. However, glutamate at high levels in the synaptic space is considered as a toxin, which causes injury and death to neuronal cells (Meldrum, 2000). MeHg causes a significant reduction in glutamate uptake in astrocyte cultures; the efflux of glutamate from astrocytes increased significantly, which lead to increased glutamate in the extracellular space and accelerated excitotoxic neurodegeneration (Aschner et al., 2007). Glutamate neurotoxicity, which is referred to as excitotoxicity, is due to over-activity of N-methyl D-asparatate (NMDA) glutamate receptors which result in an increase in Na^{+} and Ca^{+2} influx

(Pivovarova and Andrews, 2010), figure 33. Increased intracellular Ca^{+2} leads to activation of certain pathways which lead to cell death and to the generation of oxidative stress and neurotoxicity (Ceccatelli et al., 2010). In vitro co- application of nontoxic dose of MeHg and glutamate results in neuronal damage like that of excitotoxic damage (Matyja and Albrecht, 1993). Neuronal damage can be explained also by astrocytes dysfunction (Brookes, 1992).

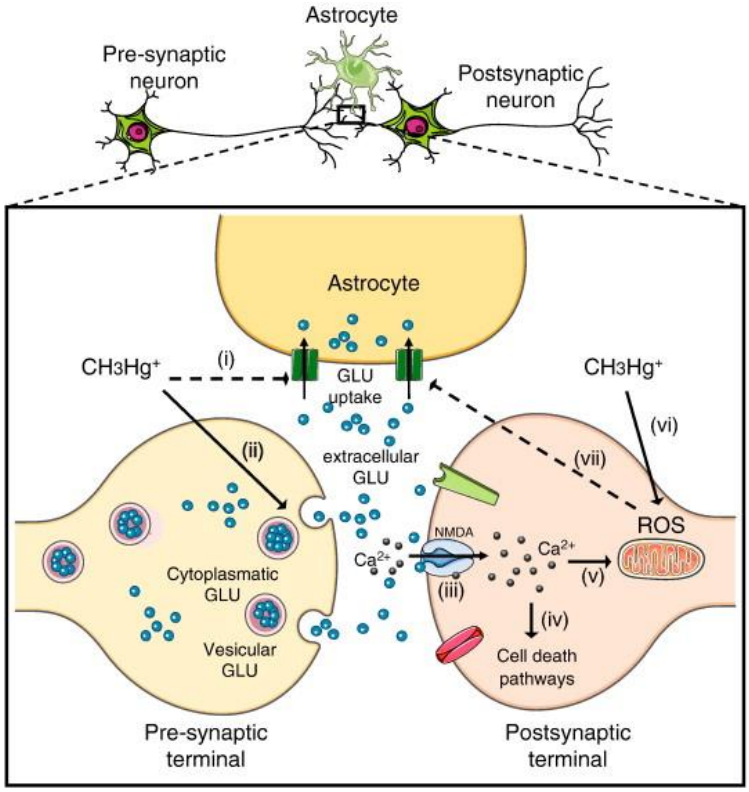


Figure 34: Mechanism of **MeHg** induced oxidative stress. It inhibit glutamate reuptake by the astrocytes which led to increase in glutamate in the extracellular spaces, and it increase Ca^{+2} and and Na^{+} influx which induce excitotoxicity by over activate NMDA receptors (Farina et al., 2011).

Our study shows that MeHg causes a dose dependant increase in astrocytes (GFAP). As MeHg is a neurotoxic chemical, this may cause astrocytes to be reactive and increase the synthesis of GFAP, which is called reactive astrogliosis, and which occurs after injury, either due to trauma, diseases, genetic disorders, or

due to chemical exposure, as in the case of MeHg (Eng et al., 2000). Astrogliosis considered as an early sign of neurotoxicity (Zurich et al., 2004, O'callaghan, 1991). Reactive astrocytes are able to release bioactive substances to modulate the neurotoxicity of these chemicals, some of them have neuroprotective while the others may have neurodegenerative potentials (Aschner et al., 2002).

5.3.2 Lead

In spite of remarkable success in abating lead (Pb) exposure, Pb remains an important health problem to children in both developed and developing countries (Bellinger, 2013). Pb is an ubiquitous environmental pollutant that can pass through the placenta and can cross the blood brain barrier to induce neurotoxicity. Exposure to Pb, even at low doses, can be hazardous (Yu et al., 2011). The results reported here show that lead decreases cell viability and total protein significantly at 50µM and more, and this can explained by Pb leading to mitochondrial and Golgi apparatus dysfunction (Strużyñska et al., 2001) and Pb exposure can result in cell death by apoptosis, which has been described before when cerebellar cells were exposed to it (Sharifi et al., 2002). Pb can affect the nervous system by several pathways; Pb is able to pass through the blood brain barrier due to its capability to substitute for calcium ions, therefore Pb gains entry into the cells through one or more of the various types of Calcium channels (ATSDR, 2007). Within the cells, Pb interferes with calcium release from the mitochondria leading to formation of a permeability transition pore which enhances programmed cell death and mitochondrial self- destruction (Mason et al., 2014). Additionally, Pb interferes with the phosphorylation of protein kinase C (PKC) which is activated due to increased intracellular Ca^{+2} concentration and Diacylglycerol (DAG), which in turn increases cell permeability to water and

other ions, which result in oedema and cell damage (Bressler and Goldstein, 1991).

Oxidative damage is another important mechanism of lead neurotoxicity; experimental evidence has shown that Pb induces oxidative stress through the disruption of pro-oxidant/anti-oxidant balance (Daniel et al., 2004, Villeda-Hernandez et al., 2006). The reduction of antioxidant activities might be due to the high affinity of Pb for sulfhydryl groups or metal cofactors in antioxidant enzymes (Gurer and Ercal, 2000). It has been shown that Pb causes significant reduction in the activity of superoxidase dismutase (SOD) and catalase (CAT) in rat and mouse brains (Nehru and Kanwar, 2004). Moreover, it has been found that Pb exposure during pregnancy reduces SOD, glutathione perioxidase (GPx) and glutathione (GSH) in rat brains (Wang et al., 2006), figure 35.

The two important pathways which are concerned with Pb are those for calmodulin and PKC. Ca^{+2} binds to calmodulin and also converts it to its active form, while Pb displace Ca^{+2} bound to calmodulin; Pb activation of calmodulin results in protein phosphorylation altering the cAMP messenger pathways (Goyer, 1997). Normally, Ca^{+2} activates PKC, which is a serine threonine protein kinase involved in dendritic branching, neurotransmitter synthesis and synaptic transmission (Bressler et al., 1999). This might explain the significant reduction in neurite length.

Several neurotransmitters also are affected by Pb interaction during development of the CNS. In animals exposed to low doses of Pb during development, high level of dopamine and catecholamine neurotransmission have been observed in the cerebral cortex, cerebellum, and hippocampus. While rats exposed to high

dose of Pb have a decrease in dopamine and catecholamine neurotransmission in the same areas of the brain (Devi et al., 2005), figure 35.

Pb enters astrocytes from receptor-operated and voltage-dependant Ca^{+2} channels which mediate Pb uptake into the cells; Pb is deposited in the lysosomes, nucleus, and other organelles of astrocytes, a process which might be occuring through Pb-binding proteins (Tiffany-Castiglioni and Qian, 2001). Astrocytes play an important role in protecting neurons against Pb neurotoxicity (Harry et al., 1996); this may explain there was no significant effect on tubulin III protein (the neuronal cells). GFAP in these result was increased significantly and in a dose dependant way. As it was suggested by O'Callaghan that this intermediate filament can be considered as an early sign of neurotoxicity (O'callaghan, 1991). The increase in this protein represents astrogliosis, in which the astrocytes react to the toxic insult by an increase in synthesis of GFAP and the astrocytes become hypertrophied and increased in number (Strużyñska et al., 2001). Several studies confirm that rat brain exposed to Pb showed an increase in the concentration of GFAP as well as the number of astrocytes (Strużyñska et al., 2001, Tiffany-Castiglioni and Qian, 2001).

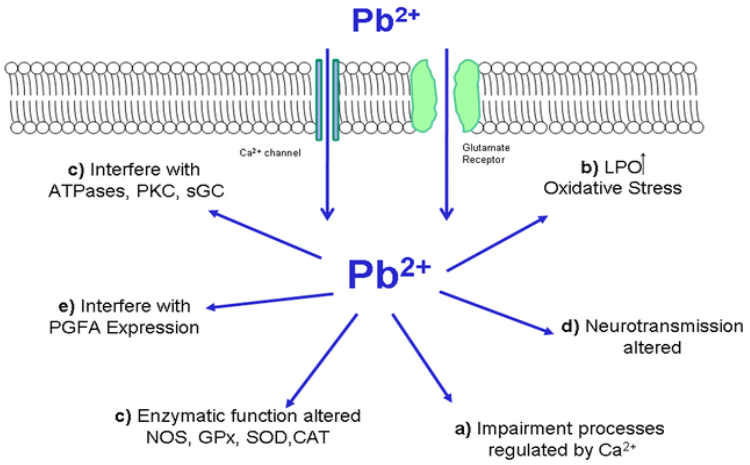


Figure 35: Mechanisms of **lead** toxicity (Nava-Ruiz et al., 2012)

It can be concluded that HNSC culture method can detect the neurotoxicity of methyl mercury at low level (1µM) in most of the end points that were tested and the neurotoxicity of lead acetate at 10µM in cell migration.

Chapter 6

Evaluation of This Model with Anti-epileptics

6.1 Anti-epileptics and Teratogenesis

Epilepsy is a common brain disorder characterize by recurrent seizures; it affects about 1% of the whole population and the highest incidence is in the first year of life (Hauser, 1994). Anti-epileptic drugs (AEDs) are used to control seizures but they are also used in the treatment of other neurological and psychiatric disorders such as bipolar disorders, migraine, movement disorders, and neuropathic pain (Rogawski and Löscher, 2004b). AEDs act on ion channels, metabolic enzymes, neurotransmitter transporters in the CNS. They change the bursting activities of neurons, prevent spread of epileptic activity and decrease synchronization (Rogawski and Löscher, 2004a). Additionally, AEDs interfere with neuronal migration, cell differentiation and plasticity, and they can lead to neuronal cell death in the developing brain in rodent (Olney et al., 2004).

About 3 to 7 out of 1000 pregnant women have epilepsy (Viinikainen et al., 2006); these women should have medication to control seizure activity; otherwise it will be harmful for both, the mother and the baby. The convulsion itself carries a lot of risks to the fetuses; it can cause intracranial haemorrhage and alteration in fetal heart rate (Fountain, 2009). Therefore; continuation of antiepileptic drugs is mandatory to save the life of the mother and her baby.

It is well known that the use of antiepileptic drugs during pregnancy can cause a wide range of congenital abnormalities (Meador et al., 2006); the original reports about the association between these drugs and congenital anomalies goes back to the 1960,s (Mullers-Kuppers, 1963). Meador et al. (2008) state that a monotherapy regimen for epileptic pregnant women can increase the incidence of congenital anomalies in their children threefold compared to that of healthy

women and polytherapy makes the incidence higher than that of monotherapy. Nearly all antiepileptic drugs are not safe and not free from teratogenic potential (Prabhu et al., 2008). The teratogenicity of these drugs is also dose dependant (Tomson et al., 2011).

6.1.1 Mechanism of action of antiepileptics

1- Interaction with ion channels

The main target of AEDs is the voltage-gated ion channel, because these channels are critical in determining the excitability of neurons and regulation of neurotransmitter release. By AEDs interaction with ion channel, they inhibit epileptic bursting activities, seizure propagation and synchronization. Sodium or calcium blockage with facilitation of potassium channels results in control of seizure activity in epileptic animal models (Tatulian et al., 2001).

Voltage gated sodium channels are composed from several subunits, which undergo conformational modification when the neuron is depolarized at the action potential threshold. This modification changes the channel into the open conductive state to allow sodium influx for only few milliseconds. Channels inactivate quickly and could be reactivated after repolarization. This quick changing between open and closed status of sodium channels is important for both the normal brain and for seizure discharge (Leach et al., 1986). Phenytoin and carbamazepine act by changing voltage gated sodium channels. Blockage of these channels can also be achieved by sodium valproate. These drugs do not affect the physiological activity of the neurons, but they inhibit the frequent repetitive firing. This means that these drugs do not make any impairment in general brain function. Also, these AEDs decrease the output of transmitter at the

synapses but they do not alter the excitatory or inhibitory synaptic responses (Prakriya and Mennerick, 2000).

Voltage gated calcium channels have two types; high voltage-activated (HVA) and low voltage-activated families. HVA can only be opened when there is a strong depolarization and can regulate calcium and neurotransmitter release from the presynaptic neuronal terminals, so it reduces neuronal excitability. Phenobarbitone blocks this type of calcium channel (Barker and Rogawski, 1993). The low voltage-activated channels (T-type) control neuronal firing and regulate bursting and intrinsic oscillations (Perez-Reyes, 2003). In 'absent seizures' there is abnormal oscillatory behaviour (Huguenard, 1996). This type of calcium channel generates a low threshold spike of calcium and stimulates a burst of action potential with the aid of sodium channels in thalamic reticular neurons (Suzuki and Rogawski, 1989). Sodium valproate is effective in the treatment of seizures by blocking this type of channel (Sankar and Holmes, 2004).

2- Interaction with neurotransmitters:

Several AEDs act by inhibition of synaptic activities or by reducing synaptic excitation. By interaction with neurotransmitter receptors and the ion channels which regulate the neurotransmitters, these drugs can reduce bursting and seizure spread. There are two neurotransmitter systems which are the target of AEDs; the inhibitory GABAergic system and the excitatory glutamatergic system. AEDs exert their anticonvulsant action either by enhancing the inhibitory system mediated by GABA (γ-amino butyric acid) receptors or blocking the excitatory glutamate receptors (such as *N*-methyl-D-aspartate – NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid – AMPA, kainite, group I

metabotropic receptors) (Kaindl et al., 2006). There are other neurotransmitter systems which are also involve in controlling seizure activities such as the glycine system, monoamines, neuropeptides, galanin, neuropeptide Y, adenosine, serotonin and histamine (Rogawski and Löscher, 2004a).

6.1.2 GABA inhibitory system

Inhibitory synapses are essential in regulating the excitatory neurons and preventing synchronized epileptiform discharge (Miles and Wong, 1987). Mediating GABA inhibition is the major mechanism of action of AEDs. GABA stimulates chloride-permeable $GABA_A$ receptors and slower metabotropic G-protein-coupled $GABA_B$ receptors. Therefore, any compound that blocks $GABA_A$ receptors can be considered as a proconvulsant. Mutations of this subunit have been associated with broad spectrum of epilepsy syndromes like childhood absence epilepsy, febrile convulsion, Dravet's syndrome, and Juvenile myoclonic epilepsy (Wallace et al., 2001, Cossette et al., 2002).

Barbiturates like phenobarbitone modulate $GABA_A$ receptors. Phenobarbitone at the clinically relevant concentration makes the channels open for a proportionally longer duration (Macdonald and Olsen, 1994), Barbiturates act on both sodium and calcium channels (Barker and Rogawski, 1993).

6.1.3 Glutamate Excitatory system

Glutamate receptors are glutamate-gated cation channels which facilitate excitatory neurotransmission in the CNS (Dingledine et al., 1999). The compounds that block NMDA, AMPA, and kainite receptors are effective anticonvulsants in epileptic animal models (Rogawski et al., 2003). AMPA and kainite receptors control seizure propagation and regulate seizure induced brain

damage (Meldrum, 1995). Kainate receptors also mediate glutamate release from excitatory afferent neurons and reduce GABA release from inhibitory interneurons (Lerma et al., 2001).

Drug	GABA system	Glutamate receptors	Sodium channels	Calcium channels
Sodium valproate	+		+	+
Phenytoin			+	
Carbamazepine		+	+	
Phenobarbitone	+	+	+	+

6.1.4 The Anti-epileptics

1- Sodium Valproate

Sodium Valproate (SV) is Valproic acid sodium salt. It was discovered by accident as an anticonvulsant in 1963 when SV was used as a solvent for other compounds which were administered to animals used in experimental models of epilepsy. Since then, SV has been used for different types of seizures. It is used for the treatment of epilepsy in more than 100 countries (Loescher, 2002). SV is also used as a mood stabilizing agent in bipolar mood disorder and it is recommended by many psychiatric and pharmacological societies as a first line drug for treatment of acute mania and as maintenance therapy for the prevention of mania and sometimes recommended for depression (Fountoulakis et al., 2005). In addition to that SV is prescribed as prophylactic drug against headaches in migraine (Chronicle and Mulleners, 2004) and has been proposed for the treatment of cancer (Blaheta et al., 2002), Alzheimer Disease (Tariot et al., 2002) and also it is used for the treatment of latent HIV infection (Lehrman et al., 2005).

6.1.4.1 Mechanisms of action of SV

1- SV administration causes an increase in the inhibitory neurotransmitter GABA by direct inhibition of GABA transaminase which is responsible for GABA breakdown (Maitre et al., 1974). Potentiation of GABA signaling can prevent seizure activity while inhibiting GABA signaling can cause seizures, therefore, this can explain the anticonvulsant property of SV

2- SV indirectly inhibits slow sodium currents and calcium currents (De Sarro et al., 1992). Sodium and calcium channels are very important in neuronal excitability. SV has been reported to block neuronal excitability and action potential firing (McLean and Macdonald, 1986). SV acts to normalize neuronal firing and to increase the threshold of neural discharge. This mechanism can explain the action of this drug in migraine (Casucci et al., 2008).

3- SV is a potent inhibitor for HDACs (Histone Deacetylase) (Phiel et al., 2001). The discovery of this fact opens a wide window to understand the teratogenecity of this drug and to explain its uses in cancer and treatment of latent HIV infection.

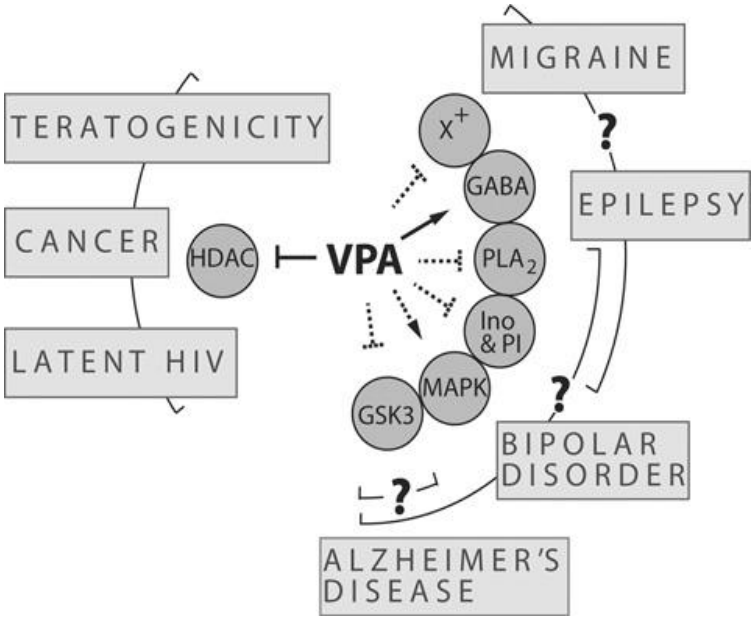


Figure 36: The targets of VPA (valproic acid) and its potential use in medicine (Terbach and Williams, 2009)

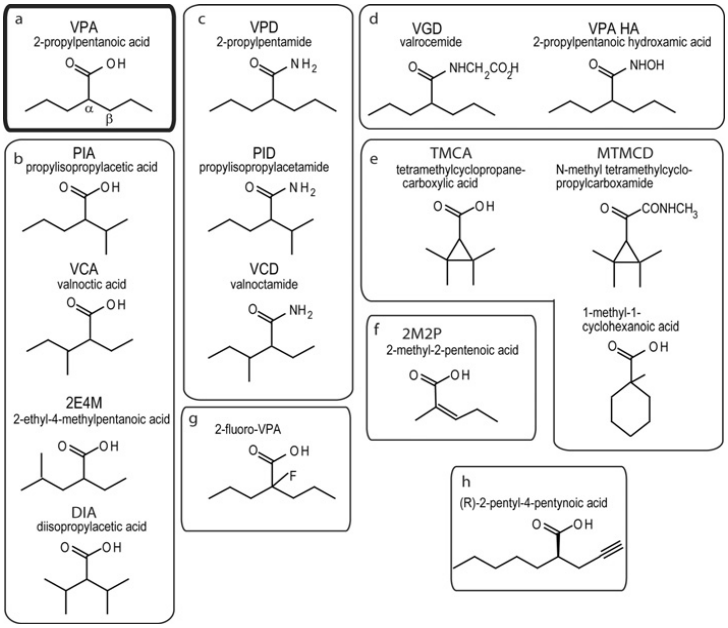


Figure 37: The chemical structure of VPA (valproic acid) and its related compounds (Terbach and Williams, 2009)

2- Phenytoin

Phenytoin-PHN (Diphenylhydantoin) is the most common drug that used for treatment of epilepsy; it was used as an anticonvulsant in the 1930s (Merrittt and Putnam, 1938). PHN exert its anticonvulsant action at serum level 10-20μg/ml,

which correspond to 40-80μM, without causing sedation or interfering with the normal CNS function (Yaari et al., 1986). It is highly effective and is the drug of choice for several types of epilepsy (Temiz et al., 2009). PHN is well known in its teratogenicity for different species such as rabbit (Danielsson et al., 1992), rats and mice (Finnell and Dansky, 1991) as well as humans (Dansky and Finnell, 1991). Because it is well documented concerning its teratogencity in humans and animals, it is placed in FDA pregnancy category D (Ozolins et al., 1995).

Maternal exposure to PHN during pregnancy increases the risk of having a child with congenital anomalies by two to three fold with a characteristic anomaly Fetal Hydantoin Syndrome (Ozolins et al., 1995), which is characterized by different signs such as facial, skull abnormalities, like short nose, low nasal bridge, hypertelorism, epicanthal fold, low hair line, hypoplasia of distal phalanges and nails. This syndrome can be observed in 10% of PHN treated mothers (Ornoy, 2006). The exact mechanism for its teratogenicity is unknown and needs to be clarified. The pattern of birth defects in humans, rabbits, rats and mice is similar; this suggest that the teratogenicity follows the same pathway (Azarbayjani et al., 2006). Azarbayjani thought that PHN teratogenicity is mostly related to excessive generation of reactive oxygen species or to impaired antioxidant defence mechanisms.

3- Carbamazepine

Carbamazepine (CBZ) is an iminostilbene structurally related to tricyclic antidepressants (Ambrósio et al., 2002) and shares some clinical similarities with structurally unrelated valproic acid, which has a short chained fatty acid structure (Beutler et al., 2005). Since its introduction in clinical practice for treating

epileptic patients in the mid-1960s, it has become the most frequently prescribed first line drug for the treatment of generalized tonic-clonic and partial seizures (Loiseau, 1995). Additionally, CBZ can be used in the treatment of neuropathic pain (Sindrup and Jensen, 1999) and psychological disorders (Albani et al., 1995). In spite of being effective and safe in treating epileptic patients, 30-40% of patients do not respond completely to CBZ treatment (Shorvon, 1996). Sometimes, CBZ may cause side effects. Acute toxicity at therapeutic doses affect the central nervous system and gastrointestinal system, causing sedation, ataxia, dizziness, nausea, vomiting, constipation, and diarrhoea. While chronic treatment with CBZ may lead to alteration in plasma lipids, hyponatraemia, increased appetite and weight gain, reduced white blood cell number and allergic reactions (Albani et al., 1995). The mechanisms by which this drug causes these side effects are not clear. The mechanism of action of this drug is still not known but it is widely accepted that this drug acts by more than one mechanism. It has been thought that it may block or enhance voltage gated Na+, Ca+2, and K+ channels, and it may modify uptake, release, receptor binding of neurotransmitters in serotonergic, dopaminergic, and glutamergic systems (Ambrósio et al., 2002). It is most commonly used in Europe by women of child bearing age; it can increase the risk of major congenital anomalies by 3.3%, the main congenital anomalies that associated with CBZ are spina bifida, cardiovascular, and other minor anomalies; but in general, it is less teratogenic than Valproate (Jentink et al., 2010b).

4- Phenobarbitone

Phenobarbitone (PHB) (phenyl-ethyl-barbiturate-acid), also known as phenobarbital, is one of the oldest anticonvulsants. It was first discovered by

German chemist Emil Fischer (Kwan and Brodie, 2004). The anticonvulsant activity of PHB was published by German physician Alfred Hauptmann in 1912 (Kumbier and Haack, 2004). At that time, bromide was used as standard drug for treating epileptic cases. Hauptmann used PHB for severe cases that were resistant to bromide, he found that PHB reduce the frequency of seizures and decrease their severity. Eventually PHB became the drug of choice in treating epilepsy around the world.

PHB was first approved by the FDA in 1939 (Salas, 2012). It is commonly used as an anti-epileptic for all the types of epilepsy except petit mal (absence seizure), as a sedative, for treatment of hyperbilirubinemia and insomnia. Animal studies on chicks and rodents have shown that maternal exposure to PHB at several times the average human dose might lead to increased risk for cleft palate, cardiac defect, and other major structural defects (Finnell and Dansky, 1991). Also, animal studies showed that the number of major structural defects was directly related to the exposure doses of PHB during pregnancy (Finnell et al., 1987).

While the animal data confirmed the teratogenicity of PHB, human data is not clear. It has been shown that in utero exposure to PHB may result in long lasting neurobehavioral effects in the human such as impairment in cognitive development (van der Pol et al., 1991) and low IQ scores (Reinisch et al., 1995). Instead of that, the embryo toxicity and teratogenicity of PHB seem to be less than that of other AEDs in animal models (Holmes et al., 2001). In vivo and in vitro animal studies demonstrated that PHB is associated with biochemical and morphological abnormalities (Bergey et al., 1981b). Several studies on the association of PHB with major congenital anomalies in the human have been published (Pennell, 2008). The same author showed also that the risk of having a

baby with a birth defect is higher when PHB was combined with another antiepileptic, polytherapy. An epidemiological study showed that PHB drug exposure during the first trimester is associated with major and minor structural defects (Salas, 2012). Postnatal exposure to PHB especially during the first 3 years of life may lead to impairment in cognitive function which persists throughout adulthood (Reinisch et al., 1995). A pattern of minor structural defects has been identified with the use of AEDs known as fetal anticonvulsant syndrome, with similar features to the syndrome being identified in infants of mothers who are exposed to PHB during pregnancy. These features include nail hypoplasia, midface hypoplasia, depressed nasal bridge, hypertelorism, and epicanthal fold (Salas, 2012). Other studies found an association between PHB exposure during pregnancy and facial clefts and cardiovascular defects (Arpino et al., 2000) and it increased the risk of having infants with a smaller head circumference and low birth weight (Dessens et al., 2000).

Mechanism of action of Phenobarbitone:

PHB acts as anticonvulsant by increasing the threshold for stimulating the motor cortex and by inhibiting seizures in the cortex, thalamus, and limbic system. PHB works by facilitating GABA, an inhibitory neurotransmitter; it stimulates GABA binding to GABA-A receptors which leads to hyperpolarization of neuronal cell membranes and thus it prevents propagation of seizure activity (Salas, 2012). Additionally, PHB decreases the effect of glutamate, the excitatory neurotransmitter in the brain (Platt, 2007). PHB increases the time of the chloride channel to be in the open state (Macdonald and Olsen, 1994) and also PHB acts on both sodium and calcium channels (Barker and Rogawski, 1993).

The anti-epileptics have been chosen because most anti-epileptics are well known in their teratogenicity, sodium valproate or phenytoin administration during pregnancy associate with high rate of congenital anomalies. While the other, (Carbamazepine and phenobarbitone), are less teratogenic. Therefore, human neural stem cell culture model was used to test if it can predict the neurotoxicity of these anti-epileptics at their therapeutic level.

6.2 Results

6.2.1 Sodium Valproate

SV at doses of 250, 500, 750 and 1000µM, which lie around the therapeutic range (400-800µM) had no significant effect on the resazurin assay when compared with the control group, (figure 38-A&B), At 1000µM, the protein content was reduced significantly, ($F(7,16)=17.27$, $p < 0.0001$, post-hoc: 1000µM vs control: $p<0.01$) and at higher doses, 1500, 2000, and 2500µM there was a highly significant reduction in both cell activity, ($F(7,16)=40.16$, $p<0.0001$, post-hoc: 1500 vs control: $p<0.0001$), and protein content when compared with the control group (1500µM vs control: $p<0.0001$), which would indicate that valproate is very toxic at these doses.

On the other hand, figure 38-C shows no significant effect of SV on the length of neuronal processes, except at the dose of 2000µM when there was significant reduction in the neuronal length when compared with the control group, ($F(5,12)=8.637$, $p=0.0011$, post-hoc: 2000µM vs control: $p<0.05$). Figures 38-D and 39 show that SV has a highly significant effect on the sizes of neurospheres through all the doses, including the therapeutic doses 500µM, ($F(5,12)=23.21$, $p<0.0001$, post-hoc: 500µM vs control: $p<0.01$, and 750µM vs control: $p<0.001$), probably due to its anti-proliferative action on the neural stem cells.

SV seems to have no significant effect on tubulin III protein in all doses in spite of the non-significant increase in therapeutic range, while SV decreases GFAP significantly at high doses 1500µM, ($F(5,12)=3.657$, $p=0.0305$, post-hoc: 1500µM vs control: $p<0.05$, and 2000µM vs control: $p<0.01$), figure 38-E and F and figure 40. In the case of cell migration, SV has no significant effect on cell migration after the 1st 24 hours at all the doses, but the detrimental effect was significant with increasing the duration, ($F(1,24)=148.6$, $p<0.0001$), and with increase concentration, ($F(5,24)=8.041$, $p=0.0001$). From the doses of 1000µM there is significant reduction in migration distance when compared with the control, (concentration vs time: $F(5,24)=1.611$, $p=0.1953$, post-hoc: 1000µM vs control: $p<0.05$), 1500µM vs control ($p<0.01$), and 2000µM vs control ($p<0.001$) after 48 hours of treatment, figure 38-G.

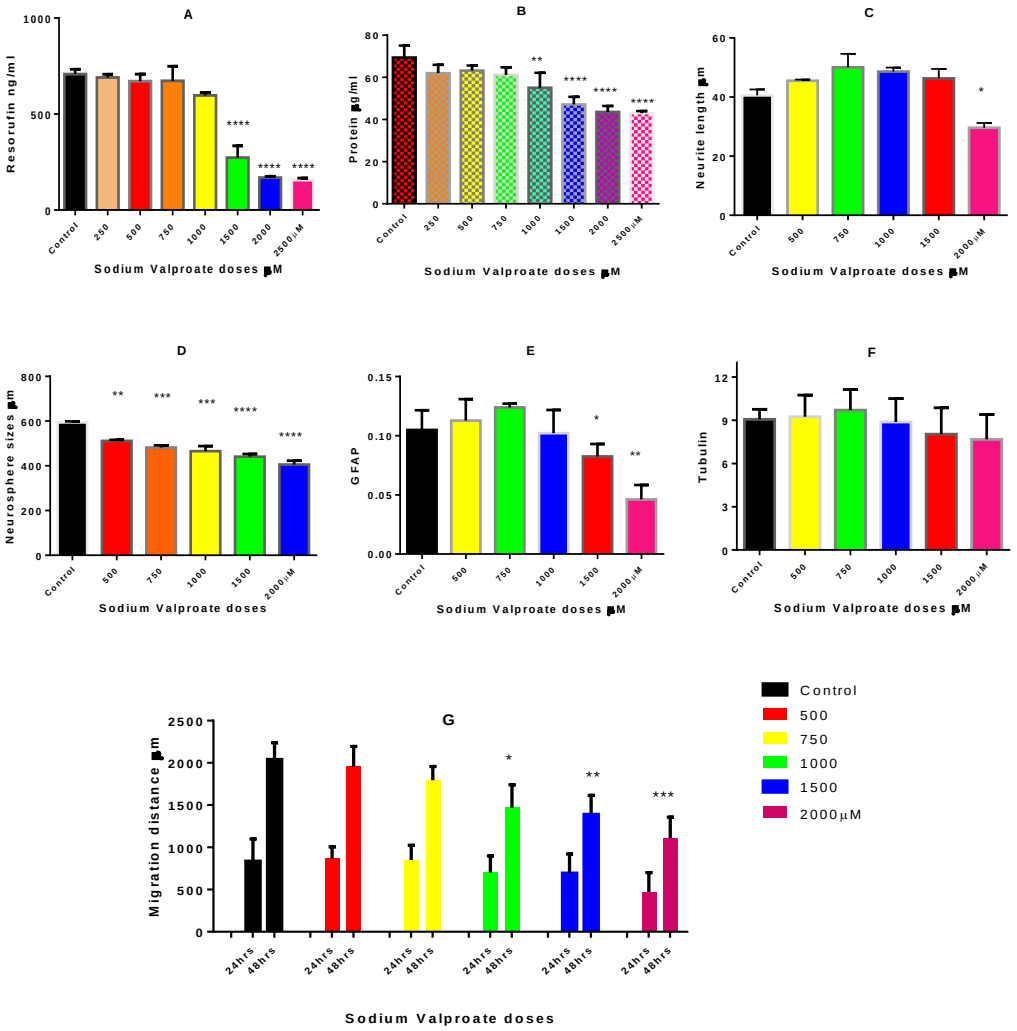


Figure 38: Effect of **Sodium Valproate** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

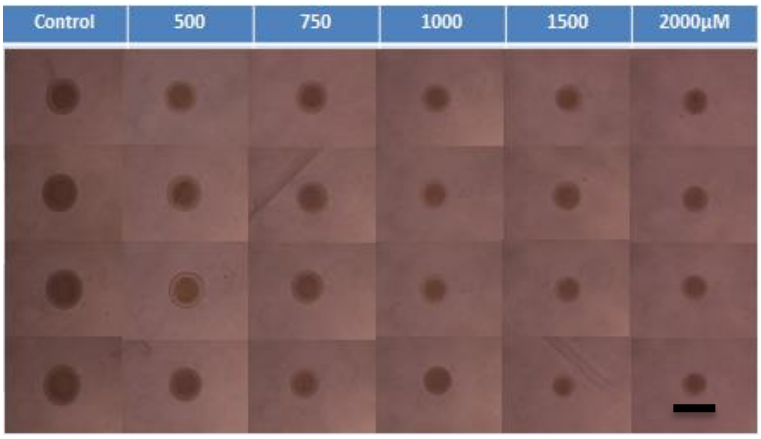


Figure 39: The effect of different doses of **sodium valproate** on the sizes of neurospheres. This figure illustrates photos of 24 wells of the same plate at the same magnification, the scale bar is 700µm.

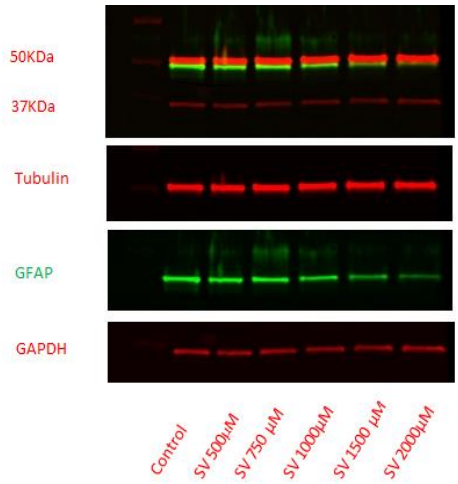


Figure 40: Western Blot analysis for the cells after **Sodium Valproate** treatment showing the bands of Tubulin III, GFAP, and GAPDH proteins, illustrating the progressive reduction in the band of GFAP with increasing the dose of the drug.

6.2.2 Phenytoin

PHN was used in doses which lie slightly below, within, and above the therapeutic range (40-80µM). Figure 41-A and B show that the doses of 20, 50, 100, and 150µM have no significant effect on both resorufin production, and protein amount, respectively when compared with the control, but, the resorufin production significantly reduced in the dose range, 200, 330, and 660µM, ($F(7,16)=43.26$, $p<0.0001$, post-hoc: 200µM vs control: $p<0.0001$), and protein amount start to be affected significantly from the dose of 200µM, ($F(7,16)=8.959$, $p=0.0002$, post-hoc: 200µM vs control: $p<0.01$). It seems that PHN has no significant effect on the length of the processes around the therapeutic doses; however, it cause highly significant reduction at the dose of 150µM, ($F(5,12)=21.54$, $p<0.0001$, post-hoc: 150µM vs control: $p<0.001$), figure 41-C.

The neurosphere sizes were affected severely by PHN in all doses, in which there is highly significant reduction in the sizes of the neurospheres from the dose of 20µM when compared with that of control group, ($F(5,12)=299.9$, $p<0.0001$, post-hoc: 20µM vs control: $p<0.001$), figure 41-D. GFAP decreased significantly at 100µM dose, ($F(5,12)=11.67$, $p=0.0003$, post-hoc: 100µM vs control: $p<0.05$), and the GFAP expression decreased significantly with increasing the doses, while tubulin III protein started to decrease significantly at 150µM, ($F(5,12)=8.953$, $p=0.0010$, post-hoc: 150µM vs control: $p<0.05$), figure 41-E and F and 42. Regarding cell migration, it appears that PHN reduces cell migration in direct proportion to the duration, ($F(1,24)=126.3$, $p<0.0001$), and to the dose, ($F(5,24)=32.83$, $p<0.0001$). Ph seems to reduce migration distance significantly at 50µM, (concentration vs time: $F(5,24)=2.475$, $p=0.0607$, post-hoc: 50µM vs control: $p<0.01$) after 24hrs and $p<0.0001$ after 48 hours, figure 41-G.

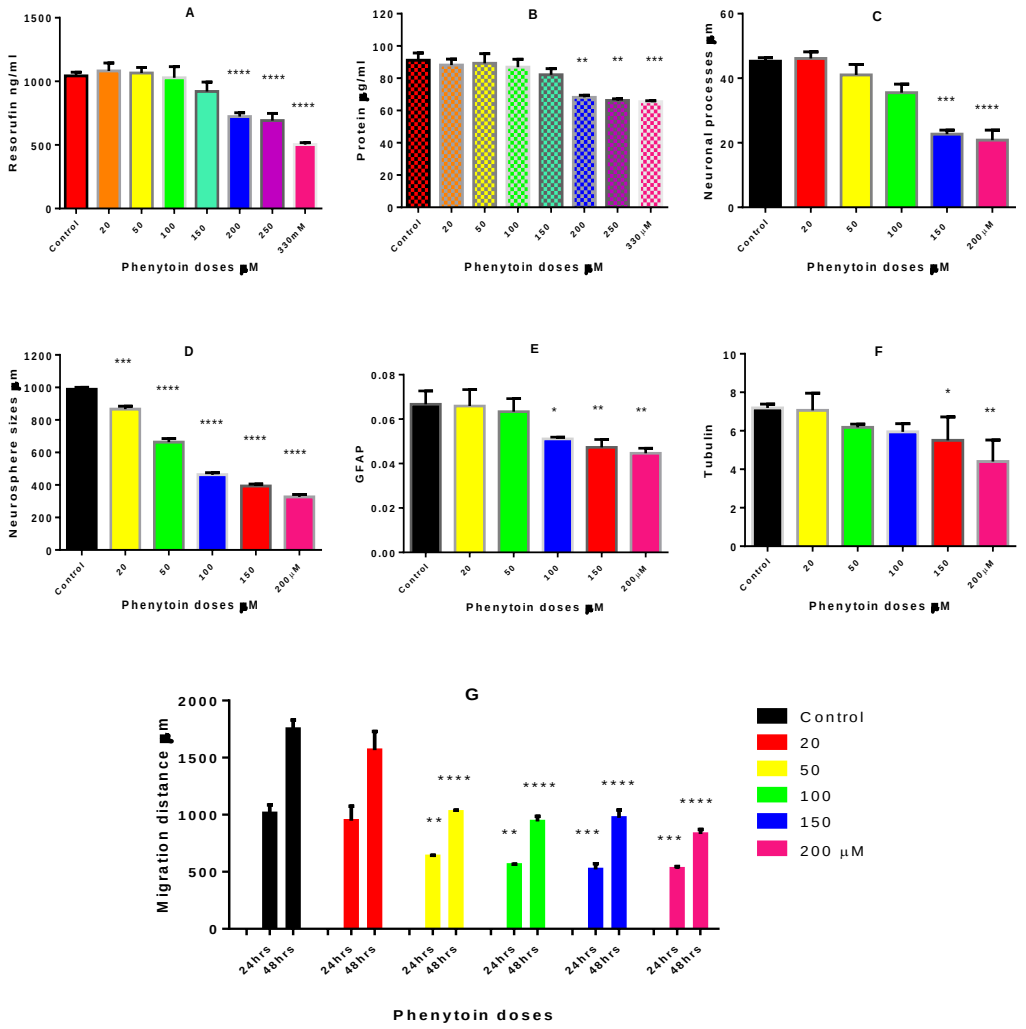


Figure 41: Effect of P**henytoin** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

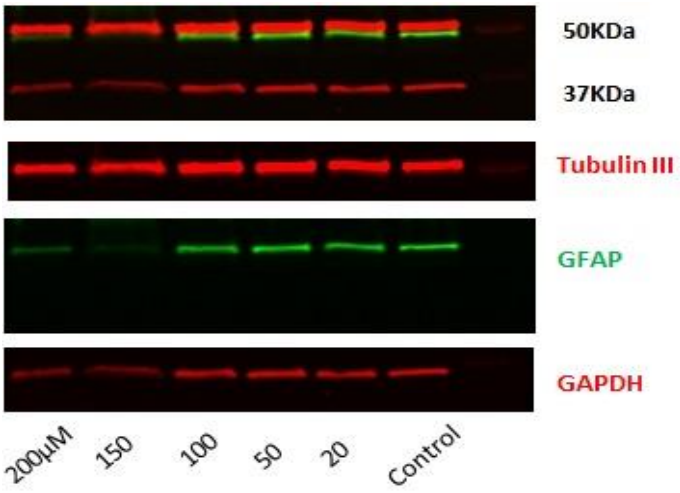


Figure 42: Western Blot analysis for the cells after **Phenytoin** treatment showing the bands of Tubulin III, GFAP, and GAPDH proteins.

6.2.3 Carbamazepine

At the dose range 5-100µM, CBZ has no significant effect on resorufin production, taking into consideration that the therapeutic dose of this drug is 25-50µM. However, at high doses of 250µM-1mM the resorufin production was reduced significantly when compared with the control, ($F(7,16)=104.9$, $p<0.0001$, post-hoc: 250µM vs control: $p<0.0001$). However, total protein amount was not significantly affected until the dose of 500µM and 1mM in which there is highly significant reduction in total protein amount when compared to control group, ($F(7,16)=18.49$, $p<0.0001$, post-hoc: 500µM vs control: $p<0.0001$), as shown in figure 43-B.

The effect on the length of neuronal processes appears to be not significant in all doses except at 200µM where they appears to be a highly significant reduction, ($F(5,12)= 30.04$, $p<0.0001$, post-hoc: 200µM vs control: $p<0.0001$), as shown in figure 43-C. The sizes of the neurospheres started to be reduced significantly also at 200µM, ($F(5,12)=8.715$, $p=0.0011$, post-hoc: 200µM vs control: $p<0.05$), figure 43-D. GFAP and tubulin III proteins was not affected significantly in all doses except in the last extreme dose 375µM at which they were reduced significantly, ($F(5,12)=4.190$, $p=0.0196$, post-hoc: 375µM vs control: $p<0.05$) for GFAP and ($F(5,12)=3.562$, $p=0.0332$, post-hoc: 375µM vs control: $p<0.05$) for Tubulin III, figure 43-E,F and 44. CBZ seems to reduce cell migration at 100µM, ($F(5,24)=0.8282$, $p=0.5422$, post-hoc: 100µM vs control: $p<0.05$), and there was no increase in significance with increasing the dose or increase the duration and its effect appears just 48 hours after treatment, figure 43-G.

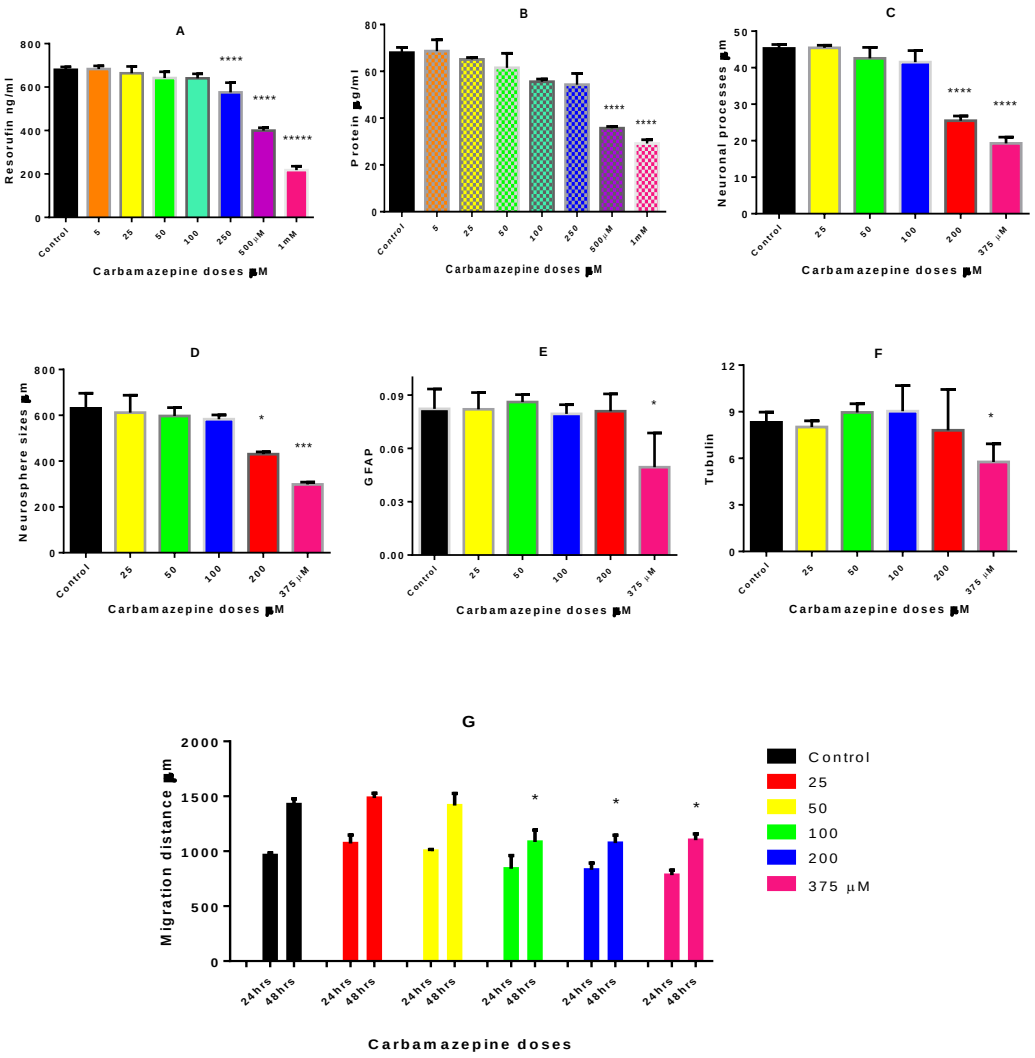


Figure 43: Effect of **Carbamazepine** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

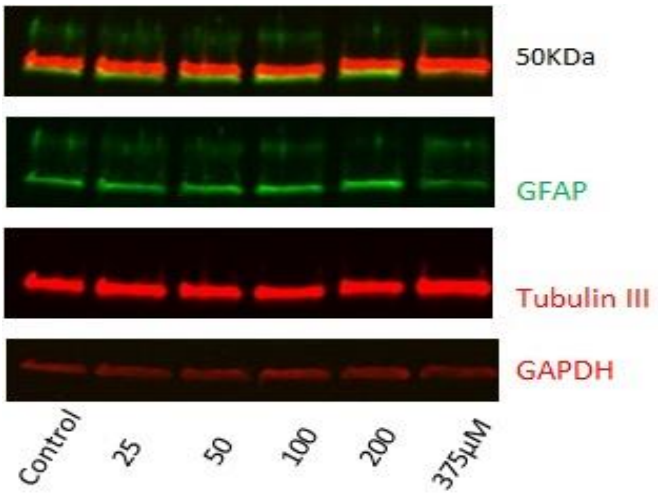


Figure 44: Western Blot analysis for the cells after **Carbamazepine** treatment showing the bands of Tubulin III, GFAP, and GAPDH proteins.

6.2.4 Phenobarbitone

It appears that PHB has no significant effect on cell viability and total protein of the cells in the dose range 100µM-2000µM. However, it reduces both these parameters at the extreme doses 3000 and 4000µM in which there is a significant reduction in cell viability, ($F(7,16)=10.97$, $p<0.0001$, post-hoc: 3000µM vs control: $p<0.05$) and total cellular protein, ($F(7,16)=3.911$, $p=0.0113$, post-hoc: 3000µM vs control: $p<0.05$), figure 45-A and B. The figure 45-C shows that PHB has no significant effect on the process length except in high dose 2000µM, in which there high significant effect on neuronal processes length, ($F(5,12)=7.598$, $p=0.0020$, post-hoc: 2000µM vs control: $p<0.01$). In other hand, PHB seems to reduce the neurosphere size at a significant rate at the doses of 1000µM, ($F(5,12)=8.715$, $p=0.0011$, post-hoc: 1000µM vs control: $p<0.05$) and (2000µM vs control $p<0.0001$) when compared with that of control group, figure 45-D.

Neuronal cell population, which is represented by Tubulin III protein, appears to be not affected in all doses of PHB, but the astrocyte population, which was marked by GFAP was reduced only at high doses 1000µM, ($F(5,12)=9.598$, $p=0.0007$, post-hoc: 1000µM vs control: $p<0.05$) and (2000µM vs control $p<0.01$), figure 45-E,F and 46. In the case of cell migration, PHB has no significant effect on migration except at high dose 2000µM and only after 48hr from drug treatment there is high significant reduction in cell migration when compared with the control group, (concentration vs time: $F(5,24)=1.057$, $p=0.4099$, post-hoc: 2000µM vs control: $p<0.001$), figure 42-G.

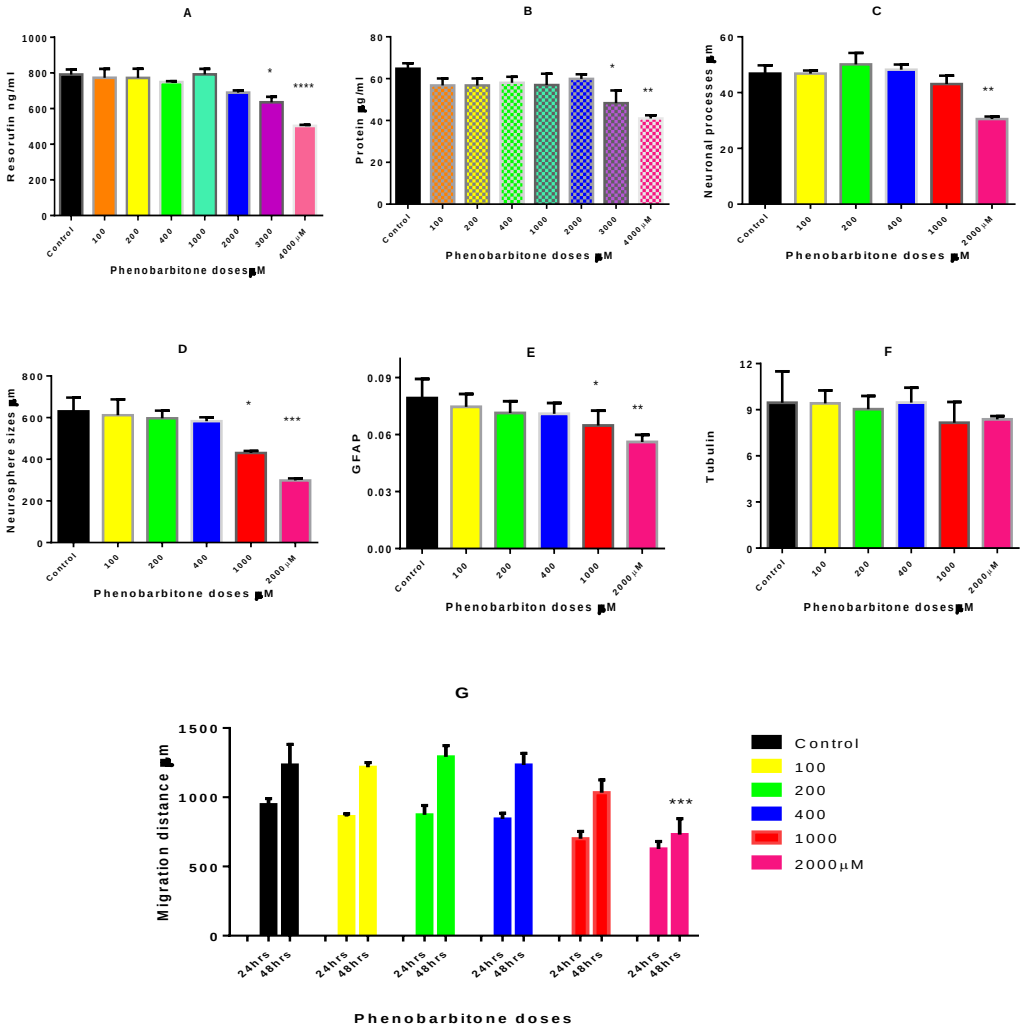


Figure 45: Effect of **Phenobarbitone** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

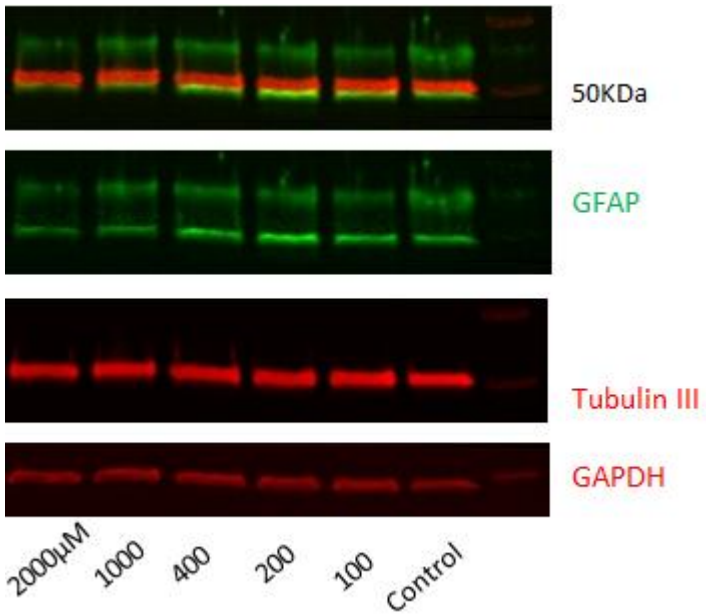


Figure 46: Western Blot analysis for the cells after **Phenobarbitone** treatment showing the bands of Tubulin III, GFAP, and GAPDH proteins.

6.3 Discussion

6.3.1 Sodium Valproate

Sodium valproate has several therapeutic applications as an anticonvulsant, anti-manic and anti-cancer drug and is also used for migraine prophylaxis. Its use in pregnancy has been shown to carry risks to the developing embryo and could result in a wide spectrum of developmental anomalies such as neural tube defects, craniofacial, cardiac and genital birth defects (Nau et al., 1991) and (Nau, 2008). The first report which suggested teratogenicity of valproate in the human was published in 1980, which demonstrated the association of sodium valproate intake during pregnancy with neural tube defects (meningocele and meningomyelocele) (Dalens et al., 1980).

SV appears to have no significant effect on cell activity or total protein levels at the doses which locate around the therapeutic range. However, at higher doses (1500, 2000, and 2500µM) SV caused a highly significant reduction in both these

parameters and appears to be very toxic. It has been reported that SV can stop the cell cycle at phase G0/1 and can arrest cell proliferation and induce apoptosis *in vitro* (Pandolfi, 2001). This may explain the reduction in cell activity and total protein only being apparent at high doses. However; Chiu et al. (2013) pointed out that SV supplement within or close to the human therapeutic dose results in hyperacetylation of histone which leads to changes in chromatin and alters transcriptional activity, modulates gene expression, enhances cell differentiation and increases apoptosis (Phiel et al., 2001). Gurvich et al. (2005) suggest that the teratogenesis of SV is through its inhibition of Histone Deacetylase (HDAC). Furthermore, SV causes demethylation of DNA which occurs due to acetylation of H3 histones. DNA demethylation leads to alteration in gene expression and various congenital anomalies (Detich et al., 2003). Therefore; it appears that these reports can explain only the results of high doses since the resorufin and protein amount was not affected in the dose range 500-1000µM. It may be that in the *in vitro* model there is no bioactivation of the drug, which make its effects appear mainly at high doses.

The results show that SV had no significant effect on the length of neuronal processes at low doses. SV acts in a way similar to the action of neurotropic factors on CNS cells by stimulating the ERK pathway (extracellular signal regulated kinase). This is involved in neurite growth, neural stem cell proliferation and maturation, neurogenesis and neural regeneration and it also regulates neural survival. Moreover, SV has been shown to increase the level of protective bcl-2 protein in the CNS which enhance cell survival when exposed to adverse stimuli and promotes neuronal regeneration (Hao et al., 2004). This may explain the tendency to increase the process length at the doses which are near the

therapeutic range especially at 750µM. Murabe et al. (2007b) also supported this finding that SV can enhance neuronal differentiation in embryonic stem cell culture. Additionally, what mentioned above might explain that SV makes no significant changes on neuronal cell population which represented by Tubulin III protein. However, it decreases GFAP protein significantly at 1500 and 2000µM doses.

This model of human neural stem cells can predict the anti-proliferative action of SV, even at its therapeutic doses, where there was a highly significant reduction in the sizes of treated neurospheres when compared with that of control group (Fig 37-D & 38). This study also shows that the neurosphere size decreased with increasing doses of SV. This may give human neural stem cell culture an advantage over other in vitro tests with its capability to predict neurotoxicity and teratogenicity of drugs.

On the other hand, SV seems to reduce cell migration significantly at 1000µM and above (figure 37-G). The inhibition of cell migration is thought to be due to the indirect inhibition by SV of Glycogen Synthase Kinase 3 beta (GSK-3β) in the Wnt signaling pathway, which is involved in neural proliferation and cell migration (Blaheta et al., 2002, De Sarno et al., 2002). This inhibition is probably due to the changes in gene expression which are involved in several cell activities(Bosetti et al., 2005). Again, this model shows its ability to detect neurotoxicity of SV through this end point, which is related to one of the important cellular events in embryonic development that might be affected to produce anatomical and/or physiological defects in the growing embryo.

6.3.2 Phenytoin

Phenytoin (PHN) was used as antiepileptic drug for more than 60 years. It acts by stabilizing the cell membrane of neurons undergoing seizure activity through inhibition of sodium influx to inside the cells and thus will prevent spreading seizure (Tunnicliff, 1996). It is well known for its teratogenicity and can cause a wide variety of anomalies in offspring. Several mechanisms have been proposed to explain this. One of these mechanisms involves the formation of toxic intermediate metabolites, when PHN is bioactivated by Cytochrome P450 to arene oxide intermediates which should be detoxified by Glutathione S-Transferases, otherwise they will covalently bind to embryonic protein and result in teratogenesis (Ozolins et al., 1995). In the second mechanism, PHN is bio-activated by peroxidase, such as Prostaglandin H Synthase and Lipoxygenase, to free radicals which form reactive oxygen species that oxidize embryonic protein, DNA, and lipid which initiate teratogenesis. Also, the anti-folate activity of PHN can explain its toxicity (ibid). Additionally, unmetabolized PHN itself may interact with some receptors reversibly and induce congenital abnormalities (Ozolins et al., 1995).

It appears from the results reported here that PHN does not affect the viability and protein content in doses from 5-200µM, taking in consideration that the therapeutic doses of PHN is 40-80µM (Yaari et al., 1986). But, it shows a significant reduction in cell viability and protein content at the dose of 250, 330 and 660 µM. It appears that these doses are extremely toxic by causing distortion in cellular morphology and cell death. The cytotoxicity of PHN can be attributed to its inhibition of Ca^{+2} influx (SOHN and FERRENDELLI, 1973) which cause severe damage to the cytoskeleton (Schlaepfer, 1977). Furthermore, it inhibits or

change the phosphorylation status of Microtubule Associated Proteins (MAPs) which are involved in cell division, movement, the growth of neural processes and axons, transport of materials along the axons and dendrites, and also participates in preserving cell morphology (Bahn et al., 1993). Perturbation of cellular cytoskeleton by phenytoin might explain the tendency of this drug to decrease the length of neuronal processes with increasing the dose to be highly significant at high doses and explain the significant reduction in migration distance even at low therapeutic doses 50µM when compared with the control group.

6.3.3 Carbamazepine

Carbamazepine (CBZ) has been used for treatment a variety of disorders such as epilepsy, neuropathic pain, mania and other psychological illnesses. It is known to be a teratogen (Shepard et al., 2002) particularly causing neural tube defects, cardiovascular and urinary tract anomalies. It appears from the results that CBZ has no effect on both the cell viability and cell proteins especially in the doses which lie below, within, and slightly above the therapeutic dose. Normally, the therapeutic serum level which used as a target for treatment in epilepsy and mood disorders is 25-50µM (McNamara, 2001). But, at the dose 250µM and more, CBZ starts to affect cell viability, in which the resorufin production reduced significantly and the protein amount decreased to high extent.

CBZ can modify the release, uptake, transmission and receptor binding of neurotransmitter in serotonergic, dopaminergic, and glutaminergic neurons (Ambrósio et al., 2002), therefore, modulation in neurotransmitters can affect critical aspects in CNS development, since neurotransmitters regulate proliferation of NSCs, control cell migration and cell differentiation (Nguyen et

al., 2001), this may explain the significant reduction in neurosphere sizes and in migration distance at 100μM dose which reside near the therapeutic dose. Also, it is well known as a HDAC inhibitor, like Sodium Valproate (Beutler et al., 2005), and its teratogenicity may be related to its anti-HDAC activity. CBZ has been identified to cause apoptosis to cultured cerebellar granular cells, by making DNA fragmentation in in a ladder pattern in a concentration dependant manner (Gao et al., 1995) and it is toxic to cultured hippocampal neuron in higher than the therapeutic doses (Araújo et al., 2004). It seems that its neurotoxicity is due to the blocking of NMDA-activated current, since this neurotoxicity can be prevented by NMDA treatment (Gao and Chuang, 1992).

The results show that CBZ has a tendency to increases tubulin III but is statistically not significant, this agrees with the finding of (Murabe et al., 2007b) who pointed out that CBZ treatment for embryonic stem cells can enhance neuronal differentiation. On the other hand, CBZ appears to be toxic on astrocytes and neurons just at extreme high doses 375μM. In general, CBZ seems to have less developmental neurotoxicity in comparison with other AEDs. Its teratogenicity and embryo toxicity appears in poly therapy especially when combined with phenytoin by which the children of epileptic mother exposing to CBZ and PHN have lower developmental and language scores (Kaindl et al., 2006).

6.3.4 Phenobarbitone

PHB is widely used in medicine for pregnant women and neonates for variable purposes including epilepsy. PHB can be used orally or by injection. The dose in adult is 60-240mg/day, which is the lowest dose to decrease its side effect. This dose is used to achieve a constant therapeutic plasma concentration at 40-130μM

(Finnell et al., 1987). Previous in vitro animal studies have shown biochemical and morphological changes in cultured neuronal cells after chronic exposure to PHB (Serrano et al., 1988). In our study, the neuronal marker, tubulin III, was not significantly affected by all the doses of PHB and the astrocyte marker, GFAP, was affected just at high doses. However, it was confirmed that PHB has neurotoxic effect in vitro and in vivo (Yanai et al., 1979, Bergey et al., 1981b). The neurotoxic mechanism of PHB is unclear, but there is a hypothesis that PHB blocks the action of trophic factors which are critical for neuronal survival, these growth factors are present in vivo and in vitro (Brenneman et al., 1987). Another mechanism is the blockage of electrical activity, which might reduce cell viability (Bergey et al., 1981a). Also, growth cone elongation process is dependent on Ca^{+2} influx (Bolsover and Spector, 1986), and as mentioned before, PHB acts by inhibiting this influx into the neurons, prolonged inhibition may explain the reduction in neuronal process growth and branching (Serrano et al., 1988). Overall, it seems that neurotoxicity of PHB appears just with chronic exposure, more than 2 weeks (Neale et al., 1985, Bergey et al., 1981b, Serrano et al., 1988) and despite of its developmental neurotoxicity, its teratogenic effect on embryos is less than any other AEDs (Holmes et al., 2001). These studies pointed out that neurotoxicity of PHB is dose and time dependant, the toxicity increase with increasing the dose and the duration. This may explain the significant reduction in cell survival, total protein amount, cell migration, and other parameters only at high doses.

Overall, human neural stem cell culture model is sensitive in detecting the neurotoxicity of sodium valproate and phenytoin within their therapeutic level,

while the neurotoxicity of carbamazepine and phenobarbitone were detected only at high levels

Chapter 7

Evaluation of This Model with Anti-oxidants and anti-oxidants with Anti-epileptics

7.1 The Anti-oxidants

7.1.1 Folic acid

Folic Acid (FA), pteroyl-L-glutamic acid, vitamin B9, folate, is made up of a 2-amino-4-hydroxypterine (purine and pyrazine parts fused to form a pterin ring). The pterine moiety is fully oxidized, in vivo is reduced to 7,8 dihydrofolate (DHF), DHF is subsequently reduced to 5,6,7,8 tetrahydrofolate (THF), which is converted enzymatically into 5-methyltetragydrifolate (5-MTHF) (Gliszczyńska-Świgło, 2007). Reduced forms of FA act as cofactors for the biosynthesis of purine, pyrimidine, and DNA (Stanger, 2002). The biochemical role of folate is well established in DNA synthesis and repair. It also, catalyses the reactions concerned with metabolism of nucleic acids and proteins (Stokstad, 1988).

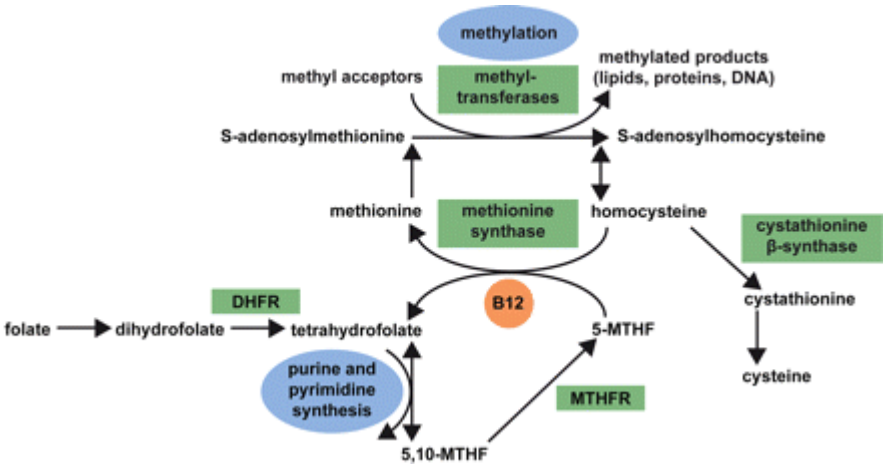


Figure 47: Folate, homocysteine, methionine metabolism; DHFR, dihydrofolate reductase; MTHF, methyltetrahydrofolte; MTHFR, methyltetrahydrofolate reductase (Van Gelder et al., 2010).

This vitamin has antioxidant properties (Gliszczyńska-Świgło, 2007) and by this activity, it has been proposed that FA can reduce the incidence of several diseases such as cardiovascular diseases (Verhaar et al., 2002), neurological and

psychiatric diseases (Alpert and Fava, 1997), and neural tube defects (Olney and Mulinare, 2002). It has been suggested that the protective properties of FA are related to its anti-oxidant activities (Joshi et al., 2001, Gliszczyńska-Świgło, 2007) and due to its free radical scavenging activity (Joshi et al., 2001), since it has been recognized that free radicals play major roles in the oxidative stress which leads to several diseases (Stocker and Frei, 1991). FA has been reported to have activity against radical mediated oxidative damage in human blood (Stocker et al., 2003). As mammals do not have the necessary enzymes to produce FA de novo (Murray, 1999, Friedrich, 1988), its presence should depend entirely on external supplementation in the diet. Good sources of FA are green leafy vegetables, mushrooms, and liver (Gliszczyńska-Świgło, 2007)

7.1.2 Melatonin

Melatonin (*N*-acetyl-5-methoxytryptamine) derived from serotonin and is the main secretory product of the pineal gland at night. It was discovered by Lerner in 1958 (Lerner et al., 1958). Its effect is well known with regard to circadian rhythmicity and reproductive function (Reiter et al., 2009, Dominguez-Rodriguez et al., 2010). Additionally, it has been discovered to have direct free radicals scavenger activities and to detoxify the highly reactive hydroxyl radical (OH) in vitro (Tan et al., 1993). Reiter (1998) reported that melatonin acts as free radical scavenger for a wide range of free radicals. Moreover, it can reduce oxidative stress by stimulating antioxidant enzymes by mechanisms which are mediated by membrane receptors or through nuclear or cytosolic binding sites (Tomás-Zapico and Coto-Montes, 2005).

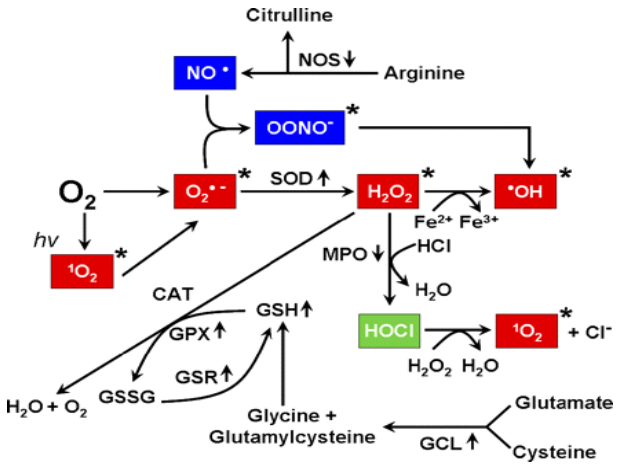


Figure 48: Mechanism of action of melatonin in nitro-oxidative stress. The oxygen reactants are in **red**, nitrogen reactant in **blue**, and chlorine reactant in **green.** Melatonin is reported to 1. Scavenge the reactants marked with an asterisk. 2. Stimulate (↑) the anti-oxidative enzymes, like glutathione peroxidase (GPx). Glutathione reductase (GRd), and superoxide dismutase (SOD). 3. Increase the level of glutathione (GSH). 4. Inhibit the prooxidative enzymes nitric oxide synthase (NOS) and myeloperoxidase (MPO) (Reiter et al., 2009, Tomás-Zapico and Coto-Montes, 2005). NO., nitric oxide; CAT, catalase; $^1O_2$, singlet oxygen; $O_2^{\cdot -}$, superoxide anion radical; GSSG, oxidized glutathione; *hy*, photic energy.

Because of these properties, melatonin treatment has been tested to know if it can reduce the oxidative damage which happens in several neurological disorders and it seems to be effective (Reiter et al., 1998). Moreover, the ability to scavenge free radicals extends into its metabolites (Galano et al., 2013). In mammals, melatonin is not only produced by the pineal gland, but also by the retina and gastrointestinal tract (Reiter, 1991). The brain tissue expresses the key enzyme which is involved in melatonin synthesis, arlyalkylamine N- acetyltransferase (AA-NAT) (Jimenez-Jorge et al., 2007) and a report suggests that its production is from glial cells (Liu et al., 2007). Its high concentration in the brain may be related to its neuroprotective function (Hardeland, 2012). Melatonin is not only a mammalian product, but could also be produced in non-mammalian vertebrates

and invertebrates, and therefore, it could be ingested (Reiter, 1998). The physiological concentration of melatonin is from 10^{-9} to 10^{-11}M and the pharmacological concentration is 10^{-7}-10^{-5}M (Hill and Blask, 1988).

Several diseases that happen during pregnancy may get benefit from melatonin treatment. Of particular note is preeclampsia, a major disorder which develops in 5-7% of all pregnancies all over the world, which is characterized by elevated systolic and diastolic blood pressure and proteinurea in the second half of pregnancy (Brown et al., 2001). Given the antioxidant activity of melatonin, and ready transfer of melatonin from maternal to foetal circulation, its administration may be helpful in reducing the systemic oxidative stress which is associated with preeclampsia and it could rescue the developing nervous system of the foetus (Wakatsuki et al., 2001).

In summary, Folic acid and melatonin have antioxidant and free radical scavenging activities; therefore, they have protective and reparative effect against several diseases, as mentioned before. And as sodium valproate and phenytoin treatment associated with oxidative stress; therefore, folic acid and melatonin will be added with them to the cells to know if they have protective effect or not.

7.2 The results

7.2.1 Folic acid

Our study shows that FA throughout all the doses which lie below and above the therapeutic level has no significant effect on all the end points except the neurosphere size, where FA appears to increase the neurosphere size significantly at doses ≥50μM when it compared with the control, ($F(5,12)=3.624$, $p=0.0314$, post-hoc: 50μM vs control: $p<0.05$), (figure 49- D).

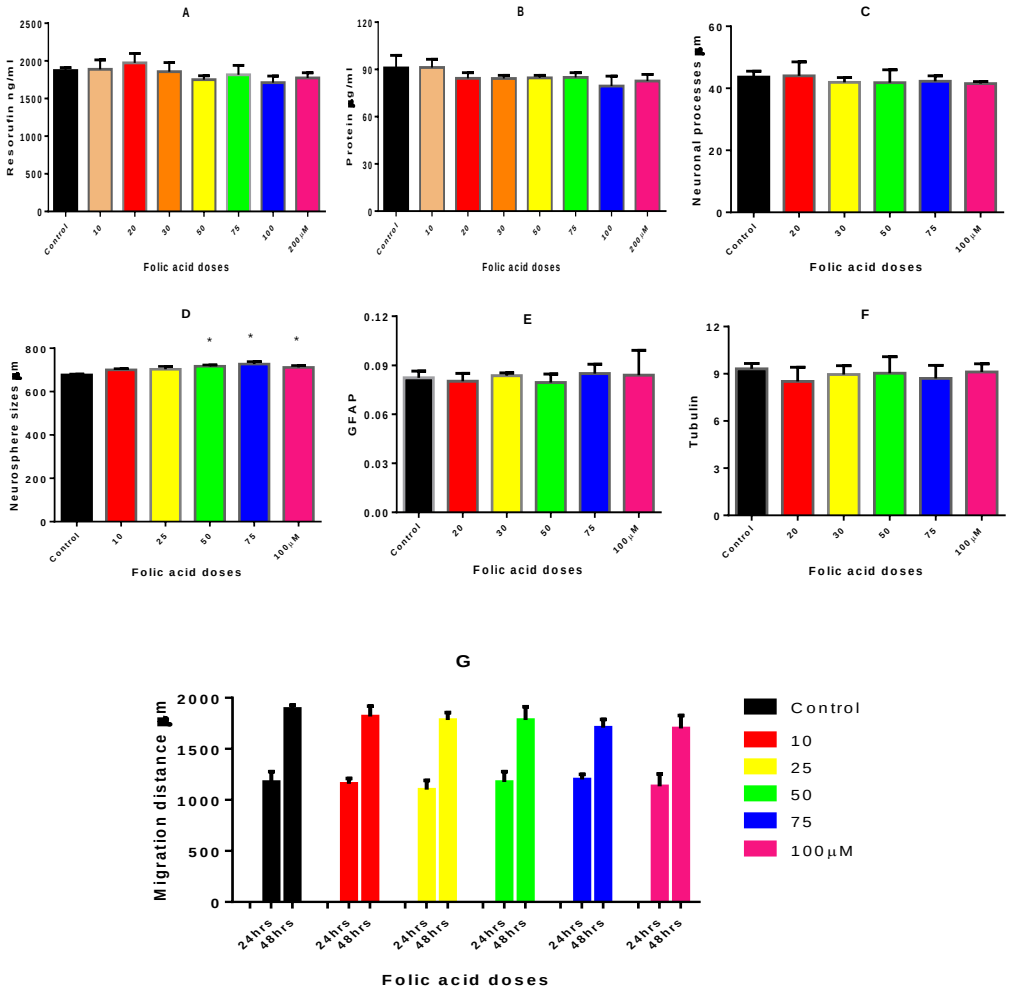


Figure 49: Effect of **Folic acid** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

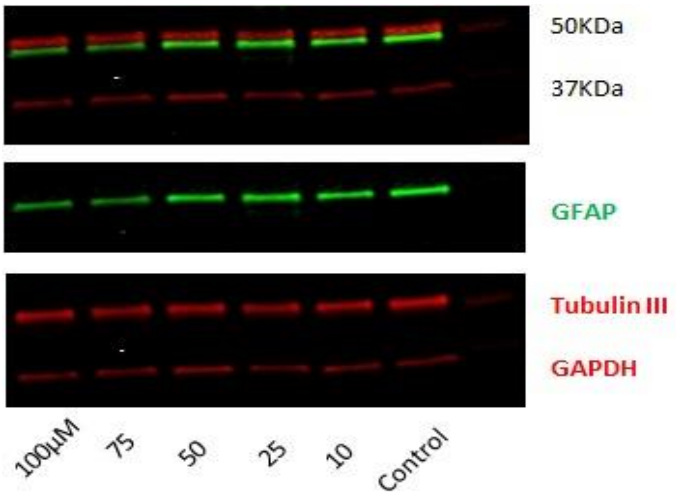


Figure 50: Western Blot analysis for the cells after **Folic acid** treatment showing the bands of Tubulin III, GFAP, and GAPDH proteins.

7.2.2 Melatonin

Melatonin treatment seems to have no significant effect on the end points except for the neurite length at the doses 10, 25µM, ($F(5,12)=3.520$, $p=0.0344$, post-hoc: 10µM vs control: $p<0.05$), and (25µM vs control: $p<0.05$), and neurosphere size at the doses 5, 10, and 25µM, ($F(5,12)=8.755$, $p=0.0011$, post-hoc: 5µM vs control: $p<0.05$), in which there are significant increase in neurite length, and neurosphere size, when it compared with control group, as shown in figure 51.

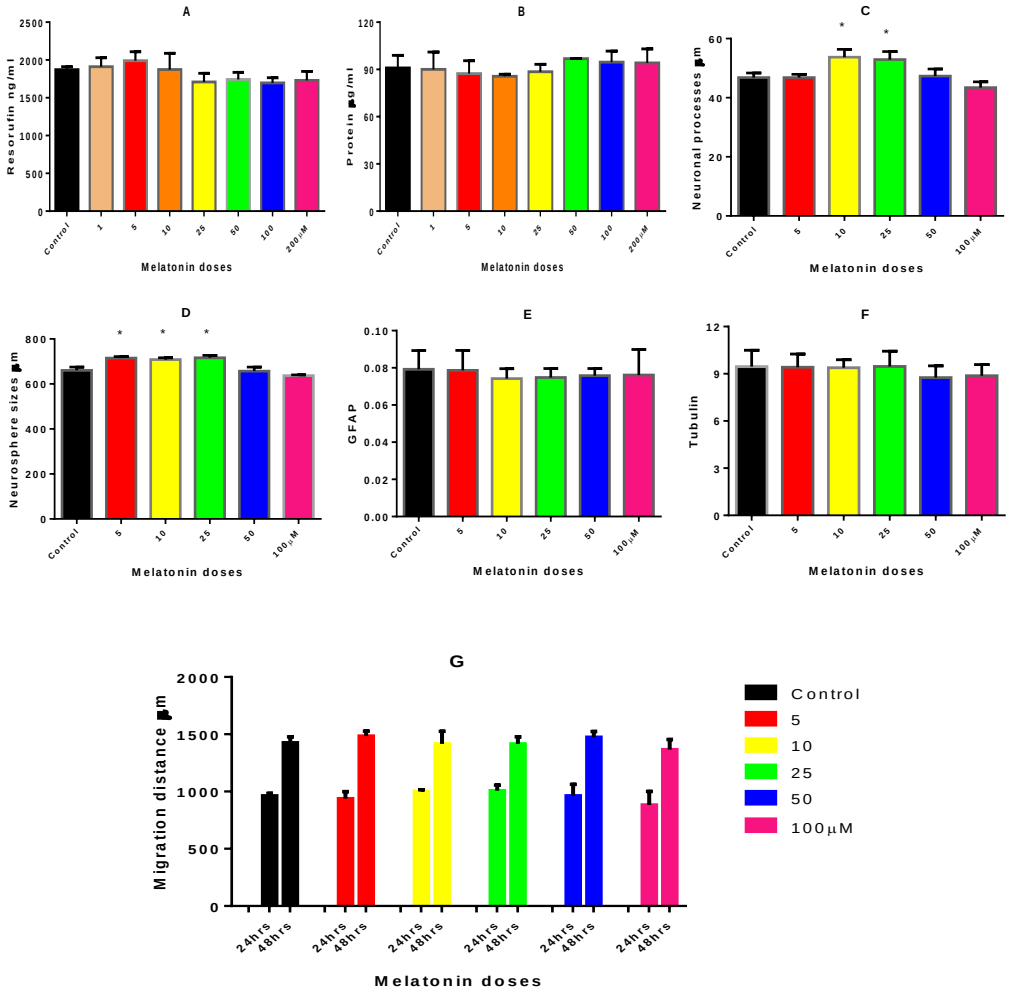


Figure 51: Effect of **Melatonin** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

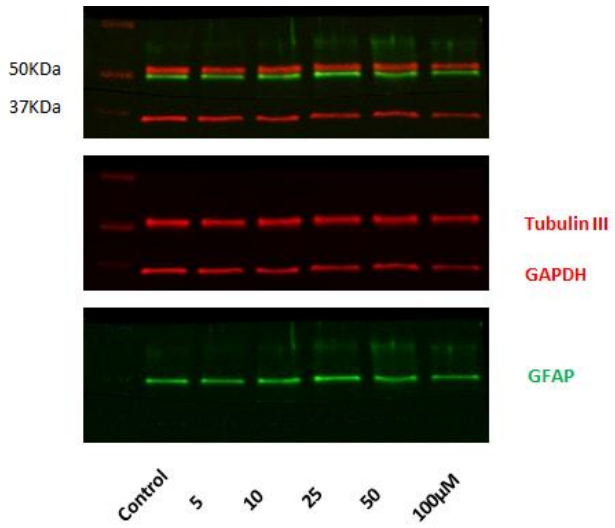


Figure 52: Western blot image showing Tubulin III, GFAP, and GAPDH after **Melatonin** treatment to the cultured cells in different doses.

7.2.3 Anti-epileptics and Anti-oxidants

7.2.3.1 Sodium Valproate

By comparing the result of SV alone treatment, figure 38, with the results of adding Folic acid (FA) to SV treated cells, it seems that adding FA makes an improvement in most of the end points. It appears that the cell survival and total protein amount reduction at 1500µM became not significance, but at 2000µM, cell viability was reduced significantly, ($F(8,18)=4.143$, $p=0.0059$, post-hoc: 2000µM vs control: $p<0.05$) and total protein amount also was reduced significantly, ($F(8,18)=9.544$, $p<0.0001$, post-hoc: 2000µM vs control: $p<0.01$) respectively. Also, the neurite length appears not significantly affected throughout all the doses. In the case of neurosphere sizes, this study demonstrated that FA supplement with SV improves neurosphere size since there is no significant change in their sizes at 500, 750 and 1000µM compared to the control, but at 1500µm the size reduction was significant, ($F(6,14)=19.85$, $p<0.0001$, post-hoc: 1500µM vs control: $p<0.01$). Additionally, GFAP protein reduction became

significant at 2000µM, (F (6,14)=2.479, p=0.0758, post-hoc: 2000µM vs control: p<0.05), and cell migration appears to be significantly affected at 1500µM, (F (6,24)=2.445, p=0.0500, post-hoc: 1500µM vs control: p<0.05), figure 53 and 54.

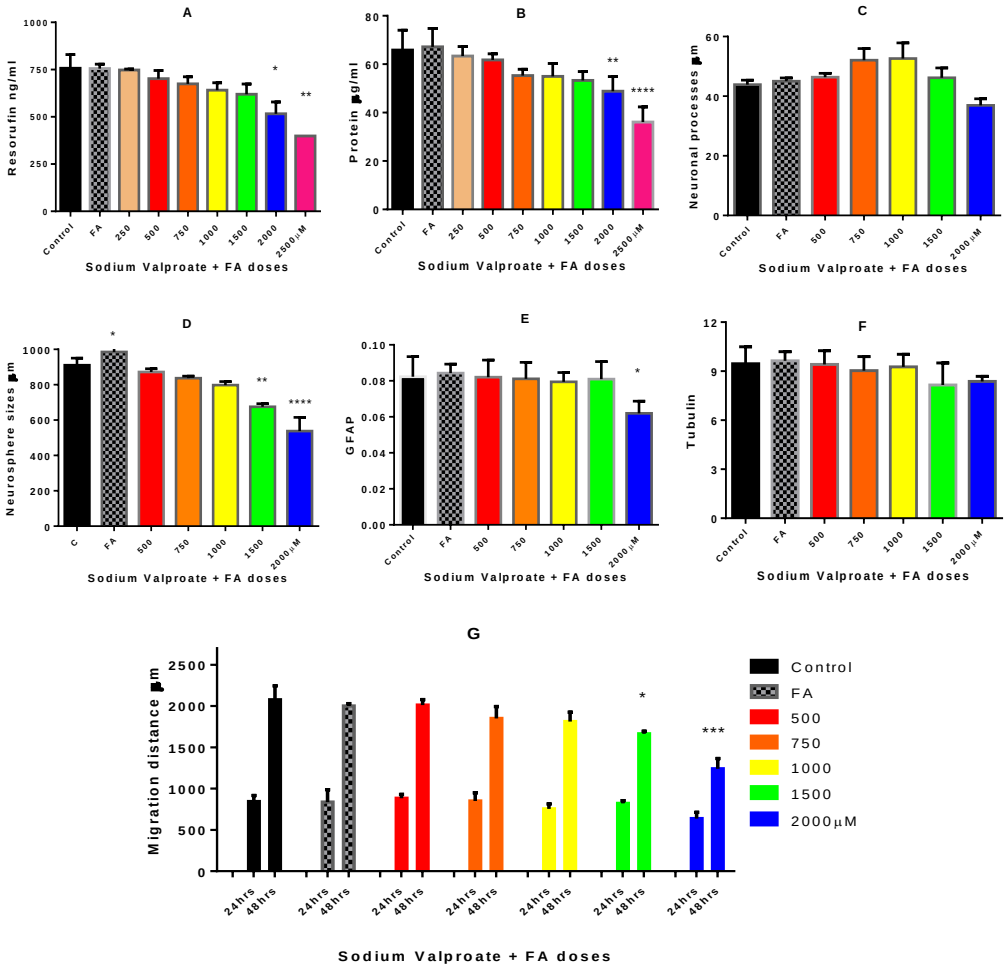


Figure 53: Effect of **Sodium valproate** at different doses **+ Folic acid (50µM)** on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

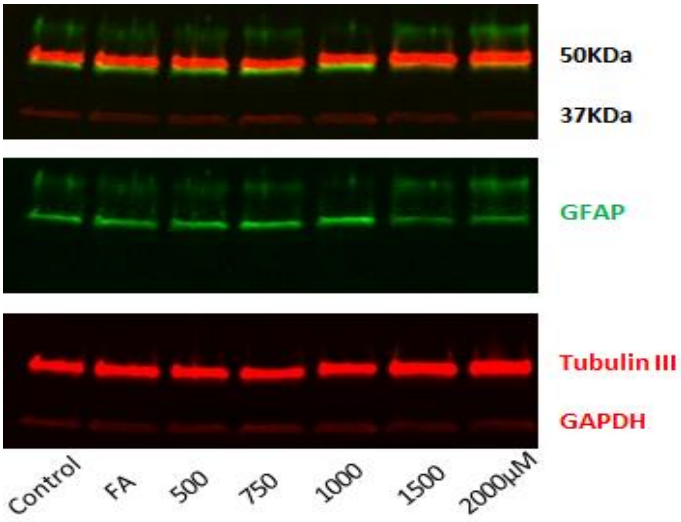


Figure 54: Western blot image showing Tubulin III, GFAP, and GAPDH after **Sodium valproate and folic acid** treatment to the cultured cells in different doses

Similarity, adding melatonin, to some extent had the same effect of adding FA to SV treated cells, but in melatonin supplement, it seems that the improvement in cell survival and total protein amount reached higher doses, 2000µM, in which, it appears to be not significant from control group, and at 2500µM, both cell viability, ($F(8,18)=8.592$, $p<0.0001$, post-hoc: 2500µM vs control: $p<0.0001$) and also the total protein amount, ($F(8,18)=4.412$, $p=0.0043$, post-hoc: 2500µM vs control: $p<0.01$), were significantly reduced figure 55.

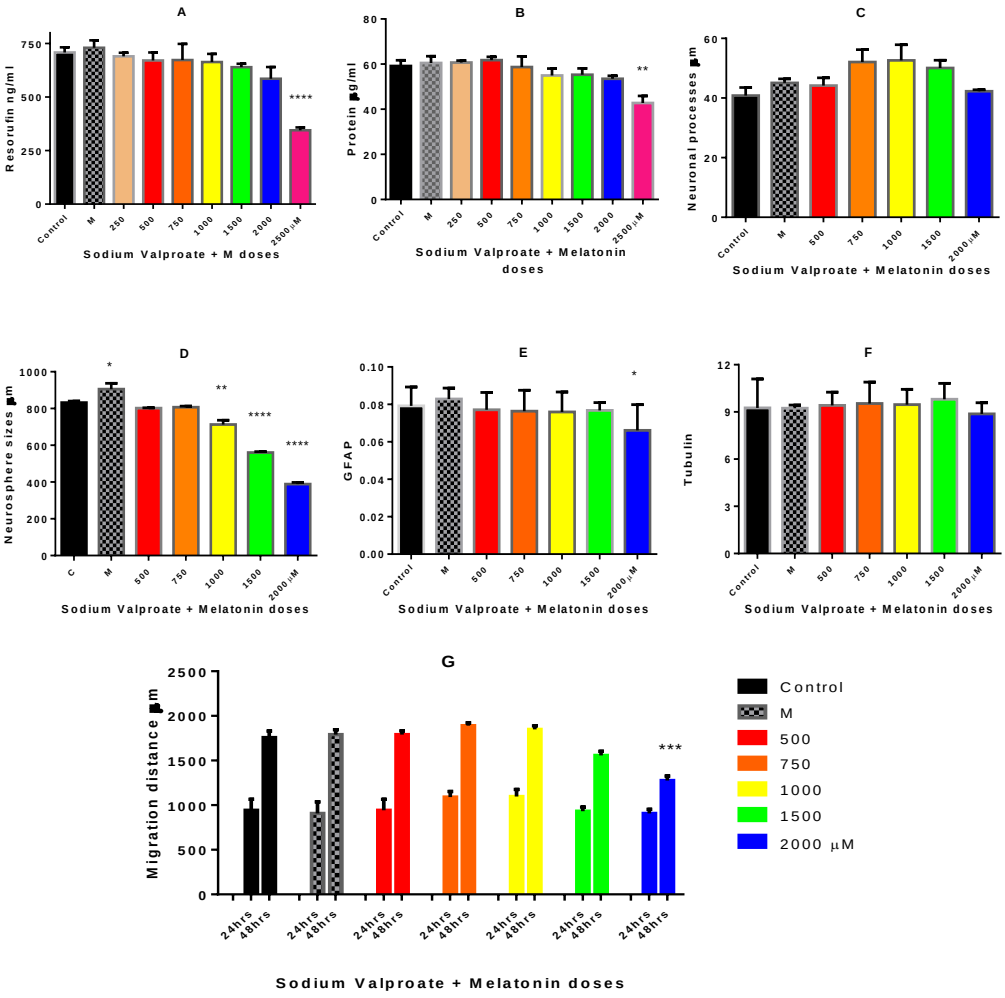


Figure 55: Effect of **Sodium valproate** at different doses + **Melatonin (10µM)** on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

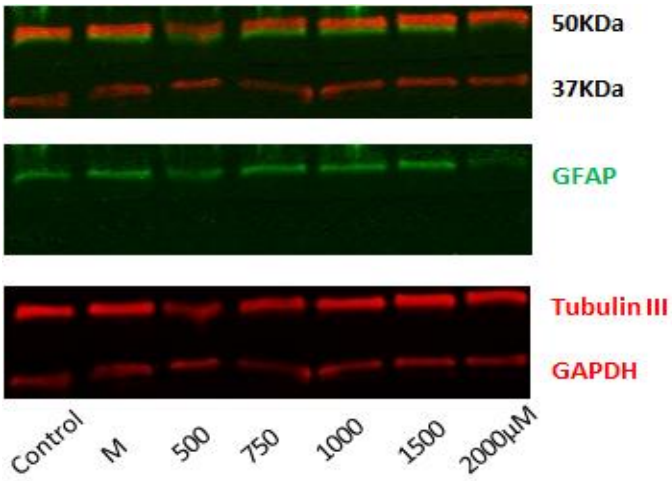


Figure 56: Western blot image showing Tubulin III, GFAP, and GAPDH after **Sodium valproate and Melatonin** treatment to the cultured cells in different doses

7.2.3.2 Phenytoin

PHN treated cells appears to get beneficial effect after adding FA to them, it seems that cell survival became not significant at 150 and 200µM unlike what happened with PHN treatment alone, however cell survival started to be significant at 250µM, ($F(8,18)=11.60$, $p<0.0001$, post-hoc: 250µM vs control: $p<0.01$), figure 41. Total protein amount appeared to be not significantly affected throughout all the doses. The neurite length reduction at 200µM seems to be significant, ($F(6,14)=4.849$, $p=0.0071$, post-hoc: 200µM vs control: $p<0.01$), also, the neurosphere size started to be significant at 100µM when it was compared to the control group, ($F(6,14)=28.85$, $p<0.0001$, post-hoc; 200µM vs control: $p<0.05$). Our results show no changes on the result of GFAP and tubulin III proteins in comparison with PHN treatment alone. Regarding the effect on cell migration, it appears that migration distance was significantly reduced at 100µM, (concentration vs time: $F(6,28)=7.628$, $p=0.0003$, post-hoc: 100µM vs control: $p<0.0001$), figure 57 and 58.

On the other hand, adding melatonin to PHN treated cells had the same changes of adding FA to the cells relatively, except the effect on GFAP was not significant at 100µM when it compared to PHN treatment alone, but significantly reduced from 150µM, ($F(4,14)=8.560$, $p=0.0005$, post-hoc: 150µM vs control: $p<0.05$), figure 59 and 60.

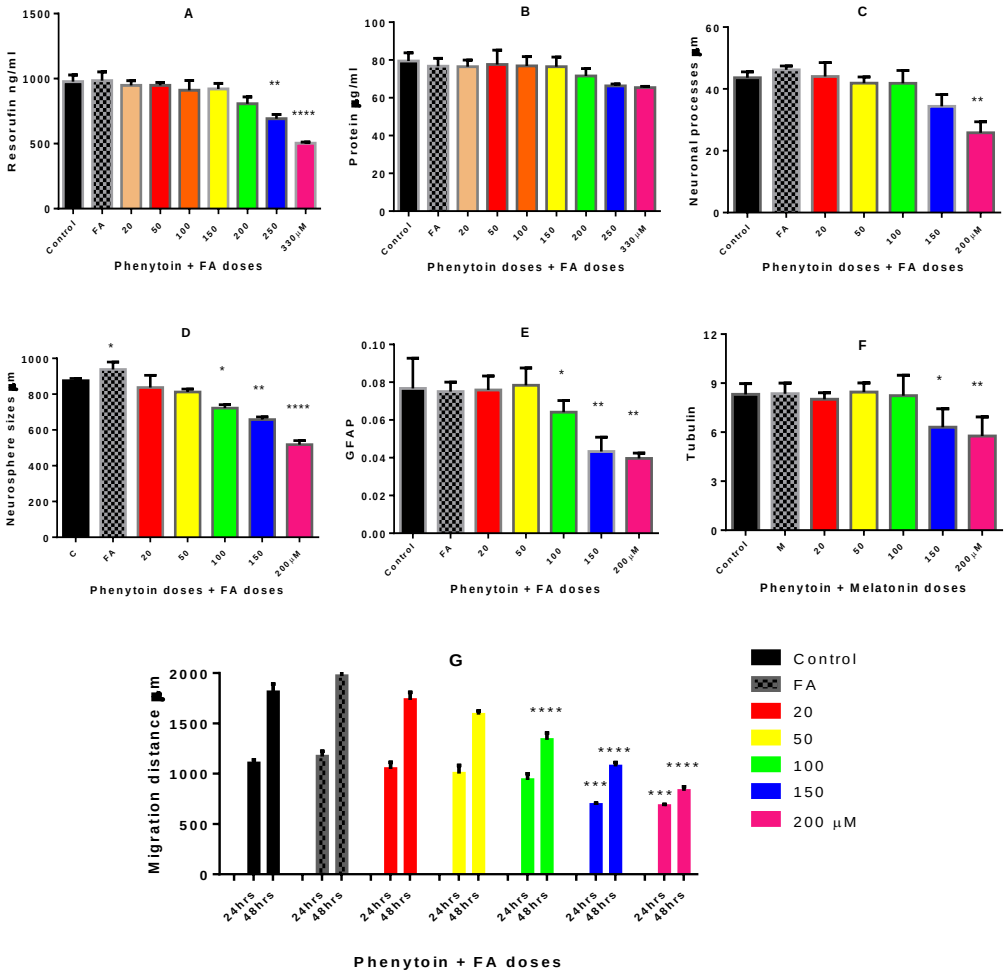


Figure 57: Effect of P**henytoin** at different doses + **Folic acid (50µM)** on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

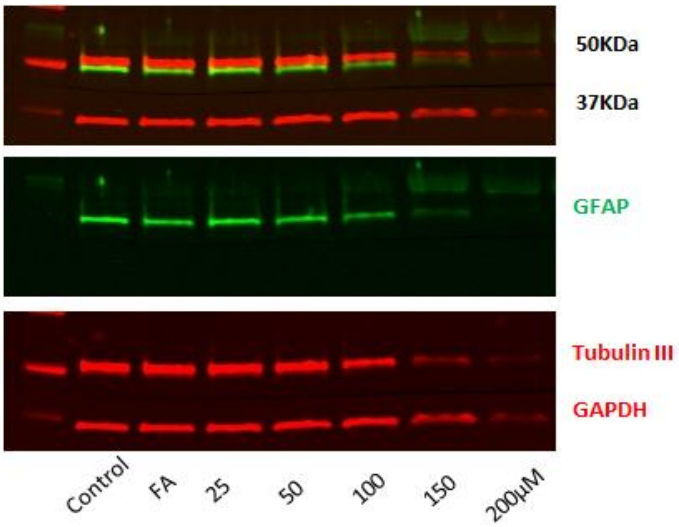


Figure 58: Western blot image showing Tubulin III, GFAP, and GAPDH after **Phenytoin and folic acid** treatment to the cultured cells in different doses

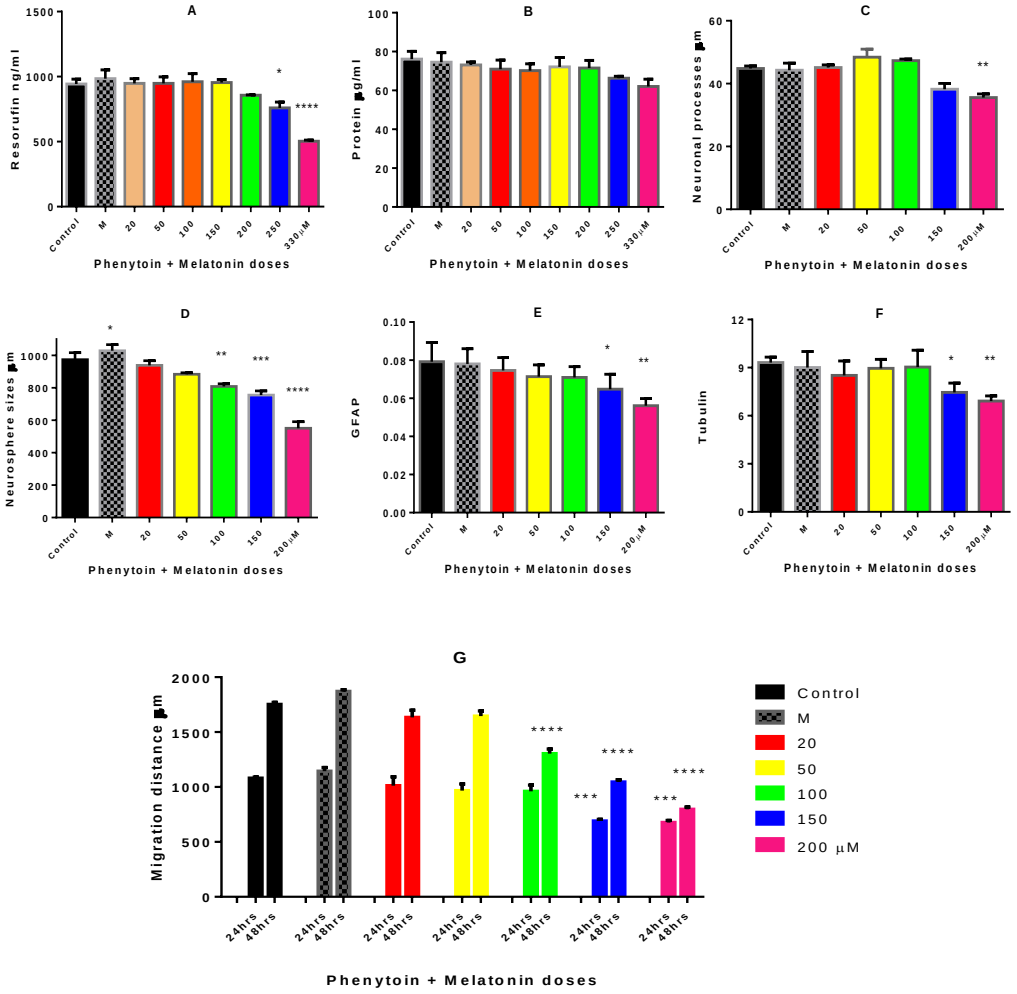


Figure 59: Effect of **Phenytoin** at different doses + **Melatonin (10µM)** on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

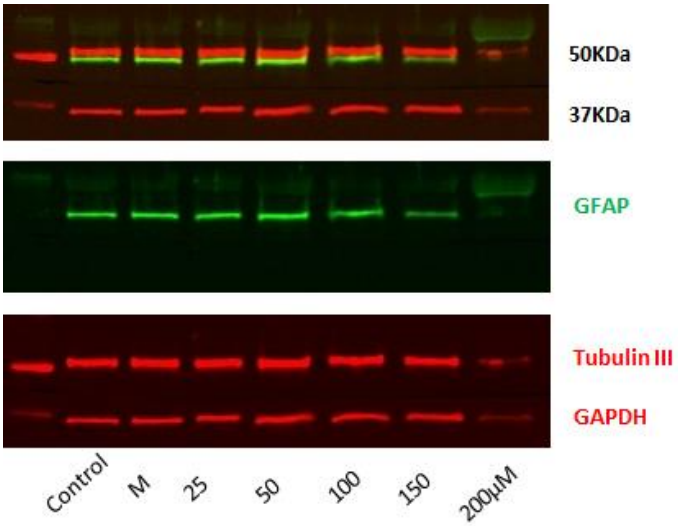


Figure 60: Western blot image showing Tubulin III, GFAP, and GAPDH after **Phenytoin and melatonin** treatment to the cultured cells in different doses

7.3 Discussion

7.3.1 Folic acid

It appears from these findings that folate administration increased the sizes of neurospheres from the dose of 25μM and more. It has been considered that 18μM of FA is low concentration while >70μM as high concentration (Liu et al., 2010, Luo et al., 2013). This means that folate supplement to the medium may stimulate neural stem cell proliferation to make the neurosphere larger than those without folate supplementation. It has been reported that anti-folate treatment like methotrexate inhibits embryonic neural stem cell proliferation in cell culture (Kruman et al., 2005), while folate treatment can enhance proliferation in these cells (Sato et al., 2006). Liu found that folic acid supplementation to rat embryonic NSCs can stimulate them to proliferate and maintain their stemness in the presence of basic fibroblast growth factor in the medium. Also, he found that FA treatment increased the percentage of nestin/BrdU double positive cells in embryonic neurospheres (Liu et al., 2010). This study shows that FA

supplementation has no significant effect on GFAP and tubulin III proteins at all doses and this may be due to the suggestion that FA supplementation may enhance both neurogenesis and gliogenesis through stimulation of Notch1 signalling pathways in embryonic NSCs (Liu et al., 2010). It has been found that genetic disruption of Notch signalling leads to a reduction in NSC markers and an increase in neuronal cell markers (Yoon and Gaiano, 2005). However, it has been shown that FA supplementation to rat embryonic NSCs enhances the stem cell differentiation into neuronal cells at the expense of astrocyte cells (Luo et al., 2013), which disagrees with our findings.

Low intake of folate during pregnancy may lead to CNS pathologies (neural tube defects) and other neurological disorders which appear later on such as seizures, dementia, and depression (Djukic, 2007, Ho et al., 2003, Clarke, 2006). Therefore, it is recommended for women who are or may become pregnant to take folic acid daily to protect the foetus from having neurodevelopmental abnormalities, since FA is involved in several processes in DNA biosynthesis such as enhancing de novo pathways (Sato et al., 2006).

It has been considered that 18μM of FA can be considered as a low concentration while >70μM as a high concentration (Luo et al., 2013, Liu et al., 2010), therefore the concentration of 50μM of FA was used here as an intermediate value between them.

It appears that adding Folic acid to SV cultured cells has to some beneficial effects on the end points that are being testing (figure 53) when compared to the results with SV treatment alone figure (38). Since SV has an anti-folate effect (Van Gelder et al., 2010), addition of FA can explain this improvement in these

parameters. Additionally, FA controls intracellular signalling pathways which regulate mitosis and apoptosis (Ho et al., 2003). It has been found that FA treatment increased mRNA expression of Notch signalling in neurospheres derived from NSCs of the rat (Zhang et al., 2008). The Notch pathway is important for cellular communication that involves gene regulation which in turn regulates cellular differentiation. The Notch gene has the ability to suppress apoptosis and stimulates cell proliferation through a growth factor- mediated survival pathway (Liu et al., 2010). This may explain the improvement against the neurotoxicity of SV.

Moreover, by comparing the results of PHN treatment alone figure (41) with the results of adding FA to PHN figure (57), it seems that adding FA also allows an improvement in the outcome of PHN treatment. PHN has a well- known mechanism of action as an anti-folate agent, as it impairs folate absorption and reduces methionine synthase activity (Van Gelder et al., 2010). Therefore adding folic acid to PHN treated cells can improve the neurological end points that are being tested. PHN as mentioned before, can lead to the generation of oxidative stress and release free radicals by bioactivation of PHN by cytochrome P450 into arene oxide intermediates (Ozolins et al., 1995), FA has antioxidant activity (Gliszczyńska-Świgło, 2007) and has free radical scavenging capacity (Joshi et al., 2001, Stocker et al., 2003) and these might explain why FA treatment with PHN could have some protective effect against PHN teratogenicity.

7.3.2 Melatonin

This study demonstrates that melatonin supplementation alone to the cultured cells has no significant effect on cell viability and total protein, but, causes a significant increase in neuronal process length. It has been assumed that

melatonin stimulates dendritogenesis in an organotypic culture of hippocampus (Domínguez-Alonso et al., 2012), and, it can promote neuritogenesis through vimentin filament reorganization by protein kinase C (PKC) activation and microfilament rearrangement, which are necessary process in growth cone formation in neurite outgrowth (Bellon et al., 2007). This study also shows that melatonin increases the sizes of neurospheres significantly, which means that melatonin stimulates NSCs proliferation. It has been reported that melatonin enhanced cell proliferation in embryonic neural stem cells (Fu et al., 2011, Sotthibundhu et al., 2010), avian astrocytes (Paulose et al., 2009), dentate neurons (Kim et al., 2004b), and mesenchymal stem cells (Zhang et al., 2013). It has been suggested that melatonin stimulates proliferation through receptor dependant signalling pathways (Fu et al., 2011) by phosphorylating the extracellular signalling regulated kinase (ERK1/2), which in turn activates several transcriptional factors that regulate the downstream proliferative activity (Pandi-Perumal et al., 2008).

By comparing the results obtained from Sodium Valproate treatment in figure (38) with the results of adding melatonin (10μM- pharmacological dose) to it (figure 55), it seems that melatonin exerts some neuroprotective effects on SV treated cells. These results show that melatonin reduced the neurotoxicity of SV in regard to cell viability, total protein amount, neuronal process length, neurosphere sizes, GFAP and migration distance. SV treatment of chick cardiac cells and embryonic stem cells can lead to generation of reactive oxygen species (Qureshi, 2012). This may explain the improvement of SV neurotoxicity on human treated cells after adding melatonin. As mentioned before, melatonin has antioxidant (Tomás-Zapico and Coto-Montes, 2005) and free radicals scavenging

activity to a wide range of free radicals (Reiter, 1998), therefore, melatonin can inhibit apoptosis (Mayo et al., 1998). Moreover, the anti-apoptotic activity of melatonin is related to increased expression of Bcl2 and inhibition of caspase-3 activation (Fu et al., 2011).

Similarly, melatonin supplementation of PHN treated cells can demonstrate beneficial effect against the neurotoxicity of PHN in cultured cells in the end points that were tested, see figure (41) for the result of adding melatonin to PHN figure (59).

Lastly, it can deduce that combining either folic acid or melatonin to sodium valproate or phenytoin has protective effect in reducing the neurotoxicity of these anti-epileptics and cause their toxicity to appear in doses which are higher than those when these anti-epileptics used alone.

Chapter 8

Evaluation of This Model with Other Neuroactive Drugs

8.1 Other Neuroactive Drugs

8.1.1 Lithium Carbonate

Lithium is a classic mood stabilizer and has been used for the treatment of bipolar diseases for more than 50 years. Now it is used for acute episodes, prevention and as a prophylactic drug. It was introduced into the psychiatric pharmacy by John Cade in 1949 (Shorter, 2009). Its efficiency in the treatment of bipolar disorder was well documented but the exact mechanism of its action in mood stabilization was still not clear. Lithium has been registered to be teratogenic since the 1960s (Lewllyn A; Stowe Z, 1998). The serum therapeutic concentration in human ranges from 600-1200μM (Su et al., 2007b) and there is only a small gap between the therapeutic and toxic doses. Chronic administration of lithium may lead to developmental malformation (Giles and Bannigan, 2006). This drug is associated with a spectrum of developmental anomalies, such as cardiac abnormalities including Ebstein anomaly, and increased baby weight. Animal studies showed that high doses of lithium can inhibit vasculogenesis (Giles and Bannigan, 2006). In mice, when lithium was given during the critical period of neural tube closure, it caused exencephaly, kinking of the spinal cord and dilatation of the ventricular system (Jurand, 1988). Recent studies showed that Lithium has a neuroprotective role in many neurological diseases including brain ischemic disease and depression. It has been shown that lithium improved the neurological deficits, behavioural disorders and can decrease the size of infarcted area in ischaemic attacks by enhancing neurogenesis and stimulating hippocampal neural progenitor cell differentiation into neural cells in vivo and in vitro (Kim et al., 2004a).

8.1.2 Diazepam

Diazepam (DZP) is one of benzodiazepine derivatives which has been used for treatment of different clinical disorders as an anxiolytic, muscular relaxant, sedative-hypnotic and as an anticonvulsant (Haefely et al., 1981). It has also been used in managing preeclampsia and eclampsia (Iqbal et al., 2014). DZP belongs to the long acting type of benzodiazepine, which has interestingly been suggested to be excreted in breast milk and can also pass smoothly through placenta to the fetus (Iqbal et al., 2014). It has been reported that this drug may associate with neural tube closure defects in explanted chick embryo (Nagele et al., 1981). Also, it can induce exencephaly in the mouse and hamster (Weber, 1984), while in human, an epidemiological study has shown that DZP may contribute to various congenital anomalies such as neural tube closure defects, cleft palate, pyloric stenosis, cardiovascular, skeletal and urogenital anomalies (Kjær et al., 2007). Another epidemiological study reported that maternal exposure to DZP might resulted in 'Neonatal withdrawal syndrome' and embryofetopathy which resembles foetal alcohol syndrome (Iqbal et al., 2014).

Both Diazepam and its metabolite, N-desmethyldiazepam, are pharmacologically active and can pass freely through the human placenta because of their lipophilicity and they are more highly bound to foetal plasma proteins than to maternal plasma proteins (Kunz and Nau, 1984). High concentrations of DZP can be found in the brain, lung and heart. Its lipophilicity allows easy penetration to the brain white matter and its long retention in the neural tissue make human brain tissue a depot for DZP. Neonates can metabolize small doses of DZP slowly and it has been shown that DZP and its metabolites can last for at least a week in

their active form after administration in a high dose to the mother. The plasma half-life in the neonate is about 31 hours (Mandelli et al., 1975)

8.1.3 Amitriptyline

Tricyclic antidepressant (TCA) drugs have been used for the treatment of depression for over 50 years. Amitriptyline (AMT) after much research since that time is still the leading antidepressant(Barbui and Hotope, 2001). Most of the antidepressants act by inhibition of reuptake of neurotransmitters (noradrenaline and serotonin) in presynaptic spaces. By this means, the extracellular content of these monoamines is increased, which occurs soon after treatment, but the clinical effect of these antidepressants occurs slowly over several weeks of continuous administration (Nestler et al., 2002). The effectiveness of TCA cannot be explained simply by their action on monoamines. The molecular and cellular mechanisms of their antidepressant action are still not fully understood. The therapeutic level of AMT in human plasma is 0.5-0.8μM (Ziegler et al., 1976) and the toxic concentration is more than 3μM (Braithwaite et al., 1979).

Women have been identified to be at risk of developing depression during pregnancy and during the postpartum period. Women who report symptoms of depression during pregnancy comprise about 12%. Additionally, of women who develop depression in the postpartum period, 40% develop depression originally during gestation (Chambers et al., 1996). Studies showed that women who are known cases of depression who discontinue treatment during pregnancy show a high relapse rate compared to those who continue treatment (Cohen et al., 2006).

There are significant maternal risks during pregnancy if depression is left untreated. Suicides have been reported as a major cause of maternal death in

several studies (Shadigian and Bauer, 2005, Oates, 2003). Maternal depression is associated with several factors which have a negative impact on foetal wellbeing, such as poor attendance at antenatal clinics, smoking, drug abuse, and alcohol (Bonari et al., 2004). Moreover, maternal depression may be associated with obstetric complications like preeclampsia (Kurki et al., 2000). Maternal depression is also associated with poor maternal-infant attachment and poor child outcome (Murray and Cooper, 1997).

These molecules lithium, diazepam, and amitriptyline are weak teratogens. We will use these molecules with our cell culture model to test their effect on the end points of our experiment.

8.2 Results

8.2.1 Lithium

Lithium treatment seemed to reduce cell survival significantly at the dose of 3000µM and more, ($F(7,16)=11.22$, $p<0.0001$, post-hoc: 3000µM vs control: $p<0.01$). On the other hand, total protein was significantly increased at 500 and 750µM, ($F(7,16)=37.69$, $p<0.0001$, post-hoc: 500µM vs control: $p<0.05$), and (750µM vs control: $p<0.05$), which is located around the therapeutic plasma level. The figure below shows there was no significant effect on neuronal process and neurosphere sizes, ($F(5,12)=3.091$, $p=0.0507$(and ($F(5,12)=4.643$, $p=0.0137$) respectively. The results demonstrated no significant effect on tubulin III protein, ($F(5,12)=0.3989$, $p=0.8404$) but caused a significant dose dependant reduction in GFAP protein started from 1500µM, ($F(5,12)=11.56$, $p=0.0003$, post-hoc: 1500µM vs control: $p<0.01$). The figure below illustrates that lithium treatment caused a significant increase in migration distance 48 hours after treatment at the

dose of 750µM, (F (5,24)=4.691, p=0.0040, post-hoc: 750µM vs control: p<0.05) and resulted in significant reduction in cell migration 24hrs after treatment, (F (5,24)=4.691, p=0.0040, post-hoc: 2000µM vs control: p<0.05), and 48hrs (P<0.0001) after treatment with 2000µM, figure 61 and 62.

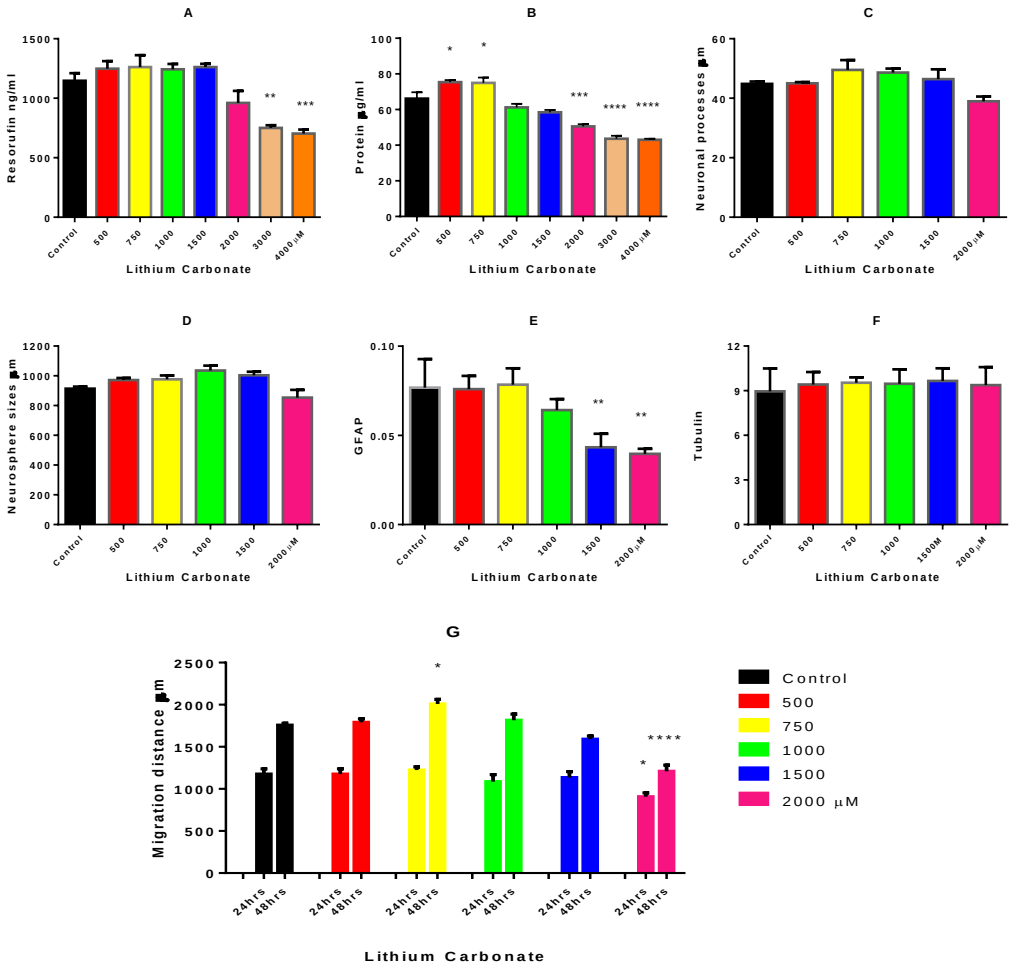


Figure 61: Effect of **Lithium** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

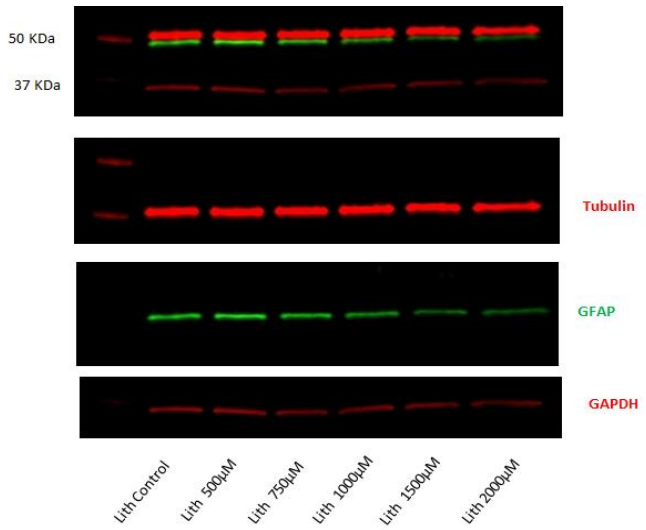


Figure 62: Western Blot Image illustrating GFAP, tubulin, and GAPDH after treating the cells with **Lithium.**

8.2.2 Diazepam

The results demonstrate that DZP treatment had no significant effect on cell survival and total protein at all doses, But from the dose of 100µM, cell survival, ($F(7,16)=6.568$, $p=0.0009$, post-hoc: 100µM vs control: $p<0.01$), total protein, ($F(7,16)=17.74$, $p<0.0001$, post-hoc: 100µM vs control: $p<0.001$), neuronal process length, ($F(5,12)=6.351$, $p=0.0042$, post-hoc: 100µM vs control: $p<0.05$), and neurosphere sizes, ($F(5,12)=127.5$, $p<0.0001$, post-hoc: 100µM vs control: $p<0.0001$) were reduced significantly when compared with the control group. There was no significant effect on both GFAP, ($F(5,12)=0.3060$, $p=0.9000$), and tubulin III proteins, ($F(5,12)=0.4165$, $p=0.8285$). While cell migration distance was not affected at any dose other than at the highest dose (200µM) and only 48 hours after treatment, ($F(5,24)=1.301$, $p=0.2965$, post-hoc: 200µM vs control: $p<0.001$), figure 63 and 64.

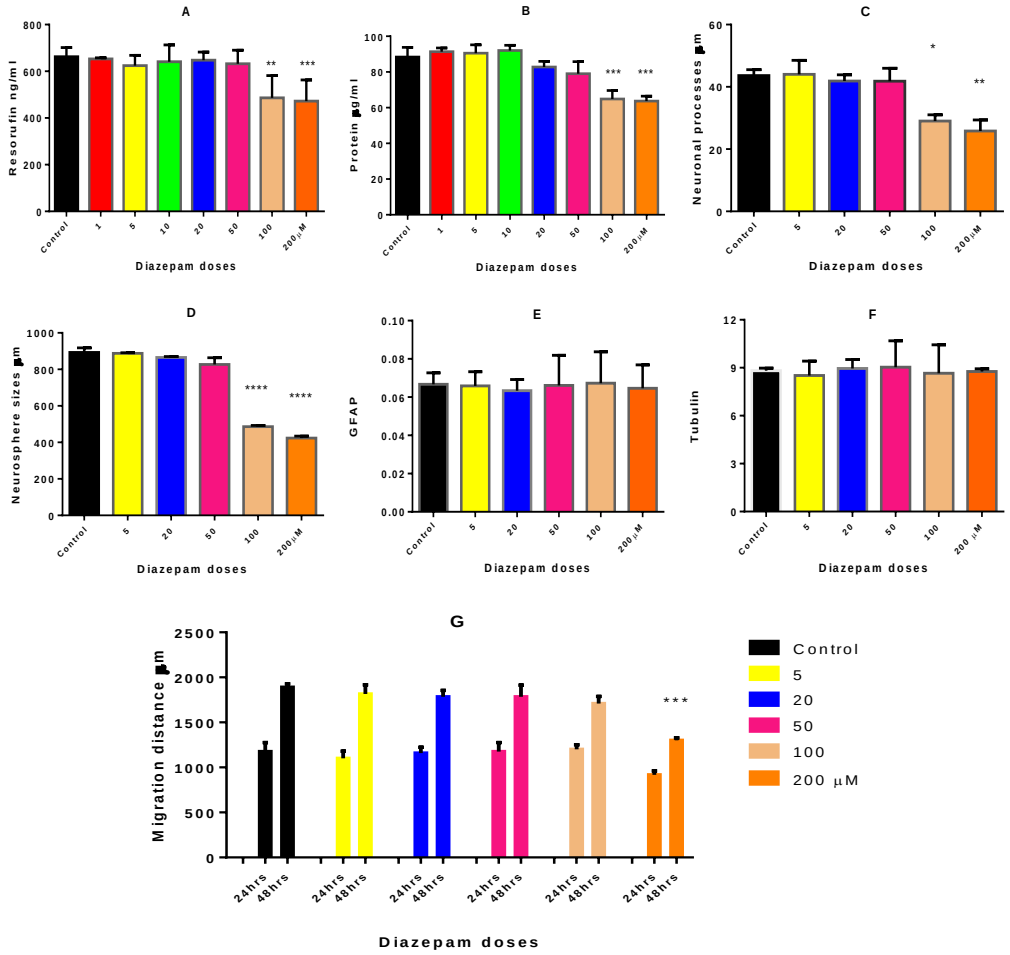


Figure 63: Effect of **Diazepam** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

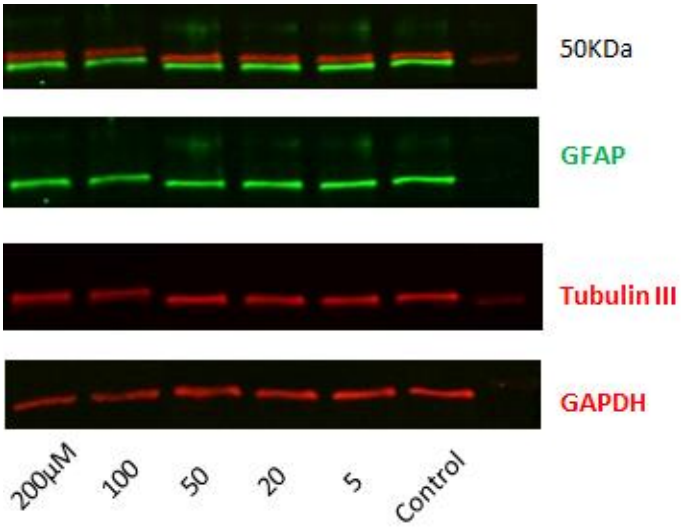


Figure 64: Western blot image showing Tubulin III, GFAP, and GAPDH after **Diazepam** treatment to the cultured cells in different doses

8.2.3 Amitriptyline

Cell survival, (F (7,16)=71.80, p<0.0001, post-hoc: 5µM vs control: p<0.001), total protein, (F (7,16)=58.27, p<0.0001, post-hoc: 5µM vs control: p<0.0001), and neurosphere size, (F (5,12)=9.015, p<0.0009, post-hoc: 5µM vs control: p<0.05) were significantly decreased at 5 µM and more. The effect of AMT treatment on neuronal process length, (F (5,12)=35.34, p<0.0001, post-hoc: 2.5µM vs control: p<0.050 appeared to be significant at lower doses; they were seen at 2.5µM and more. Also, AMT reduce cell migration distance in time dependant way, (F (1,24)=106.9, p<0.0001), and dose dependant, (F (5,24)=7.306, p=0.0003). At 2.5µM, AMT reduce and cell migration significantly, (concentration vs time: F (5,24)=1.031, p=0.4222, post-hoc: 2.5µM vs control: p<0.05). Additionally, AMT appeared to have no significant effect either on GFAP, (F (5,12)=0.4591, p=0.7992) or tubulin III proteins, (F (5,12)=0.1086, p=0.9883), when compared with the control group, figure 65 and 66.

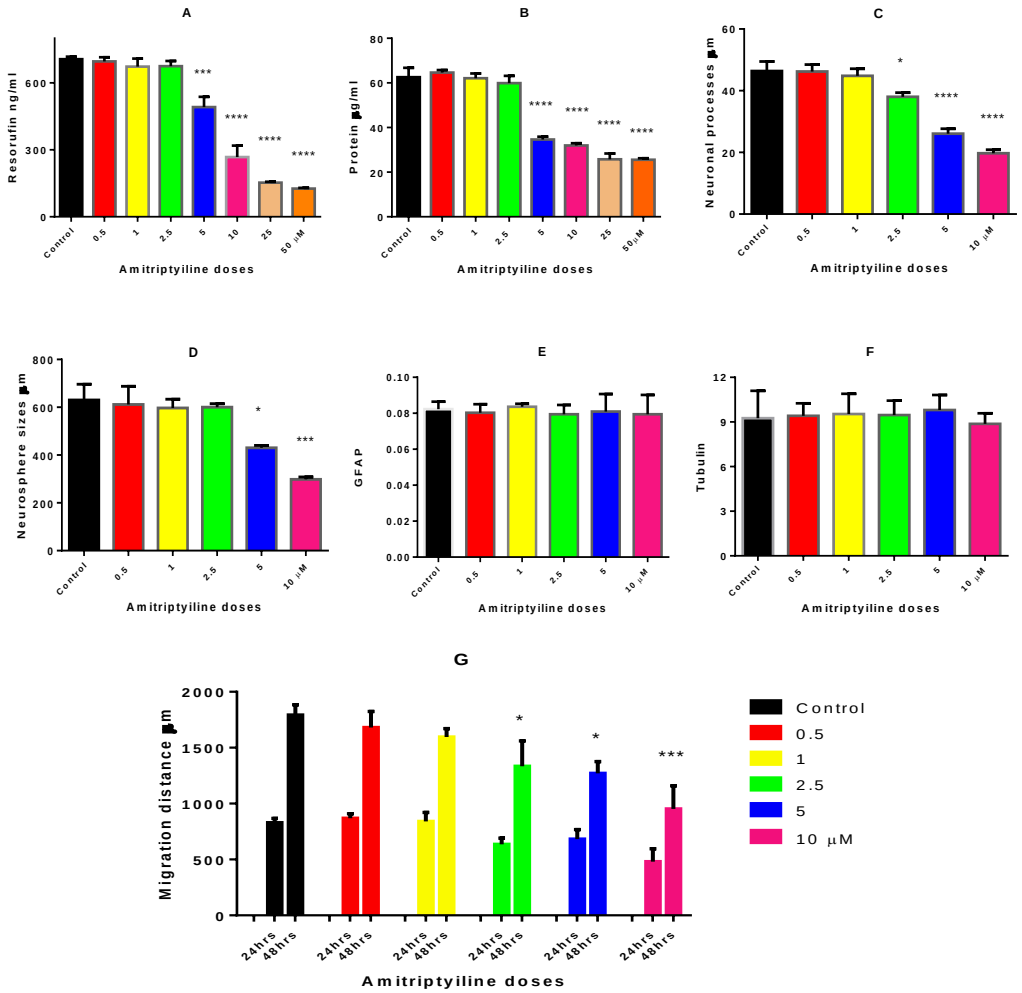


Figure 65: Effect of **Amitriptyline** at different doses on A: Resorufin production, B: Total protein. Mean± SE. n=3 (average 6 wells from each experiment), C: The length of neuronal processes. Mean ± SE. n=3 (average 35 neurons from each experiment), D: The neurosphere size changes with different doses. Mean ± SE, n=3 (average 10 from each experiment), E: GFAP (Western Blotting); n=3, F: Tubulin protein (Western Blotting); n=3, G: Cell migration 2 days after drug treatment, mean ± SE. n= 3 (average 6 from each experiments).

Significance: * when P<0.05, ** when P<0.01, *** when P<0.001, and **** when P<0.0001.

A-F statistical analysis was performed by using One way ANOVA, while in G, two way ANOVA was performed, then Dunnett multiple test was used to compare the mean of each group with that of control group.

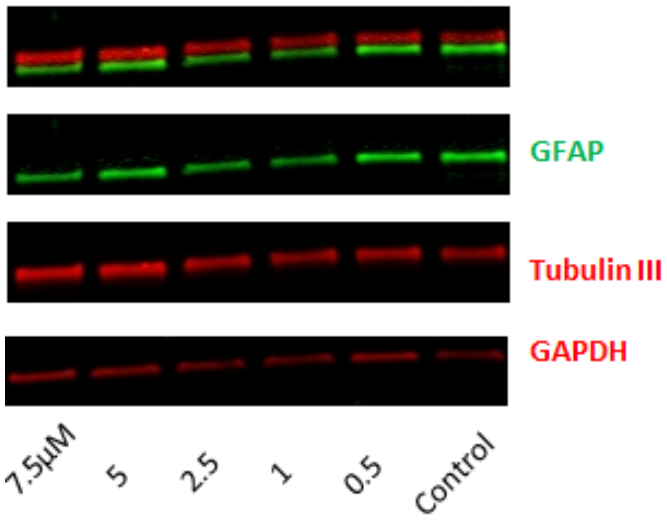


Figure 66: Western blot image showing Tubulin III, GFAP, and GAPDH after **Amitriptyline** treatment to the cultured cells in different doses

8.3 Discussion

8.3.1 Lithium Carbonate

Lithium Carbonate was considered to be a weak teratogenic substance that is associated with several congenital anomalies, especially cardiovascular (Giles and Bannigan, 2006) and nervous system malformation (Jurand, 1988). Animal studies showed that treatment with lithium at a dose close to the human therapeutic dose did not demonstrate any congenital defects but higher doses were associated with a lot of skeletal, neurological, vascular and cardiac abnormalities (Giles & Bannigan, 2006).

The results reported here reveal that Lithium Carbonate has no significant effect on the cell viability at the doses which are close to the therapeutic range, while it seems that lithium treatment leads to a significant increase in total protein at the doses 500 and 750µM. It has been suggested that Lithium has a neuroprotective

effect in mood disorders and has been used for the treatment of these disorders for several decades. It acts by inducing several molecular and biochemical effects on neurotransmitter signalling, signalling cascades, gene expression, ion exchange and hormonal and circadian regulation (Manji and Lenox, 2000). Lithium treatment shows an increase in the volume of the grey matter in many areas in the brain which are involved in mood regulation, by stimulating proliferation of neural progenitor cells and enhancing neurogenesis through different mechanisms (Su et al., 2007b). Lithium inhibits GSK directly by binding to its magnesium-sensitive site (Klein and Melton, 1996) and indirectly by enhancing phosphorylation of this kinase (Chalecka-Franaszek and Chuang, 1999). It has been postulated that GSK dysfunction is involved in pathogenesis in mood disorders (Jope, 2011).

Moreover, lithium exerts its neuroprotective effect through induction of BDNF and stimulation of its receptor. Lithium protects primary cortical neurons from glutamate excitotoxicity and using a BDNF neutralizing antibody deprived the cells from neuroprotection (Hashimoto et al., 2002). Additionally, Lithium controls GDNF in vivo and in vitro, which is involved in maintaining cell survival, axonal growth, chemo- attraction, and cell migration (Paratcha and Ledda, 2008). This drug has an anti-apoptotic activity by upregulation of mRNA expression of the Bcl2 anti-apoptosis protein, decreasing the expression of P53, which is a pro-apoptotic protein and reducing mitochondrial release of cytochrome c (Chen and Chuang, 1999). Lithium prevents the activation of caspase-3 which leads to mitochondrial release of cytochrome c which induces apoptosis. Also it can modify NMDA receptor activity (Hashimoto et al., 2003). Another mechanism of action for lithium, is activation of PI3K/Akt, MEK/ERK,

Wnt/β-catenin pathways which enhance release of anti-apoptotic factors and induce growth production (Chiu et al., 2013). These mechanisms may explain the significant increase in total protein, neurosphere size, and migration distance in the treated cells.

Lithium appears to not affect the length of neuronal processes even at a high dose (2000μM) and this agrees with (Jeerage et al., 2012) who pointed out that lithium has no effect on neurite length of rat cortex progenitor cells in this range of doses, but at high doses (10mM and more) lithium treatment resulted in significant reduction in neurite length. The effect on GFAP and tubulin III proteins would indicate that lithium is toxic to astrocytes since GFAP expression decreased in a dose dependant way, which may explain its teratogenicity, but had no significant effect on tubulin III, since it has neuroprotective effect.

8.3.2 Diazepam

Diazepam (DZP) acts in various ways to exert its actions. Its anticonvulsant property is explained by reducing action potential firing (Drexler et al., 2010), and it enhances GABAergic inhibition in dose dependant way (Skerritt et al., 1984). A study on genetically modified mice showed that DZP treatment resulted in hypnosis and sedation by acting on $GABA_A$ receptors, which are located in glutamatergic cortical neurons (Baur et al., 2008). Additionally, it caused Na^+ current disruption in the neurons of the CNS (Nagele et al., 1981). DZP has been shown to inhibit Ca^{+2} uptake by synaptosomes; therefore, it may result in a reduction of neurotransmitter release which results in suppression of spontaneous firing (Skerritt et al., 1984). DZP also, enhances the inhibitory transmission of synapses and reduces neuronal activity and cerebral cortex excitability, this may

explain the key mechanism of DZP to produce sedation and hypnosis (Baur et al., 2008).

The therapeutic plasma level of DZP as an anxiolytic agent is 0.35-6.0μM (http://www.nhtsa.dot.gov/people/injury/research/job185drugs/diazepam.htm) and as an anticonvulsant is around 50μM (Regan et al., 1990). The results show that DZP had no effect on cell viability and total cellular proteins at the doses 1, 5, 20, 50μM, but, there is significant reduction in those parameters at doses 100μM and 200μM. Regan et al. (1990) pointed out that DZP has an anti-proliferative action on cells lines derived from mouse cultured brain cortex at a concentration 2-3 fold higher than the therapeutic concentration. In addition to that, this drug can suppress the proliferation of human Glioblastoma cells in a dose dependant way, by inducing Go/G1 phase arrest (Chen et al., 2013). This may explain the significant reduction in neurosphere size at the doses 100 and 200μM. Regarding the effect of DZP on the length of neuronal processes, the results demonstrate that DZP has no significant effect statistically on the length, except at high doseswhere the length was reduced significantly. Additionally, it appears that DZP has no significant effect on both GFAP and tubulin III proteins when compared with that of control group.

In general, DZP is considered to be safe and studies have reported that there is no association between DZP exposure during pregnancy and major congenital malformation even in the first trimester of gestation (Bellantuono et al., 2013). However, another study suggested that that DZP would be safe if it had been used in small dose and for short duration and it recommended to avoid DZP administration at high doses for long duration (Iqbal et al., 2014).

8.3.3 Amitriptyline

As there are high risks on both mothers and babies if depression is left untreated during pregnancy, clinicians have become aware of the importance of treating depressed mothers. Several studies have shown fetal exposure to antidepressants during pregnancy by placental passage (Hendrick et al., 2003) and through amniotic fluid analysis (Loughhead et al., 2014). In general it seems that the association between prenatal exposure to antidepressants and abnormal development is not clear and needs more investigation and studies.

It appears that AMT has no significant effect on all the parameters tested at the doses which are around the therapeutic level (0.5-1μM). Cell survival and total protein were reduced significantly after 5μM. This agrees with (Braithwaite et al., 1979), who reported that AMT is toxic >3μM. It has been proposed that AMT triggers apoptosis in neuronal cells, glioma C6 cells, and even lymphocytes (Spanova et al., 1996, Xia et al., 1998, Post et al., 2000). AMT has been shown to increase the generation of reactive oxygen species and decrease intracellular glutathione levels and consequently it reduces the antioxidant capacity of the cells. Also it has been suggested that AMT activates NF-kB subsequently, which is a transcriptional factor that when activated induces cell death and it is considered as a pro-apoptotic factor (Post et al., 2000). AMT treated cell culture showed mitochondrial depolarization, release of cytochrome c and activation of caspase-3, which are markers of apoptosis (Lirk et al., 2006). This may explain the significant reduction in cell survival, total protein and neurosphere size.

Moreover, AMT treatment resulted in a significant reduction in the length of neuronal processes at the dose of 2.5μM and neurite length reduction was dose dependant. This agrees with the studies on chick and rat cerebrum (Farbman et

al., 1988). It has been believed that AMT reduces adenylate cyclase, which is crucial for neurite growth (Wong et al., 1991). The results also show that AMT has no significant effect on both tubulin III and GFAP proteins. This is may be due to this drugs is toxicity which may and trigger cell death for both neuronal (Post et al., 2000) and glial cells (Spanova et al., 1996) equally.

It seems that these neuroactive molecules do not have any toxic effect within their therapeutic level and the toxic effect appeared only at high doses which are higher than the therapeutic level.

Chapter 9

General Discussion

General Discussion

The development of the nervous system includes a series of sophisticated and critical events such as gene expression, neural stem cell proliferation, cell interactions, cell differentiation, cell migration and neural process formation and elongation (Sanes, 2006). Early exposure of the developing nervous system to chemicals which interfere with these events may result in developmental neurotoxicity (DNT). There is increasing concern about the association between environmental exposure to chemicals and a broad spectrum of congenital abnormalities including learning difficulties and neurodevelopmental disorders including autism, attention deficit and hyperactivity syndrome (Grandjean and Landrigan, 2006). Presently, there are more than 30000 chemicals in the markets of Europe, Canada and the USA for which there are little toxicity data (Judson et al., 2009). The USA and European Regulatory Agencies developed guidelines for in vivo studies to evaluate the developmental neurotoxicity of chemicals (USEPA, 1998) and (OECD, 2007); these guidelines specify studying behavioural and neuropathological endpoints in the offspring of rodents treated with these chemical during pregnancy and lactation.

These traditional in vivo studies (animal based) are impractical for the huge number of chemicals that are produced annually. It would be expensive, time consuming, requiring a large amount of chemical to be used in the experiments and needing a large number of animals to be sacrificed. Therefore, according to the 3R principle (Reduction, Replacement, and Refinement) of Russell and Burch 1959 (Russell WMS, 1959), alternative testing strategies are required to reduce animal numbers and to establish other methods which should be cheaper, quicker, and yet still sensitive. In 2007 the National Research Council's (NRC) Committee

on Toxicity Testing and Assessment of Environment Agents adopted this strategy and cited the need for in vitro tests which can prioritize the chemicals that need further investigation (NRC, 2007). REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) legislation also prompted the need for a new strategy (Hartung, 2009). Until recently, in vitro tests for studying the effects of chemicals on the development of the nervous system were mainly limited to transformed cell lines from human or rodents, these cell lines being derived from tumours and not necessarily reflecting normal neural cells.

Neural tissue culture was used for the first time more than 100 years ago for the purpose of studying neural process growth (Harrison et al., 1907). In order to achieve an *in vitro* test with a high level of predictability for developmental neurotoxicology, a test should ideally recapitulate the whole process of development of the human nervous tissue, including proliferation, migration, differentiation, and synaptogenesis. Deriving such a test is challenging. Human neural stem cells have been proposed for this purpose; they can offer several advantages over the other *in vitro* tests in providing a good source of normal cells which have the ability to divide and differentiate into the other cells of the central nervous system. Also these cells may have a better predictive power in detecting human neurotoxicity, since the extrapolation of the results is less (Breier et al., 2010) as species differences are circumvented (Coecke et al., 2007).

The end points in neurotoxicology are divided into two groups: general and specific. The general tests are related to the basal level of cell functioning which are indicators for cell survival. These tests are nonspecific and could be the end points for other non-neuronal cells and may be for all eukaryotic cells. These include: test for mitochondrial function, total cellular proteins, reactive oxygen

species, cell-cell communications, energy regulation (oxidation-reduction status), ion transport, cell respiration, …etc. (Harry et al., 1998).

On the other hand, there are specific end points which are peculiar to the cells of the nervous system, such as differentiated neural cells. Among these tests is the measurement of process outgrowth which receive the most attention since axonal and dendritic processes are the hallmarks of neural morphology and critical in cell connectivity and function. Neurite outgrowth is a critical event in nervous system development, in which neurons extend specialized processes to establish contacts (synapses) and facilitate the flow of information throughout the neural network (Sanes, 2006). Neurites refer to both axons and dendrites that are sprouted from the cells growing in culture; they are critical in defining the morphology of neurons. Abnormalities in the morphology of the neurites have been found after in vivo exposure of laboratory rodents to developmental neurotoxicants (Morrow et al., 2005). Neurite outgrowth has been studied in vitro using a variety of neural cell culture models such as tumour derived or transformed neural cell lines (e.g. PC-12) and dissociated primary cultures from developing animals (Radio and Mundy, 2008). In the present study, primary neural stem cells have been used as a new model to study neurite outgrowth after exposure to chemicals which are known to be teratogenic, such as sodium valproate.

Neuronal proliferation is another event in nervous system development; alteration in this by genetic, environmental or chemical influences can result in developmental abnormalities and neurotoxicity (Barone Jr et al., 1999). Therefore, cell proliferation can be a target for neurotoxicant chemicals and could be incorporated as an endpoint in in vitro screening batteries. Neural cell proliferation has been used in several studies as an end point to detect the

neurotoxic potential of chemicals (Costa et al., 2007, Jacobs and Miller, 2002). Cellular proliferation is controlled by multiple factors which affect the cell cycle (Ohnuma and Harris, 2003) and represents a potential site of action for chemicals. Since the mechanism(s) of action of different chemicals on cell proliferation are often unknown, detecting the effect of these chemicals regardless the site of action would be a suitable endpoint for screening purposes.

Cell migration is another essential process in development of the nervous system. Neural cells migrate within the developing brain to reach their final position and form the correct connections with other cells in the brain. Disturbed neural migration during development of the nervous system may result in improper neural outcome, as in foetal alcohol syndrome (Guerri, 1998), epilepsy and mental retardation (McManus and Golden, 2005).

Several in vitro methods have been used in studying cell migration such as the scratch method in confluent cell monolayers, the transwell method, video microscopy, live cell imaging, or hippocampal slice cultures and these have been used to observe and track migrating cells. In these techniques, the source of the cells is usually either animal or tumour cells, which may not resemble normal human cells. Therefore, species differences and the discrepancies between the normal and tumorigenic cells are disadvantages of using these techniques in neural cell migration assays.

Levels of GFAP and tubulin III proteins were measured and considered as end points. In the case of GFAP, it is well known that this protein is a sensitive and early bio marker of neurotoxicity when studied quantitatively and qualitatively (O'Callaghan and Sriram, 2005). Injury to the brain causes astrocytes to transform

into reactive astrocytes and start to proliferate and result in a state called astrogliosis, and this can occur before the appearance of the toxic effect on neurons (O'callaghan, 1991). Astrogliosis appears clearly with methyl mercury and lead acetate and a reduction in tubulin III protein (neuronal cells) appears obvious with methyl mercury. This indicates that this model has the capability to detect chemical toxicity.

Physiological cell death, which is the deletion (by apoptosis) of redundant or unsuccessful neurons in the developing nervous system, has been considered as a regular phenomenon in brain development. It has been reported that drugs like sedatives or anticonvulsants, can stimulate a wave of neuronal apoptosis in the developing brain when administered to immature rodents (Bittigau et al., 2002, Ikonomidou et al., 1999), such compounds include, phenytoin, valproate, GABA receptor agonists (benzodiazepine and barbiturate), and NMDA receptor blockers. The developmental period of the human brain which is vulnerable to drug induced neuro-apoptosis extends from the 6^{th} month of gestation to several years after birth. This coincide with a brain growth spurt as shown in the figure (16) (Ikonomidou and Turski, 2010). Exposure to these compounds at therapeutic doses during the vulnerable period in the rat, which is the first two postnatal weeks, triggers immature neurons to commit cell suicide (apoptosis) (Bittigau et al., 2002).

It is well known that anti-epileptic administration during pregnancy is associated with a broad spectrum of congenital anomalies (Meador et al., 2006) and most of the anti-epileptics in use are not safe during pregnancy (Prakash et al., 2008) and the teratogenicity is dose dependant (Tomson et al., 2011). It appears from this study that human neural stem cell culture is a sensitive model to predict the

teratogenesis of these compounds, especially phenytoin and sodium valproate, in which the end points tested, neuronal process length, neurosphere sizes and cell migration, were significantly reduced at the doses which lie within therapeutic range. However, phenobarbitone and carbamazepine seem to be toxic only at high doses and have no significant effect at therapeutic doses. It has been pointed out that carbamazepine is less teratogenic than sodium valproate (Jentink et al., 2010a). The teratogenicity of carbamazepine appears if it is combined with other anti-epileptic (like phenytoin) and it has been found that epileptic mothers who received carbamazepine and phenytoin had children with low developmental and language scores (Kaindl et al., 2006). Regarding phenobarbitone, it has been shown that it is less toxicity than other anti-epileptics (Holmes et al., 2001).

Additionally, the anti-oxidants, folic acid and melatonin appear to have no significant effect on most of the end points, however, they enhance neural stem cell proliferation by increasing the sizes of neurospheres, this agree with previous study about folic acid (Liu et al., 2010) and about melatonin (Fu et al., 2011), which reported that these drugs stimulate neural stem cell proliferation, but adding them with sodium valproate and phenytoin has to some extent some beneficial effect on the end points by making their toxic effect appear at doses which were higher than those when these drugs were used alone

On the other hand, regarding the effect of other neuroactive drugs (lithium, diazepam, and amitriptyline) on the neural stem cell culture, this study indicates that these drugs may be safe within their therapeutic doses and the toxicity appears only at high doses which are away from the therapeutic doses. Lithium has been considered as a weak teratogen and animal studies showed that lithium treatment did not result in developmental abnormalities (Giles and Bannigan,

2006), but it has a narrow gap between the therapeutic and the toxic dose (Su et al., 2007a). Diazepam treatment also has no significant effect on the cultured cells, other than at extremely high doses. It has been suggested that diazepam is safe even when administered to pregnant women during the first trimester (Bellantuono et al., 2013), however, another study showed that benzodiazepine treatment could be safe if it has been used in small dose and for a short duration (Iqbal et al., 2002). Similarly, amitriptyline appears safe during the therapeutic dose, but its association with developmental anomalies remains unclear.

Future work

Study the effects of chemicals and drugs for longer duration, more than 2 weeks, to study their effect at a chronic level. We recommend also to study synaptogenesis, which is an important end point in neurotoxicology, and to study cellular distribution within the neurosphere whether it is affected or not. Also, the effects of these chemicals on myelination process and microglia should be considered. Lastly, Study this model with other models such as neural stem cells from mice or rats to compare the sensitivity of this model with the others.

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Appendices

Appendix 1: Preparation of solutions

Full culture medium	**500ml**
Dulbecco modified eagle medium-DMEM	440ml
10% heat inactivated foetal bovine serum	50ml
2oomM Glutamine	5ml
50µg/ml penicillin/streptomyecin	5ml

Resazurin stock solution

Resazurin 5mg added to 5ml of HBSS to give the Resazurin stock (5ml, 1mg/ml in HBSS) then the solution was filter sterilized in to a 50ml universal container. HBSS (45ml) with calcium and magnesium was added to create a working dilution of 100µg/ml (This stock solution was wrapped with aluminium foil and stored at -20° C). This was subsequently diluted 1:10 in HBSS and the tube also wrapped in aluminium foil to avoid photo reduction. Resazurin solution (10µg/ml) was stored at 4^{o}C and used within 2 weeks. Resazurin solution was warmed to 37^{o}C prior to exposure to cells.

Kenacid blue fixative	**1 litre**
Glacial Acetic acid	10ml
Ethanol	500ml
DH2O	490ml

Prepared and kept at room temperature

Kenacid blue stock solution	**890ml**
Kenacid blue	0.4g

Ethanol and	250ml
DH2O	630ml

Prepared and kept at room temperature

Kenacid blue working solution	**50ml**
Acetic acid to	6ml
Stock solution	44ml

It was made up immediately prior to use.

Kenacid blue washing solution	**1 litre**
Glacial acetic acid	50ml
Ethanol	100ml
DH2O	850ml

Prepared and kept at room temperature

Kenacid blue desorb solution	**1 litre**
Potassium acetate	98.15g
Ethanol	700ml
DH2O	300ml

Prepared and kept at room temperature

Neural Stem cell maintenance media	**100ml**
DMEM (Dulbecoo's Modified Eagle Medium)-Sigma	47.5ml
Ham's Nutrient F12 -Sigma	47.5ml
200mM L- Glutamine- Sigma	1ml
100X N2- Invitrogen	1ml

50X B27- Invitrogen	2ml
5µg/ml Heparin- Sigma	100µl
10ng/ml bFGF (basic Fibroblast Growth Factor)-Gibco	100µl
20ng/ml EGF (Epidermal Growth Factor)- Gibco	100µl
Neural Stem Cell Differentiation Media	**100ml**
DMEM (Dulbecoo's Modified Eagle Medium)	47.5ml
Ham's Nutrient F12	47.5ml
200mM L- Glutamine	1ml
100X N2	1ml
50X B27	2ml
10X electrophoresis buffer:	**1 litre**
Tris	30.3gm
Glycine	144gm
SDS	10gm
Distilled water	1 litre
Prepared and kept at room temperature	
Transfer buffer:	**10 litres**
Tris	30.3gm
Glycine	144gm
Methanol	2 litres
DH2O	8 litres
Prepared and kept at 4° C	

TBST:	**10 litres**
Tris	30.3gm
NaCl	73.12gm
DH2O	1 litre

The pH should be adjusted to 7.6 by using Hcl. Then 9 litres and10 ml tween were added to the final solution. Prepared and kept at room temperature

Lysis Buffer:	**500ml**
Tris	12.1gm
EGTA	1.9gm
Sucrose	51.7gm
Triton X100	500µl
NaF	0.021gm
Betaglycerophosphate	1.08gm
DH2O	500ml

Prepared and kept in fridge at 4°C

100µl of protease inhibitor was added to 10ml of lysis buffer to be used within 2 weeks

6X Solubilisation Buffer	**10ml**
SDS	2.4gm
Glycerol	3.0ml
Betamercaptoethanol	3ml
BPB	240µl

Tris Hcl	2.5ml of 1.5M

Prepared and kept in freezer at -20°C

Lowry A Solution	**500ml**
NaoH	2gm
SDS	1gm
NaCO3	10gm
DH2O	500ml

Prepared and kept at room temperature

Lowry B Solution

CuSO4	1.0% 100µl in 20ml DH2O
NaK Tartrate	2.0% 100µL in 20ml DH2O

Prepared and kept in fridge at 4°C

Appendix 2: Materials

Plastic and Glass Wares:

100 mm round Petri dishes	Sterilin, UK
24 well plates	Nunc, UK
48 well plate	Nunc, Uk
96 well plate (ultra-low attachment)	Corning
96 well plates	Costar and Nunc, UK
Bijou tubes	Sterilin, UK
Eppendorf tubes	Sterilin, UK
Serological Pipette	Gilson, France
25 cm^2 Tissue Culture Flasks (untreated)	SPL Life Sciences- Korea
75 cm^2 Tissue Culture Flasks (untreated)	SPL Life Science- Korea
Universal tubes, 20 and 50 ml	Sterilin, UK
Conical centrifugation tubes, 15 ml	BD Falcon, USA
Filter 0.22	Sartorius Stedim, Germany

Equipments:

FLUORStar Galaxy	BMG Cambridge, UK
ASYS HITEC EXPERT 96	SLS, UK
Balance	A &D Intsrument, UK
Centrifuge (Centaur 2)	Fisons and Sigma, UK
Class 1 laminar flow hood	Faster, Italy
Class 2 Laminar flow hood	Heraeus Instrument, UK
CO2 Incubator	Sanyo, Japan and Heraeus, UK

Cooled Incubator	Gallenkamp, UK
Egg Incubator	ChickTec, UK
Haemocytometer	Hawksley, UK
Inverted phase microscope	Zeiss, Germany and Olympus, Japan
Plate shaker	Luckham Ltd, UK
Microtome	Leica, Germany
Automatic processor	Leica, Germany
Florescent microscope	Leica, Germany

Cell culture reagents:

100% Ethanol	Sigma-Aldrich, UK
DMEM (Dulbecco's Modified Eagle Medium)	Sigma-Aldrich, UK
Ham's Nutrient F12	Sigma-Aldrich, UK
0.05% Trypsin -0.02% EDTA	Sigma-Aldrich, UK
B27 supplement	Invitrogen, UK
N2 supplement	Invitrogen, UK
bFGF (Basic Fibroblast Growth Factor)	Gibco by Life technology, UK
EGF (Epidermal Growth Factor)	Gibco by Life technology, UK
Heparin	Sigma-Aldrich, UK
Foetal Calf Serum (heat Inactivated)	Sigma-Aldrich, UK
Hank's Balanced Salt Solution (HBSS)	Sigma- Aldrich, UK
Dimethylsulfoxide (DSMO)	Sigma- Aldrich, UK
Horse Serum	Sigma-Aldrich, UK
L- Glutamine	Sigma- Aldrich, UK

Penicillin-Streptomycin	Sigma- Aldrich, UK
Accutase	Sigma-Aldrich,UK
Phosphate Buffer Saline tablet (PBS)	Sigma- Aldrich, UK
Paraformaldehyde PFA	Sigma, UK
Trigene	Medichem International
Poly D Lysine	Sigma- Aldrich, UK
Poly L Lysine	Sigma- Aldrich, UK
Collagen I rat tail	Sigma- Aldrich, UK
Laminin	Sigma- Aldrich, UK

Drugs:

Methyl mercury	Sigma- Aldrich, UK
Lead acetate	Sigma- Aldrich, UK
Sodium Valproate	Sigma- Aldrich, UK
Phenytoin	Sigma- Aldrich, UK
Phenobarbitone	Sigma- Aldrich, UK
Carbamazepine	Sigma- Aldrich, UK
Lithium Carbonate	Fischer Scientific, UK
Diazepam	Sigma- Aldrich, UK
Amitriptyline	Sigma- Aldrich, UK
Folic acid	Sigma- Aldrich, UK
Melatonin	Sigma- Aldrich, UK

Chemicals for Cell Viability Assay:

Resazurin	Sigma- Aldrich, UK

Resorufin 95% dye colour	Sigma- Aldrich, UK

Chemicals for protein detection assay:

Bovine Serum Albumin	Sigma- Aldrich, UK
Ethanol	Sigma- Aldrich, UK
Glacial Acetic acid	Fischer Scientific, UK
Kenacid Blue dye	Sigma- Aldrich, UK
Potassium Acetate	Fischer Scientific, UK

Western Blot Analysis:

Precast gel	Biorad
Tris	Invitrogen
Glycine	Fisher Scientific
SDS	Acros Organics
Methanol	Sigma
Nacl	Fisher Chemicals
EGTA	Sigma
Sucrose	Fisher Scientific
Triton X100	Sigma
NaF	Sigma
Betaglycerophosphate	Sigma
Glycerol	Fisher Scientific
Betamercaptoethanol	Fisher scientific
Bromophenolblue	BDH
NaOH	Fisher Scientific

NaCO3	Fisher Chemicals
CuSO4	Fisher Scientific
NaK Tartrate	Fisher Scientific

Software:

Endnote X6	Thomas Reuters, USA
Prism statistical software version 6.0	Graph pad, USA
Volocity version 6.3.1	PerkinElmer, USA
Odyssey	Li-Cor BioSciences, UK

Appendix 3: The Antibodies

1. Primary and secondary antibodies in Western blot:

	Primary Antibodies	M.W	Dilution	Sources	Cat. no
1	Mouse monoclonal Anti-Tubulin III antibody	50Kda	1:2000	Abcam	ab78078
2	Rabbit polyclonal Anti-GFAP antibody	55Kda & 48Kda	1:20000	Abcam	ab7260
3	Mouse monoclonal Anti-GAPDH antibody	37Kda	1:5000	Sigma	G8795

	Secondary Antibodies	Dilution	Sources	Cat. no
1	Goat Anti-mouse	1:30000	Li-Cor Odyssey	926-32211
2	Goat Anti-rabbit	1:30000	Li-Cor Odyssey	926-68020

2. Primary and secondary antibodies in Immunocytochemistry and Immunohistochemistry:

	Primary Antibodies	Dilution	Sources	Cat. no
1	Mouse monoclonal Anti-Tubulin III antibody	1:500	Abcam	ab7751
2	Rabbit polyclonal Anti-GFAP antibody	1:800	Abcam	ab7260
3	Rabbit polyclonal Anti-SOX2 antibody	1:2000	Abcam	ab97959
4	Mouse monoclonal Anti-Nestin Antibody	1:1000	BD Biosciences	611658

	Secondary Antibodies	Dilution	Sources	Cat. no
1	Goat polyclonal secondary antibody to mouse IgG, Alexa Fluor 488	1:500	Abcam	ab150113
2	Goat polyclonal secondary antibody to rabbit IgG, Alexa Fluor 555	1:500	Abcam	ab150098