CHAPTER I

INTRODUCTION

Introduction

Natural processes of differentiation, development and aging cause apoptotic cell deaths which are caused due to the formation of free radicals (OH^-, O^-) through the different metabolisms (Halliwell and Gutteridge, 1985; Tyurin, 2000). The free radicals generation can be induced, enhanced by exposure of organisms to various natural and other products that lead to cell deaths as well other cell pathological effects leading to tissue injury and in acute conditions may resulted into animal death. In developing animal, it may develop the abnormalities at subacute concentrations (Choudhary, 2006).

In case of brain, free radicals during natural aging or pathological conditions lead to various neurological disorders Viz. Parkinson's disease (impaired motor control), Alzheimers disease (degeneration of brain).

During development if the balance of free radical generation lead to higher levels of free radicals it also causes many abnormalities, series conginitive deficits or behavioural abnormalities (Sarah *et. al.* 1999).

In recent years exposure of animals and humans to polluting agents is being continuously increased through normal living or through occupational compulsion, it is time that these types of studies be performed in developing animals. The abuses of which, that are caused in incoming generations be conveyed to the society at large so that alert and precondotinary measures can be though off.

Reasons to select the problem:

For the reasons stated above the present project was designed. Thus to study the developmental alterations *in ovo* developmetnal model of chick was selected. Since, brain is the organ of immense importance to human which involves characters that give identity as human, in additon to its other functional roles brain developmental has studied. As stated earlier free radicals viz. O^-, OH^- are generated in development and their balance is important for normal development. Therefore, in the project, by introduction of H_2O_2, its balance was disturbed to increase the free radicals during early development of brain.

Early development of brain was studied because it is early phase when subtle changes in free radicals may be leading minor to serious consequences on full development of brain.

It was decided to study glycosaminoglycans and sialic acid because glycosaminoglycans are known to play role in differention and development and sialic acid is a main constituent of gangliosides, which are membrane glycolipids of major brain cells neurons. Study of effect of vitamin C was included so that the regulatory role of naturally occuring antioxidants (Rondst, 2000) can be made out which can help the precuationary efforts to manage the free radical induced damage to brain in early development.

In this light, present project was designed and accordingly worked out. Thus, H_2O_2 and vitamin C modulated effects on distribution of GAGs and sialic acid was studied during early development of chick brain.

Reasons to select chick embryo as a model:

An avian embryo is a valuable model system for vertebrate embryology. Easy availability, accessibility to various developmental stages and amenability of organ makes the chick embryo one of the favored model systems. Seminal discoveries regarding organogenesis and vertebrate morphogenesis have been made using chick embryos as a model (Ghatpande *et al.*, 2008). Experimental studies on animal models play essential roles in the development of preventive, diagnostic and therapeutic procedures for diseases in a wide spectrum of fields including neurological sciences. The goal of this study was to demonstrate the effect of free radical and it's scavenger on the chick embryo as a potential experimental model in the field of developmental neurobiology.

Chick embryo is used as a model for differentiation and developmental studies and is used to determine the neuroteratogenic effects of nicotine (Wielgus, 2004) and is free of confounds related to mammalian maternal effects (Wormer, 2005).

There have been plenty of researches reportedly using it as a model for radiation effect study (Ingole and Ghosh, 2006). Chickens chorioallantoic membrane (CAM) is used to predict pro/anti-angiogenetic chemical (Nishikawa *et al.*, 1987).

Present project was designed to study H_2O_2 induced alterations in brain (early development) and its possible management by vitamin C and therefore it was used for the same purpose.

Reason to select brain as an organ of study:

Brain may be especially at risk from free radical attack, because this tissue characterized by a low content of natural antioxidants (Surai *et al.*, 1996) and generates a greater amount of free radicals per gram of tissue than any other organ (Reiter, 1995). Reactive oxygen metabolites (ROM) have also been implicated in the aetiopathogenic processes of a number of pathological conditions of the brain. While primarily indirect, evidence for this view is accumulating, and credence for the participation of free radical oxidative interactions in promoting tissue injury in such conditions as brain trauma, ischemia, and toxicity, and in neurodegenerative diseases such as Parkinson's disease, Alzheimer's dementia, multiple sclerosis, and lipofuscinosis, is growing. Concomitant with this new understanding of the injurious role of free radical oxidants in neural pathology, there is the increasing appreciation for the need for both fundamental and clinical research into the development of the potential preventive and therapeutic benefits that are now being foreseen for a variety of antioxidant nutritional and pharmacological interventions (Evans, 1993). Besides oxidative stress is an early feature after cerebral ischemia (Blomgren and Hagberg, 2006) that lead to neuropathological alterations.

Reasons to study early development of brain:

In late developmental stages, free radicals generation and its management by Vitamins have been studied as realized by the following review. In most of the work pieces of brain were used while neurons were used majority of times therefore to know the details of *in vivo* early chick brain development, this period of development was selected and it was decided to study *in vivo*. The increased levels of free radicals like H_2O_2 generated in early stages of brain differentiation and development and its management with one of the vitamins, ascorbic acid; which is known to influence in advanced stages of development will provide the consequences (mortality/abnormality/protection) in ROS stressed brain in early development.

For this reason, different stages of brain differentiation and development were used to design the experimental work in the present project, which has been given in detail under chapter Material and Methods.

Reasons to take H_2O_2 as a neuronal toxicant:

Hydrogen peroxide is easily accessible chemical that can generate the free radicals in the adult as well as embryonic organs. Hydrogen peroxide causes toxicity via three main mechanisms: corrosive damage, OH^-, O^- free radical generation through tissue metabolisms leading to lipid peroxidation which can cause necrotic cell death in various tissues as well as embryos. Besides Rebeca (1997) had stated that, neuronal vulnerability to H_2O_2 during *in vivo* development is unknown and workouts investigations.

Reasons to study Vitamin C:

As stated earlier vitamin C is one of the antioxidant used widely. Vitamin C is easily available and accessible material from the natural diet. It can also a part of regular food in the daily diet of humans (Cadenas and Packer,19996).

Reasons to study Glycosaminoglycans (GA):

Glycosaminoglycans are a family of sulfated polysaccharides involved in diverse biological processes such as neuronal development, tumor growth and metastasis, viral invasion and spinal cord injury (Capila, 2002; Sasisekharan *et al.*, 2002; Laabs *et al.,* 2005). For example, glycosaminoglycans modulate key signaling pathways essential for proper cell growth and angiogenesis (Sasisekharan *et al.,*; 2002; Laabs *et al.*, 2005). They are also important for axon path finding in the developing brain and have been linked to the pathology of Alzheimer's disease (Merry *et al.,* 2001; Bulow and Hobert, 2002; Li *et al.*, 2005). The remarkable ability of glycosaminoglycans to regulate various processes is only beginning to be understood at a molecular level. Increasing evidence suggests that glycosaminoglycans encode information. The mechanism by which it coordinates biological events is crucial for understanding diverse aspects of biology and could reveal new therapeutic opportunities. *In vitro* it has been shown that external administration of glycosaminoglycans to cultures of fibroblast in which H_2O_2 toxicity was induced, showed their antioxidant effects on fibroblast (Campo *et al.,* 2004).

There is increasing evidence that different proteoglycans act as regulators of cell migration, axonal path finding, synoptogenesis and structural plasticity

(Represa, 1993; Bandtlow and Dieter, 2000). These being the events involved in development of brain it was decided to study H_2O_2 and vitamin C influenced behavior of GAGs in early brain development in chick.

Reasons to select the histochemical techniques:

Bioassay of any of the chemical component of tissue gives the picture of total amount of the respective chemical. But, histochemical techniques provide information about their localization, any quantitative change associated with any of the physiological or pathphysiological alterations which can be related to the tissue within which the alterations occurs. Under light microscope partial visible quantization can be conducted. Besides the early chick brain development involves differentiation, development and histogenesis of brain wall and its ventricles therefore the localization of these components can reveal the role of GAGs in histogenesis and morphogenesis as well.

Reasons to use window method:

Since the effect of doses that allow the animal survival and without any abnormality in brain needed to be selected in present project, window method was used for introduction of H_2O_2 and/or vitamin C. HBSS was used as the medium, which is also used *in vivo* and in vitro chick embryo studies (Korn and Cramer, 2007).

Reasons to select 0.5 mM H_2O_2 dose for study:

Hydrogen peroxide treatment was initiated as a single dose at different hrs of early development that show morphologically and histochemically detectable events using HH stages (Hamilton and Hamberger, 1951). The doses were continued for successive developmental hours. At hrs of incubation 1 ml in HBSS containing varied concentrations viz. 0.05 mM, 0.5 mM and 1.5 mM H_2O_2 were tested. Exposure period of doses was terminated at various intervals to study the intermittent effect. The highest interval was of 144 hrs. Twenty four hrs earlier to this brain compartments are completed and growth begins. In preliminary experiments mortality induced by H_2O_2 doses was selected with different doses on hatching. Similarly abnormalities were also studied on this data. Data is presented in the section I Mortality observations. Average 50% mortality was shown with 0.5 mM in all the experiments conducted. Therefore, this dose was selected for the

further vitamin C mediated experimental studies and histochemical distribution of GAGs and SA was studied in brain.

Reasons to select the 3 mg/egg dose of vitamin C for study:

Three mg/egg ascorbic acid dose which is known to improve hatchability of broiler egg (Ipek *et al.,* 2004) was selected to scavenge the free radicals generated by H_2O_2 treatment. Besides 4 mg/egg and 5 mg/egg dose of vitamin C was also used considering the increased need of free radical scavengers in H_2O_2 treated embryos. Tree mg dose alone had been shown to prevent 0.5 mM H2O2 induced mortality (50%) without any abnormalities on hatching. Both the higher doses showed abnormal development of brain and neck region and death in following 1 to 3 days. For these reasons 3 mg vitamin C dose was selected for antioxidant protection studies.

Chick brain development:

Fertilization

Fertilization of the chick egg occurs in the oviduct, before the albumen and the shell was secreted upon it. The egg is telolecithal, with a small disc of cytoplasm sitting atop a large yolk. The yolky eggs of birds undergo discoidal meroblastic cleavage to give blastomeres (8 cells). Further successive cleavage furrows divide the central cells and other radial furrow divides peripheral cells.

Blastulation and Gastrulation:

The blastomeres form blastoderm. It is 5 -6 layers thick where cells are linked by tight junctions. A closely packed mass of blastomeres is formed called as Morula.

At the time of egg laying the blastoderm contains some 20,000 cells and called as hypoblast. Gastrulation occurs in first few hours after the egg is laid. The two-layered blastoderm (epiblast and hypoblast) is formed consequently.The avian embryo comes entirely from the epiblast (Rosenquist,1966; 1972). Three germ layers of the embryo proper (plus a considerable amount of extraembryonic membrane) are formed from the epiblastic cells. Fate maps of the chick epiblast were studied by Schoenwolf (1991).

The Primitive streak:

At 12 hrs thickened area of the epiblast is formed called primitive streak. The streak elongates forwards to form head and secondary hypoblast cells continue to migrate anteriorly from the posterior margin of the blastoderm. The primitive streak defines the anterior posterior axes of the embryo. At 16 hrs of incubation, a depression forms within the streak. This depression is called the primitive groove, flanked on either side by primitive ridge. It is the primitive groove, along which cells migrates to blastocoel.

Formation of endoderm and mesoderm:

Epiblast formed the head process. The head of the avian embryo forms anterior (rostral) to Hensen's node. The cells that follow the above cells on migration route form chordamesoderm (notochord) cells and extend up to the presumptive midbrain, to meet the prechordal plate. The hindbrain and trunk form from the chordamesoderm at the level of Hensen's node and caudal to it.

At 24 hrs development primary neurulation results in constriction of anterior portions of the neural tube, while secondary neurulation results in the formation of neural tube caudal to the twenty-seventh somite pair (i.e., everything posterior to the hindlimbs) (Pasteels,1937; Catala *et al.,* 1996).

Chick neurulation :

By 33 hours of incubation, neurulation in the chick embryo is continued. The neural folds migrate toward the midline of the embryo, and fuse to form the neural tube beneath the overlying ectoderm. The cells at the dorsalmost portion of the neural tube become the neural crest cells.

Primary neurulation:

The head and trunk regions both undergo variants of primary neurulation, and this process can be divided into four distinct but spatially and temporally overlapping stages: (1) formation of the neural plate; (2) shaping of the neural plate; (3) bending of the neural plate to form the neural groove; and (4) closure of the neural groove to form the neural tube (Smith and Schoenwolf, 1997).

Formation and shaping of the neural plate:

At about 18 hrs of incubation areas of greater density arise and extend rostral to the cephalic end of the notochord. The cells of the ectoderm at the midline region and is termed as neural plate/ medullary plate. Edges of neural plate thicken and move upward to form the neural folds, while a U-shaped neural groove appears in the center of the plate.

Neural plate formation is induced by the action of chordamesoderm or (notochord) in ectoderm. Induction results in transformation of unspecialized ectodermal cells to primordium of central nervous system. The cell divisions of the neural plate cells are preferentially in the rostral-caudal (beak-tail; anterior-posterior) direction (Jacobson and Slater, 1988; Schoenwolf and Alvarez 1989; Sausedo *et al.*, 1997).

Bending of the neural plate:

In the embryo of 22 hrs, the neural plate becomes longitudinally folded to establish a trough known as the neural groove. On either side of neural groove formed are neural folds. These are the differentiation stages of the central nervous system. The bending of the neural plate involves the formation of hinge regions where the neural tube contacts surrounding tissues.

Closure of neural tube:

The folding of the neural plate takes place at 24 hrs. By 27 hrs the neural folds fuse with each other and the neural tube is formed. The neural tube cells that expressed E-cadherin, they start producing N-cadherin and N-CAM.

Neurulation in the cephalic (head) region is well advanced and closing of the neural tube occurs i.e. neuromery. It is the process of segmental arrangement of transverse bulges along the neuraxis which defines the various brain regions. The two open ends of the neural tube are called the anterior neuropore and the posterior neuropore.

Secondary neurulation:

Secondary neurulation is usually seen in the neural tube of the lumbar (abdominal) and tail vertebrae. It can be seen as a continuation of gastrulation.

Differentiation of the Neural tube:

The neural tube and its lumen bulges and constricts to form the chambers of the brain and the spinal cord. The cell populations within the wall of the neural tube rearrange themselves to form the different functional regions of the brain and the spinal cord. These neuroepithelial cells differentiate into the numerous types of nerve cells (neurons) and supportive cells (glia).

Differentiation of brain regions:

The thickened wall and dilated lumen which develops into brain while undilated posterior part gives rise to spinal cord. Initial neural tube gives 11 neuromeres which were first time analyzed in series of stages in chick (Kallen, 1955).

There are three primary brain vesicles. The fore brain (prosencephalon), posterior constriction is mid brain (mesencephalon), posterior to the mesencephalon, a very slight constriction is hind brain (rhombencephalon). It continuous posteriorly with cord region of neural tube.

Divisions of brain and closure of neuropore:

Prosencephalon form pair of primary optic vesicles. Lumen of optic vesicle communicate with the lumen of prosencephalon. By 33 hrs optic vesicles comes to lie near the superficial ectoderm. Depression in the prosencephalon is infundibulum.

38 hrs of development:

The prosencephalon divides into the anterior telencephalon and caudal diencephalon. Telencephalic vesicles contain the rudiments of the cerebral cortex. The diencephalon contains the rudiments of the thalamus and hypothalamus, as well as a pair of lateral optic cups from which the neural portion of the retina will form. The median enlargement extending rostral beyond the level of the optic vesicles

indicates the establishment of telencephalon. The optic vesicles and the prosencephalon lying between them go into the diencephalon.

The mesencephalon and its lumen eventually becomes the cerebral aqueduct. The rhombencephalon is subdivided into metencephalon and myelencephalon which becomes the medulla oblongata. The metencephalon gives rise to the cerebellum. The rhombencephalon shows segmental pattern specifies to the origin of nerves. The rhombencephalon divides into rhombomeres, (Guthrie and Lumsden, 1991). Each of it will form ganglia, the clusters of neuronal cell bodies, whose axons form a nerve, which generate the cranial nerves.

At 3-4 day, the brain volume expands thirty-fold. This rapid expansion is due to positive fluid pressure exerted against the walls of the neural tube by the fluid within it. When the neural folds close in the region between the presumptive brain and the presumptive spinal cord, the surrounding dorsal tissues push into constrict the neural tube at the base of the brain (Schoenwolf and Desmond, 1984; Desmond and Schoenwolf, 1986; Desmond and Field, 1992).

Flexion and Torsion: At 38 hrs flexion and torsion is set in.

Flexion:

The direction of the flexion is ventrally towards the yolk and is carried out so that the bend is towards the anterior of notochordal end.

Torsion:

Telencephalic region of embryo is twisted so that left side lies next to the yolk and right side is away from the yolk. At about 38 hrs, the cranial flexion and torsion are evident in the head region of embryo. At 43 hrs, the further progress of both flexion and torsion is well marked. The entire embryo comes to lie with its left side on the yolk. The progress of torsion, flexion makes spinal axis C shaped and head and tail lie close to each other.

50-55 hrs of development:

The mesencephalon remains most anterior to prosencephalon and myelencephalon that lie opposite to each other. The prosencephalon is now in close proximity to the heart, optic vesicle and the auditory vesicle are brought opposite to each other at nearly the same antero-posterior level.

The flexion in the body and in the brain region is marked at the heart in the region of transition from myelencephalon to spinal cord. This is the future neck region of the embryo and the flexion is the cervical flexion. Thus torsion and flexion is completed.

Growth of telencephalon region:

At 40 and 50 hrs the prosencephalon is divided into telencephalon and diencephalons. In the floor of the diencephalons, the infundibular depression has become deepened.

Posterior part of the brain and cord region of the neural tube:

The mesencephalon enlarges to separate cephalic diencephalons and caudal metencephalon. In myelencephalons dorsal wall becomes much thicker. In spinal cord region of neural tube lateral walls have become thickened so that the neural canal appears slit like.

72 and 96 hrs of development:

Formation of the telencephalic vesicles:

The antero-lateral walls of the primary fore brain are lateral telencephalic vesicles. The lumen of telencephalon has three divisions, a median telocoele, posterior diocoele and two lateral telocoele. The telencephalic vesicles becomes the cerebral hemisphere.

Diencephalon:

The lateral walls of the diencephalons at this stage shows little differentiation except ventrally where the optic stalks merge into the wall of the brain. It also gives

epiphysis as a median invagination. Later in development the lateral walls of diencephalons becomes greatly thickened.

The boundary between the diencephalons and the mesencephalon is an imaginary line drawn from the internal ridge formed by the original dorsal constriction between the primary forebrain and mid brain.

Mesencephalon:

The dorsal wall of the mesencephalon thickens rapidly and becomes the corpora quadrigemina of the adult brain.

Metencephalon:

The boundary between the mesencephalon and metencephalon is indicated by the original interneuromeric constriction. The metencephalon shows practically no differentiation in 4 day chick. Later this extends ventrally and laterally and extensive growth of fibers tracks gives rise to the pons and the cereberal peduncles. The roof of metencephalon undergoes extensive enlargement.

Myelencephalon:

The dorsal myelencephalic wall reduced in thickness and myelocoel. The ventral and lateral myelencephalic wall becomes the floor and side walls of the medulla.

All the developed parts are further enlarged and advance towards completion of brain development (Pattern, 1952).

Histogenesis:

Initial ectoderm of open neural groove or neural tube is made up of pseudostratified cylindrical epithelium, the neural epithelium/ primitive ependymal layer or the matrix layer. It contains tall neuroepithelial cells arranged in different planes and are with degree of mitotic activity (Sawer, 1993) and are placed at different positions (Langman *et al.*, 1966). Nuclei of cells that are located to external limiting membrane are in S phase. They move towards lumen of the neural tube and undergo mitotic divisions.

During closure of neural tube, neuroepithelial cells move and repeat germinative cycle and move beneath the membrane as neuroblasts and produce processes, which are forunners of axons and dendrites.

a) In later stages, a dense cell layer called the mantle layer develops laterally. It is also referred as inner ventricular or intermediate layer.
b) Still lateral to this, the cell free marginal layer can be seen. It is outer layer with numerous cell processes but few cell bodies.
c) Ependymal cells are formed in the gradually disappearing neural epithelium. It is innermost layer and composed of columnar epithelium of central canal, which are still in mitotic cycle. This layer is also referred as ventricular and ultimately becomes ependyma.

Neuroblasts:

Some cells of neuroepithelium loose ability to undergo mitosis and become neuroblasts. Actually, the mantle cells differentiate into neuroblasts and neurons, and into spongioblast and neuroglial cells which later form the astrocytes and oligodendrocytes. Neuroblasts initially are bipolar with slender processes. It becomes unipolar by retraction of processes. Unipolar cells accumulate large amount of ER (Nissl substance) and send several processes and becomes multipolar.

Neuroglia divide further even in adult. These glioblasts gives three types of cells viz. astrocytes, oligodendroglial cells and microglia. Astrocytes and oligodendroglial cells may arise from glioblasts or glial progenitor cell. Glioblasts develops long, five processes and differentiste into astrocytes. The processes of astrocytes becomes closely associated with capillaries and it serves as a transit system for metabolites within the substance of central nervous system. Oligodendroglial cells are smaller and more simpler than astrocytes and become recognizable later in development. They appear as satellite around the cell bodies of neurons and are also involved in the mylination of nerve fibers within the white matter of central nervous system. The third type, the microglia, are active in phagocytosis after damage to the nervous system and are presumably of mesodermal origin (possibly a form of macrophage). The neuroblasts of the mantle layer are not evenly distributed; they aggregate into clusters, which form the brain nuclei, or the strata of the cortical layers. Maturing neuroblasts gives nerve fibers. They do so in distinct patterns, the fibers giving rise to well defined fascicles, growing in specific direction at specific locations. They terminate at specific regions of the central

nervous system, or outside it. The formation of these connections- the wiring of the CNS produces the functional qualities of the CNS, and turns the brain and spinal cord into a working unit (Sperry, 1988). The process of neurogenesis is complicated and not a one step process. It has to be analyzed on various levels.

Early morphogenesis:

Closing of neural tube (neuromery) is segmental arrangement of transverse bulges along the neuraxis (Baer, 1928) develop in an orderly way in chick embryo (Kallen and Lindskog, 1989).

The bulges developed begin to disappear in the region caudal to the optic vesicles and referred as proneuromery, neuromery, and post neuromery respectively which continue to form brain walls. The brain wall begins to thicken and fissures become indistinct.

The floor of the neural plate folds down around the notochordal tip to form the hypothalamic protuberances. In the roof of the third and fourth ventricles ependymal tela develop. In other parts of the brain, transformation of neural to ependymal tissue occurs.

Histogenesis of neural tube wall in early stages:

The wall is of a pseudostratified cylindrical epithelium, the neural epithelium. Mitotic figures restrict to the ventricular surface. The nucleus moves to the ventricular lumen, there enters mitosis, and the daughter cells again move to later side.

The lateral part of the neural wall is the stratum zonate. This is a cell free zone composed of cytoplasmic extensions from the epithelium and continue to grow. In chicks, it is formed at HH stage 13-25 varying with the part of the brain. Then cells detach from the neural epithelium and migrate radially into the zonal layer. It begins early in the rhombencephalon and spinal cord, where as it is late in the dorsal parts of the hemispheres and the mesencephalic bulges.

Cell migration in chick embryo:

The radial migration from the neural epithelium is a continuous process, to produce a gradually thickening layer of migratory cells. This occurs in wave and produces a stratification of the migrated cells into concentric layer. With each wave of migration, the formation of each layer corresponds to a peak of mitotic activity.

The migrated cell layer increases in thickness, while the neural epithelium decreases and finally disappears.

Radial migration does not take place in random fashion in the brain:

The migrated layers of the cells are later transformed to nuclear rudiments or cortical layers after histodifferentiation. The layers can be traced in stained sections through a series of subdivisions or fusions until cell groups which corresponds to the adult brain nuclei, can be identified.

In embryos brains during 2.5-5.5 days no tangential migration was seen in the ventricular zone it was only radial. The tangential migration began just beyond the basal limit of the ventricular zone. On embryonic days 6 and 7, this spanned from the ventricular to the pial surface. Where, axonal tracts are present and are aligned with the migrating cells, and support the non-radial cell migration in diencephalon (Golden *et al.*, 1997).

Molecules involved in cell differentiation, migration and differentiation:

Neural cell adhesion molecules (N-CAM), the neuroglia cell adhesion molecule (Ng-CAM), and extracellular matrix protein, cytoactin are the molecules involved in brain development and differentiation (Chuong *et al.,* 19887). Among them N-CAM was found at all external granular layer and it was enriched in the molecular layer as development proceeded. Ng-CAM appeared in premigratory granular cells just before migration and though disappeared from cell bodies remained in the early period (36 hrs in culture) and major effects at a later period (18-72 hrs in culture).

Thus, Ng-CAM appears on postmitotic neurons. They appeared on 4D development on neuronal processes. They appears on cell body from neuroectodermal development and plays role in polarity development and network building. (Thiery *et al.,* 1985). Neuron-glia-related cell adhesion molecules (Nr-CAM) are another member of CAM family, Ng-CAM molecules though they may have independent bonding specifications, they have related functions and they act synergistically in development of neurons system (Krushel *et al.,* 1993). The chick axons associated surfaces glycoprotein (neurofascin) and its heterogeneity is generated by O and N-linked oligosacharides (Vokmen *et al.,* 1992).

The expression of the endocannabinoid receptor, CB1, initiates in the first born neurons of the CNS and plays an important role in directing axonal outgrowth in newly born neurons (Begbie *et al.,* 2004).

Chondroitin sulfate proteoglycans have been implicated in the regulation of cell migration and pattern formation in the developing peripheral nervous system. It blocks the migration pathways of neural crest cells or motor and sensory axons. And play a functional role in the guidance of migratory neural crest cells and outgrowing axons, (Landolt *et al.,* 1995). Versican is a large chondroitin sulfate proteoglycan belonging to the lectican family. Alternative splicing of versican generates at least four isoforms named V0, V1, V2, and V3. Versican V1 isoform not only enhanced cell proliferation, but also modulated cell cycle progression and protected the cells from apoptosis but inhibited cell proliferation (Sheng *et al.,* 2005).

H_2O_2 generated free radicals:

Free radicals are generated *in vivo* as byproducts of normal metabolism. They are produced in the catalytic action of a variety of cellular enzymes and electron transport processes and are implicated in a number of physiologic and pathological processes (Dianzani, 1992). Hydrogen peroxide (H_2O_2) is one of the free radical producing chemical. It is a pale blue liquid which appears colorless in a dilute solution, slightly more viscous than water. It is a weak acid. It has strong oxidizing properties and is therefore a powerful bleaching agent. It has also be used as a disinfectant, as an oxidizer, as an antiseptic, and in rocketry (particularly in high concentrations as high-test peroxide or HTP) as a monopropellant, and in bipropellant systems. The oxidizing capacity of hydrogen peroxide is so strong that the chemical is considered a highly reactive oxygen species. Hydrogen peroxide is naturally produced as a byproduct of oxygen metabolism, and virtually all organisms possess enzymes known as peroxidases, which harmlessly catalytically decompose low concentrations of hydrogen peroxide to water and oxygen. The most important oxygen-based free radicals are superoxide and the hydroxyl radical, produced from the reduction of molecular oxygen. These free radicals are extremely reactive and are often associated with cell damage, mutations, and even malignancies. Cellular targets at risk from free radical damage depend on the nature of the radical and its site of generation. Cellular sources of free radicals and the reactions they can undergo cellular defenses and adaptive mechanisms (Freeman and Crap, 1982).

Free radicals can damage cell, membranes molecule by molecule, making holes. The cells leak and lose their chemical balances. Subsequent free radicals are also able to damage DNA, making cells dysfunctional. Aging is simply the progression of damage, caused by metabolic free radicals. The body has developed a number of mechanisms to minimize free radical damage and even repair damage. Enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, as well as antioxidants such as vitamin A, vitamin C, vitamin E, and polyphenols play important roles in protecting the body from free radical damage. Superoxide dismutase (SOD) is present in both the mitochondria and the cytosol. SOD reacts with 2 superoxide anions to form 1 molecule of hydrogen peroxide. Hydrogen peroxide can be easily transformed into the dangerous hydroxyl radical via reaction with Fe^{2+}. Catalase reacts with hydrogen peroxide to form water and oxygen. Glutathione peroxidase can also react with hydrogen peroxide, by reducing hydrogen peroxide and oxidizing glutathione. Glutathione is an antioxidant. Antioxidants are an important part of the free radical defense mechanism along with the enzymatic processes. Hydrogen peroxide is produced *in vivo* by many reactions. Hydrogen peroxide is unique in that it can be converted to the highly damaging hydroxyl radical or be catalyzed and excreted harmlessly as water. Glutathione peroxidase is essential for the conversion of glutathione to oxidized glutathione, during which H_2O_2 is converted to water (Alessio and Blasi, 1997). If H_2O_2 is not converted into water, $1O_2$ is formed. Singlet oxygen is not a free radical, but can be formed during radical reactions and also cause further reactions. Singlet oxygen can then transfer the energy to a new molecule and act as a catalyst for free radical formation. The molecule can also interact with other molecules leading to the formation of a new free radical (Karlsson, 1997).

Hydrogen peroxide and nervous system:

Oxyge radicals, the biproduct of the biochemical and physiological reactions are known to damage cellular lipids, proteins and nucleic acids and initiate cell signaling pathways after cerebral ischemia. Genetic manipulation of intrinsic antioxidents and factors in the signaling pathways has provided substantial understanding of the mechanisms involved in cell death/survival signaling pathways and the role of oxygen radicals in ischemic cerebral injury (Atsushi and Carolina, 2005).

Hydrogen peroxide (H_2O_2) accumulates during hypoxic-ischemic brain injury and may mediate neurotoxicity in the immature brain. It is determined at that H_2O_2 causes aturation specific neurotoxicity. Primary neuronal cultures were exposed to H_2O_2 (25, 50, 100 μM) for 5 min or 24 h during *in vitro* development, and toxicity assessed showed immature neurons incurred marked and dose dependent injury after both brief and prolonged H_2O_2 exposures, and marked dose dependent death following prolonged H_2O_2 exposures. Mature neurons incurred marked injury following prolonged but not brief H_2O_2 exposures, and were relatively resistant to H_2O_2 induced death following both brief and prolonged exposures. Thus, H_2O_2 is selectively toxic to immature neurons *in vitro*. Neuronal vulnerability to H_2O_2 during *in vivo* development is unknown and warrants investigation. (Rebecca *et al.,* 1997). Concentrated hydrogen peroxide is caustic and exposure may result in local tissue damage. Ingestion of concentrated (>35%) hydrogen peroxide can also result in the generation of substantial volumes of oxygen. Where the amount of oxygen evolved exceeds its maximum solubility in blood, venous or arterial gas embolism may occur. The mechanism of CNS damage is thought to be arterial gas embolisation with subsequent brain infarction. Rapid generation of oxygen in closed body cavities can also cause mechanical distension and there is potential for the rupture of the hollow viscus secondary to oxygen liberation. In addition, intravascular foaming following absorption can seriously impede right ventricular output and produce complete loss of cardiac output. Hydrogen peroxide can also exert a direct cytotoxic effect via lipid peroxidation.

From the above data hydrogen peroxide can cause the damge to the body organs General Accounting Office (GAO, USA) that lists 30 chemicals of concern because of widely acknowledged reproductive and developmental consequences. But reports were located on the developmental or reproductive effects of hydrogen peroxide in humans. The data was also small concerning the other animals.

Hydrogen peroxide and the Glycosaminoglycans:

Acid glycosaminoglycans (GAGs) antioxidant activity was assessed in a fibroblast culture system by evaluating reduction of oxidative system-induced damage. Three different methods to induce oxidative stress in human skin fibroblast cultures were used. In the first protocol cells were treated with $CuSO_4$ plus

ascorbate. In the second experiment fibroblasts were exposed to $FeSO_4$ plus ascorbate. In the third system H_2O_2 was utilized. The exposition of fibroblasts to each one of the three oxidant systems caused inhibition of cell growth and cell death, increase of lipid peroxidation evaluated by the analysis of malondialdehyde (MDA), decrease of reduced glutathione (GSH) and superoxide dismutase (SOD) levels, and rise of lactate dehydrogenase activity (LDH). The treatment with commercial GAGs at different doses showed beneficial effects in all oxidative models. Hyaluronic acid (HA) and chondroitin-4-sulphate (C4S) exhibited the highest protection. However, the cells exposed to $CuSO_4$ plus ascorbate and $FeSO_4$ plus ascorbate were better protected by GAGs compared to those exposed to H_2O_2. These outcomes confirm the antioxidant properties of GAGs and further support the hypothesis that these molecules may function as metal chelators (Campo *et al.,* 2004) Oxidative stress, inflammation and apoptosis play a critical role in the onset and progression of cellular damage. It was previously reported that hyaluronan (HA) and chondroitin-4-sulphate (C4S) were able to protect human skin fibroblasts from oxidative stress. This antioxidant activity is due to the chelation of transition metal ions. Nuclear factor kB (NF-kB), complexed with the inhibitory protein IkB alpha, is an ubiquitous response transcription factor involved in inflammatory reactions and acts by inducing cytokine expression, chemokines and cell adhesion molecules. Caspases are specific proteases responsible for the regulation and the execution of apoptotic cell death. The damage caused by free radicals may be amplified greatly by the activation of these factors. According to these finding the use of HA and C4S could be positive both as tool to clarify the exact mechanism of GAGs/ROS interaction needed to be studied before this drug therapy be developed (Campo *et al.,* 2008). Hydrogen peroxide which is generated by inflammatory cells may be important in suppression of GAG synthesis in inflammatory conditions (Matsubara *el al.,* 1992) is also supportive observation. To manage the H_2O_2 induced free radical toxicity naturally occurring vitamin C was selected as an antioxidant its potency as free radical scavenger being known (Balz Frei, 1999). Following is the review on vitamin C.

Vitamin C:

Vitamin C is an unstable, easily oxidized acid and can be destroyed by oxygen, alkali and high temperature. Ascorbic acid occurs physiologically as the

ascorbate anion: a water-soluble antioxidant that is found throughout the body. It acts as part of the intracellular antioxidant network, and as such is normally neuroprotective and neuromodulator. A possibly unique role it might have is as an antioxidant in the brain extracellular microenvironment, where its concentration is modulated by glutamate–ascorbate heteroexchange at glutamate uptake sites (Kiyatkin and Rebec, 1998).

Vitamin C is a water-soluble, hexonic sugar acid, with a molecular weight of 76.13. It is purely the L-enantiomer of ascorbate; the opposite D-enantiomer has no physiological significance. Both forms are mirror images of the same molecular structure. L-ascorbate is a strong reducing agent when it carries out its reducing function, it is converted to its oxidized form, L-dehydroascorbate (Vitamin C – Risk Assessment. UK Food Standards Agency). L-dehydroascorbate can then be reduced back to the active L-ascorbate form in the body by enzymes and glutathione (Meister,1994).

At physiological pH vitamin C exists as monovalent anion, ascorbate (Davies *et al., 1991).* Its enediol structure enables it to be an electron donor, via loss of two electrons to form its final oxidation product, dehydroascorbate. Free radicals with unpaired electron generated by biological systems can lead to the one-electron oxidation of ascorbate to form semi-dehydroascorbate or ascorbyl radical. This radical intermediate is also formed in enzymatic reactions that involve ascorbate as an electron-donating co-factor (Diliberto *et al.,* 1987).

Vitamin C is a cofactor and as a nutrient it is also used as antioxidant and acidity regulator. Vitamin C exists in different form according to chemical structure viz. Ascorbic acid (E300), Sodium ascorbate (E301), Calcium ascorbate (E302), Potasium ascorbate (E303), ester of Ascorbyl palmitate and Ascorbyl stearate (E305) and Erythorbic acid (E315). It is synthesised internally by almost all organisms. The human body does not synthesize them; therefore, they must be supplied by the diet in the required amount.

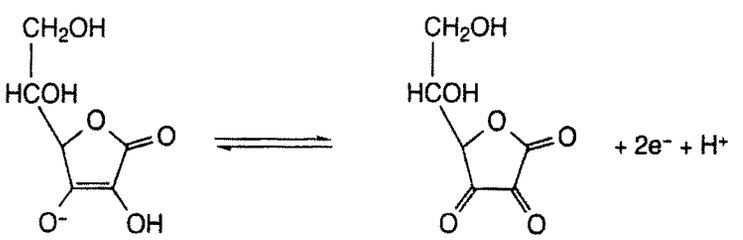
People consuming diets rich in ascorbate from natural foods, showed lower mortality from a number of chronic illnesses. However, there are scientists of opinion that additional ascorbate from supplements may not be highly beneficial (Bjelakovic *et al.,* 2007). Vitammin C is known to cure the common cold and reduce colon cancer (Iqbal *et al.,* 2004). The optimal daily dose of Vitamin C is in a range

of 200 to 400 mg per day. Safe doses of Vitamin C are less than 1000 mg daily (Levine *et al.*, 1996). The effects of a combined supplement of Vitamin E, Vitamin C and beta-carotene (Redoxon protector-75 mg, 150 mg, 15 mg respectively) improved the antioxidant activity (Calzada *et al.*, 1995). Vitamin C is used to treat Osteoarthritis and maintains cartilage. Its treatment improved whole body glucose disposal, non oxidative glucose metabolism and showed beneficial effects upon glucose and lipid metabolism in aged non-insulin dependent (type II) diabetic patients (Paolisso *et al.*, 1995).

In patients with diabetes mellitus inactivation of endothelium-derived nitric oxide by oxygen-derived free radicals contributes to impaired endothelium-dependent vasodilation. Vitamin C (24 mg/min) improved endothelial dysfunction in patients with non-insulin-dependent diabetes mellitus (Ting *et al.*, 1996).

Vitamin C also showed reduced sorbitol and glucose ration in human erythrocytes *in vitro* indicating inhibition of polyol pathway and its direct effect on aldose reductase activity (Eriksson and Kohvakka, 1995). Vitamin C and beta-carotene reduced risk of death in middle-aged men (Pandey *et al.*, 1995). Consumption of an antioxidant-rich diet (Vitamins C and E, beta carotene, and soluble dietary fiber) may reduce the plasma levels of lipid peroxide and cardiac enzyme and increase the plasma level of ascorbic acid. Antioxidant-rich foods reduce myocardial necrosis and reperfusion injury induced by oxygen free radicals (Singh *et al.*, 1995).

Ascorbate:



Ascorbate Dehydroascorbate

Antioxidant property of ascorbate is due to its properties as an electron donor. Ascorbate is a broad-spectrum radical scavenger that is effective against peroxyl- and hydroxyl-radicals, superoxide, singlet oxygen, and peroxynitrite (Nishikimi,1975; Bodannes,1979; Machlin, 1978 and Vatassery, 1996). Although ascorbate reactions occur in the aqueous phase, this can prevent oxidation of lipid-

soluble vitamin E (α-tocopherol), which in turn stops peroxidation of cell membranes (Seregi *et al.*, 1978 and Niki, 1991). Oxidized ascorbate can be reduced and thus recycled by glutathione (GSH), other intracellular thiols (Meister, 1994 and Winkler, 1949) and in some cells by a GSH-dependent dehydroascorbate reductase (Rose, 1993 and Fornai, 1999). The presence of this intracellular enzyme in brain was confirmed recently, with regionally distinct levels confined to gray matter (Fornai, 1999).

Ascorbate serves as an electron-donating enzyme co-factor, the most important ascorbate-dependent enzyme processes are collagen biosynthesis, via hydroxylation reactions (Barnes, 1975) and noradrenaline–adrenaline synthesis by dopamine-β-hydroxylase (Diliberto *et al.*, 1987).

Vitamin C and chick embryo:

Biosynthesis of ascorbic acid was found in the kidneys (mesonephros and metanephros) of the chick embryo as well as in the yolk sac membrane (Peterkofsky, 1991). The activity of L-gulonolactone oxidase in the yolk sac membrane suggested that it was the major source of ascorbic acid in the chick embryo (Yew, 1985). The distribution of ascorbic acid in the cells and tissues of chick embryos was shown by Bourne (1992) in a small area near the nucleus (probably the Golgi material), round the whole of the nuclear surface, or in the axon and axon hillock; or it may be diffusely distributed through the cytoplasm. On fourth day, especially in the axons of certain cells of the brain and spinal cord; later, localization in the Golgi substance becomes very general in most parts of the brain and cord. In ganglion cells, the meninges and the choroid plexuses.

In chick with induced hypoxia increased cerebral vitamin C it result, in part, from inhibition of cellular ascorbic acid transport. Changes in ascorbic acid concentration occur in response to oxidative stress, consistent with a role for the vitamin in the detoxification of oxygen radicals in fetal tissues. Changing O_2 levels have less effect on ascorbic acid concentration in brain than in plasma, indicating that brain cells regulate the vitamin (Wilson and Jaworski, 1992).

The effects of oxygen on ascorbic acid concentration and transport were studied by Wilson and Jaworski (1992) in chick embryo (*Gallus gallus domesticus*). During normoxic incubations, plasma ascorbic acid concentration peaked on fetal day 12 and then fell, before increasing again on day 20 when pulmonary respiration

began. In contrast, cerebral ascorbic acid concentration rose after day 6, was maintained at a relatively high level during days 8–18, and then fell significantly by day 20. Exposure of day 16 embryos for 48 h to 42% ambient O_2 concentration decreased ascorbic acid concentration by four-fifths in plasma and by one-half in brain, compared to values in normoxic (21% O_2) or hypoxic (15% O_2) controls. Hyperoxic preincubation of embryos also inhibited ascorbic acid transport, as evidenced by decreased initial rates of securable and Na^+-dependent [^{14}C]ascorbic acid uptake into isolated brain cells. These changes in ascorbic acid concentration occur in response to oxidative stress, consistent with a role for the vitamin in the detoxification of oxygen radicals in fetal tissues. However, changing O_2 levels have less effect on ascorbic acid concentration in brain than in plasma, indicating regulation of the vitamin by brain cells. Furthermore, the effect of hyperoxia on cerebral vitamin C may result, in part, from inhibition of cellular ascorbic acid transport (Wilson and Jaworski, 1992).

In ovo injection of ascorbic acid was used to eliminate stress caused by the increase in metabolic heat of the embryo during incubation and by providing glucose as a supplementary energy source to the embryo prior to hatching and its effects on embryonic mortality, hatchability and chick hatch weight was determined by Ipek *et al.,* (2004).

Glycosaminoglycans (GAGs):

The glycosaminoglycans (GAGs) are abundant heteropolysaccharides in the body. These molecules are long branched polysaccharides containing a repeating disaccharide unit. The disaccharide units contain either of two modified sugars N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) and a uronic acid such as glucuronate or iduronate. GAGs are highly negatively charged molecules, with extended conformation that imparts high viscosity to the solution. GAGs are located primarily on the surface of cells or in the extracellular matrix (ECM). Because of low compressibility these molecules are ideal as a a lubricating fluid. Their rigidity provides structural integrity to cells and provides passageways between cells, allowing cell migration. The physiologically significant GAGs are dermatan sulfate, chondroitin sulfate, heparin, heparan sulfate, and keratan sulfate. Hyluronic acid is a glycan sugars present in the make-up of any given class of GAG.

All GAGs possess N- and/or O-sulfate groups distributed on their disaccharide building blocks. Most sulfated GAG chains are covalently linked to core proteins to form proteoglycans, which are strategically located at the cell surface and in the extracellular matrix (Bernfield *et al.*, 1999; Kato *et al.*, 1994). In addition to their structural functions, proteoglycans and GAGs play different roles in diverse processes, such as enzyme regulation and cellular adhesion, growth, migration, or differentiation (Lyon and Gallagher, 1998; Selleck, 2000; Turnbull *et al.*, 2001). These processes are mediated by their binding capacities and focal sequestering of a number of biologically active proteins (David and Bernfield, 1998). Among them, growth factors are important to cause many biological events, such as cell activation in response to injury.

Classification for GAGs:

Members of the glycosaminoglycan family vary in the type of hexosamine, hexose or hexuronic acid unit they contain (e.g. glucuronic acid, iduronic acid, galactose, galactosamine, glucosamine).

They also vary in the geometry of the glycosidic linkage.

Examples of GAGs include:

Name	Hexuronic acid / Hexose	Hexosamine	Linkage geometry between predominant monomeric units	Unique features
Chondroitin sulfate	GlcUA or GlcUA(2S)	GalNAc or GalNAc(4S) or GalNAc(6S) or GalNAc(4S,6S)	-4GlcUAβ1-3GalNAcβ1-	Most prevalent GAG
Dermatan sulfate	GlcUA or IdoUA or IdoUA(2S)	GalNAc or GalNAc(4S) or GalNAc(6S) or GalNAc(4S,6S)	-4IdoUAβ1-3GalNAcβ1-	Distinguished from chondroitin sulfate by the presence of iduronic acid, although some hexuronic acid monosaccharides may be glucuronic

				acid.[1]
Keratan sulfate	Gal or Gal(6S)	GlcNAc or GlcNAc(6S)	-3Gal(6S)β1-4GlcNAc(6S)β1-	Keratan sulfate type II may be fucosylated.[2]
Heparin	GlcUA or IdoUA(2S)	GlcNAc or GlcNS or GlcNAc(6S) or GlcNS(6S)	-4IdoUA(2S)α1-4GlcNS(6S)α1-	Highest negative charge density of any known biological molecule
Heparan sulfate	GlcUA or IdoUA or IdoUA(2S)	GlcNAc or GlcNS or GlcNAc(6S) or GlcNS(6S)	-4GlcUAβ1-4GlcNAcα1-	Highly similar in structure to heparin, however heparan sulfates disaccharide units are organised into distinct sulfated and non-sulfated domains.[3]
Hyaluronan	GlcUA	GlcNAc	-4GlcUAβ1-3GlcNAcβ1-	The only GAG that is exclusively non-sulfated

Abbreviations

GlcUA = β-D-glucuronic acid

GlcUA(2S) = 2-O-sulfo-β-D-glucuronic acid

IdoUA = α-L-iduronic acid

IdoUA(2S) = 2-O-sulfo-α-L-iduronic acid

Gal = β-D-galactose

Gal(6S) = 6-O-sulfo-β-D-galactose

GalNAc = β-D-N-acetylgalactosamine

GalNAc(4S) = β-D-N-acetylgalactosamine-4-O-sulfate

GalNAc(6S) = β-D-N-acetylgalactosamine-6-O-sulfate

GalNAc(4S,6S) = β-D-N-acetylgalactosamine-4-O, 6-O-sulfate

GlcNAc = α-D-N-acetylglucosamine

GlcNS = α-D-N-sulfoglucosamine

GlcNS(6S) = α-D-N-sulfoglucosamine-6-O-sulfate

Chondroitin-sulfate

Chondroitin-4-sulfate: R_1 = H; R_2 = SO_3H; R_3 = H. Chondroitin-6-sulfate: R_1 = SO_3H; R_2, R_3 = H.

Chondroitin sulfate is a sulfated glycosaminoglycan (GAG) composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid). It exist in a proteoglycan. A chondroitin chain can have over 100 individual sugars, each of which can be sulfated in variable positions and quantities. Chondroitin sulfate is an important structural component of cartilage and resist compression. It is widely used dietary supplement for treatment of osteoarthritis.

Letter identification	Site of sulfation	Systematic name
Chondroitin sulfate A	carbon 4 of the N-acetylgalactosamine (GalNAc) sugar	chondroitin-4-sulfate
Chondroitin sulfate C	carbon 6 of the GalNAc sugar	chondroitin-6-sulfate
Chondroitin sulfate D	carbon 2 of the glucuronic acid and 6 of the GalNAc sugar	chondroitin-2,6-sulfate
Chondroitin sulfate E	carbons 4 and 6 of the GalNAc sugar	chondroitin-4,6-sulfate

(Davidson and Meyer, 1954).

Sulfate is covalently attached to the sugar at physiological pH. It is a cation. Chondroitin sulfate marketed as sodium salt (Barnihill *et al.,* 2006).

Structure of chondratin sulfate:

They are unbranched polysaccharides of variable length containing two alternating monosaccharide of D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc). Some GlcA residues are epimerized into L-iduronic acid

(IdoA); the resulting disaccharide is then referred to as dermatan sulfate. Chondroitin sulfate chains are O-linked to serine of certain proteins.

Attachment of the GAG chain is linked with tetrasaccharide Xyl - Gal - Gal - GlcA which added in ER (Xyl) and golgi apparatus (Silbert and Sugumaran, 2002). Each monosaccharide is unsulfated, sulfated once, or sulfated twice. Commonly the hydroxyls of the 4 and 6 positions of the N-acetyl-galactosamine are sulfated, with some chains having the 2 position of glucuronic acid. Sulfation is mediated by specific sulfotransferases in golgi apparatus. Sulfation in these different positions confers specific biological activities to chondroitin GAG chains.

Functions of chondratin sulfate:

Functions depends on the properties of the overall proteoglycan of which it is a part. These functions can be broadly divided into structural and/or regulatory roles. However, chondroitin sulfate is a major component of extracellular matrix, and maintains the structural integrity of the tissue. The large aggregating proteoglycans: aggrecan, versican, brevican, and neurocan i.e. the lecticans form the components of extracellular matrix.

Aggrecan (chondroitin sulfate) is a major component of cartilage. GAG loss is a major cause of osteoarthritis. Their interactions are regulatory in diverse array of cellular activities. The lecticans of the brain extracellular matix, where the chondroitin sugar chains function to stabilize normal brain synapses as part of perineuronal nets. Chondroitin sulfate proteoglycans are increased after injury to the CNS where they prevent regeneration of damaged nerve endings. But their role remains to be revealed, new roles continue to be discovered for them.

Dermatin sulfate:

Dermatin sulfate is a glycosaminoglycan (formerly called a mucopolysaccharide) found mostly in skin, but also in blood vessels, heart valves, tendons, and lungs.

It is also referred to as chondroitin sulfate B, (Levene and Forge, 1913) although it is no longer classified as a form of chondroitin sulfate by most sources.

Function of Dermatin sulfate:

Dermatin sulfate may have roles in coagulation, cardiovascular disease, carcinogenesis, infection, wound repair, and fibrosis. Dermatan sulfate accumulates abnormally in several of the mucopolysaccharidosis disorders.

Keratan Sulfate (KS):

Keratin sulfate is a sulfated polylactosamine. KS chain identical to the type found on conventional glycoproteins and mucins. Their linkage to protein distinguishes two types of KS, KS I originally described in cornea, is linked through a core glycan structure found in the N-glycosylated glycoproteins and KS II (skeletal KS) is an O-glycan linked through GalNAc to Ser/Thr.

Heparan sulfate (HS):

Heparan sulfate (HS) is a linear polysaccharide found in all animal tissues. It occurs as a proteoglycan (PG) in which two or three HS chains are attached in close proximity to cell surface or extracellular matrix proteins (Gallagher and Lyon, 2000). It is in this form that HS binds to a variety of protein ligands and regulates a wide variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumour metastasis.

HS biosynthesis:

Many different cell types produce HS chains with many different primary structures. Therefore there is room for a great deal of variability in the way HS chains are synthesised. The pathways for HS/heparin or chondroitin sulfate (CS) and dermatan sulfate (DS) biosynthesis diverge after the formation of this common linkage structure. After attachment of the first N-acetylglucosamine (GlcNAc) residue elongation of the tetrasacchride linker is continued by the stepwise addition of GlcA and GlcNAc residues.

As the chain polymerises it undergoes a series of modification reactions in HS N-sulfated residues are mainly grouped together and separated by regions of N-acetylation where GlcNAc remains unmodified (Toida *et al.,* 1997).

GAGs in the chick embryo:

The sulfation profile and activities progressively descreased with development and these alterations were precisely coordinated. The proportion of

both Chondratin- 6-sulfate to chondratin-4-sulfate and C6ST to C4ST activities progressively decreased with development. The developmentally regulated expression of the sulfotransferases a prodominent factor for stage specific regulation of chondratin sulfate. (Hiroshi *et al.,* 1997)

Sulphated GAGs were present from the earliest stages. They were more abundant than the non-sulphated forms and showed stage-related changes. Chondroitin sulphate and especially dermatan sulphate appeared to be the predominant GAGs in embryos at stage XIII+. Dermatan sulphate was about three times as abundant as chondroitin sulphate at stage XIII+. In contrast, embryos at the definitive streak stage (stage HH4) produced about twice as much chondroitin sulphate as dermatan sulphate. At the head process stage (stage HH5), the level of chondroitin sulphate was reduced and its relative content in the embryo was about the same as dermatan sulphate. Levels of dermatan sulphate were more than five times those of heparan sulphate from stage XIII+ through to stage HH5 and three times more at stage HH7-9. The 4- and 6- sulphation of chondroitin sulphate increased 14- and 10-fold respectively, from stage XIII+ to stage HH 7-9. The sulphation pattern of chondroitin sulphate had a di-4S: di-6S molar ratio ranging from 4 to 8:1 and a Adi-4S:Adi-OS molar ratio ranging from 9 to 16:1 and was developmentally regulated. Thus, chondroitin sulphate in the early chick embryo was sulphated predominately in the 4-position in all stages studied. The presence of both 4- and 6-sulphated disaccharides in chondroitin sulphate indicated that both 4 and 6 sulfotransferases were active in the early embryo. Hyaluronate and sulphated GAG content increased markedly at gastrulation when the first major cellular migrations and tissue interactions begin. (Sandaliss *et. al.* 2003).

Hyaluronan (HA):

Structure and properties:

The HA disaccharide consists of GlcNAcβ1-3GlcAβ1-4 and is repeated many times in each chain. The HA chain is polymerized by a plasma membrane bound enzyme and is not subjected to any type of covalent modification during its synthesis. Nuclear magnetic resonance studies of the shape of the HA chain was performed by Scott (Scott, 1991).

GAGs content was examined in chick embryo brain starting from the 9th day of incubation to the 4th post-hatching day. Chondroitin 6-sulfate, hyaluronate and

heparan sulfate were recovered at any developmental stage examined. C6-S was the main GAG (except on the 15th day), while HS was the least represented. The highest differences in the relative amounts of GAGs are observed on the 9th day. C6-S shows high developmental relative changes, while HA and HS exhibit a similar pattern (Pane and Wegelin, 1990).

HA is unique in the GAG family by lacking covalent linkages to proteins thus not being able to form proteoglycans. The only exception to this rule is the ester linkage between the C-terminal aspartic acid of the heavy chains of pre--trypsin inhibitor/inter-trypsin inhibitor and the C6-hydroxyl group of an internal GlcNAc residue in the HA chain (Zhao *et. al.*, 1995),although the functional role of this linkage is not clear.

HA is found mainly in the extracellular space where it accumulates, but the polymer can also be bound to the cell surface or be located intracellularly around in the nucleus and in the lysosomes (Ripellino *et. al.*, 1988; Laurent and Fraser, 1991; Eggli and Graber, 1995). The largest storage of HA in the human body is in the skin, constituting about 50 % of the total HA supply (Ripellino *et. al.* 1988).

High molecular weight HA have been ascribed an essential structural role in the extracellular matrix of the connective tissue where the polymers create a mesh-like structure (Laurent, *et. al* 1992; Scott, *et. al.* 1991).The viscous hydrated HA gel provides resistance to compressive forces and acts as a biological lubricant. During embryonic development the deposit of HA creates a cell free area by expansion of the extracellular space which facilitates cell migration (Toole, 1982). Besides being a structural element, HA has been reported to be involved in several specific processes such as lympocyte extravasation (DeGrendele *et. al.* 1997), cancer metastasis (Sherman *et al.*, 1994) and angiogenesis (Rooney *et. al.* 1995)probably through interactions with HA binding proteins. Since the HA polymer itself does not exhibit any structural diversity, its function is in part due to the chain length. The inductive role of HA in angiogenesis could be ascribed to HA oligosaccharides (West *et. al.*, 1985) whereas high molecular weight HA exerted an inhibitory effect (Feinberg, and Beebe1983).

HA binding proteins:

The fact that HA can form several specific interactions with proteins, serves as indirect evidence that HA is more than a structural component of the extracellular

matrix. These HA binding proteins are mainly found extracellularly, but can also be bound to the cell surface or be located intracellularly. New HA binding proteins are currently under characterization.

Hyalectans:

The hyalectans are a family of proteoglycans interacting with HA and lectins and have currently four members: versican, aggrecan, neurocan and brevican (Iozzo and Murdoch, 1996). Versican has been implicated in regulation of neural crest cell migration by exerting a barrier role for the axonal outgrowth (Landolt *et al.,* 1995). Aggrecan secreted by chondrocytes aggregates extracellularly but can also bind to the cell surface (Kimura *et al.,* 1979; Sommarin et. al., 1983). Aggrecan, link protein and HA forms a ternary complex (Heinegard and Oldberg 1989) which, inside cartilage, occupies a large hydrodynamic volume making the tissue elastic to compressive forces. The affinity of the aggrecan link complex for HA is quite high (Kd 1 nM). Neurocan is developmentally regulated, and its interactions with its ligands may be confined to restricted areas and a relatively brief stage in development (Milev *et. al.,* 1996). Brevican is the smallest member of the hyalectans (protein core 100 kDa). The molecule can be synthesized in a soluble extracellular form or as a GPI-anchored protein (Seidenbecher *et al.,* 1995), which localizes to the plasma membrane. Brevican has been reported to inhibit neurite outgrowth *in vitro* thus maybe controlling infiltration of axons and dendrites *in vivo* (Yamada *et. al.* 1997).

Structure and ligand binding CD44 is the best characterized HA receptor (Tiedemann, 1997; Lesley *et al.*, 1998). Besides HA, the CD44 receptor can bind a number of other extracellular matrix components such as collagen I (Faassen *et. al* 1992), fibronectin (Jalkanen *et al.,* 1992), HS, CS and heparin (Sleeman et. al 1997). CD44 is widely expressed, and can be found on many cells including neural cells (Lesley and Hyman, 1998)). CD44 is a type-1 transmembrane glycoprotein and exists in isoform. This proximal region also contains O-glycosylation sites and sites for attachment of GAG chains. The existence of CD44 molecules on the cell surface does not automatically convey HA binding but depends on the "activation state" of the cell.

Binding cells have less N-glycosylation than non-binding cells and treatment of these non-binding cells with N-glycosylation inhibitors rendered them active. O-

glycosylation of CD44 has also been reported to have a negative effect on HA binding.

Cell migration and cell locomotion involves a series of complex interactions between cytoskeleton, cell surface receptors and matrix components. Several examples from the field of embryonic development implicate HA and CD44 as positive elements in cell migration. The migration of cells into the developing chick embryo cornea and heart coincided temporally and spatially with the synthesis of HA and the migration decreased as the hyaluronidase activity in the tissues increased. *In vitro* these matrices consist either of newly synthesized HA chains, still attached to the HAS protein (Heldin and Pertoft, 1993),or of exogeneously added HA which is bound to HA receptors (CD44) (Knudson and Knudson, 1991; Knudson *et. al.*, 1993). The matrix formation can be promoted by HA binding proteoglycans, which also increase their density and stability .Experimental evidence supporting this hypothesis has been presented where neural crest cell migration was inhibited by HA matrix promoting chondroitin sulfate proteoglycans (i.e.aggrecan).

It is predicted that HA binding with different growth factors may lead to modulation of various types of effects during embryonic growth also (Piek *et al.*, 1999).

Knudson and Knudson (1993) have presented the hypothesis where the establishment of a pericellular HA matrix would interfere with and disrupt normal cell migration. This model has to some extent been experimentally supported by Perris *et al.* (1996), where HA binding proteoglycans that promote pericellular matrix formation was shown to inhibit migration in neural crest cells.

Sialic acid:

There are many factors that may support brain growth. One of which is sialic acid (also known as *N*-acetylneuraminic acid), a nine-carbon sugar that is a structural and functional component of brain gangliosides. Sialic acid may be a conditionally essential nutrient in infancy, if demand outstrips the rate of endogenous synthesis (Friede, 1989; Uauy and Peirano, 1999; Uauy *et al*, 2001).

Sialic acids comprise 43 family of naturally occurring derivatives of the nine-carbon sugar neuraminic acid (Schauer *et al*, 1995; Schauer and Kamerling, 1997).

Sialic acid is important for brain development, learning and memory and cognitive performance. Sialic acid is found in whey protein isolate or concentrate and in concentrated amounts in eggs. Sialic acid is found mainly in the glycoproteins and glycolipids. It is also found in many fluids including saliva, urine, cerebrospinal fluid, amniotic fluid and breast milk. During pregnancy, sialic acid levels are raised, suggesting its importance in the immune system along with other physical and mental development systems in infants. Disrupted sialic acid metabolism is seen in infants who are developmentally delayed. Like the other glyconutrients, sialic acid is important for cellular communication and is an immune system modulator.

Sialic acid-rich oligosaccharides on the glycoconjugates found on surface membranes help keep water at the surface of cells. The sialic acid-rich regions contribute to creating a negative charge on the cells surface. Since water is a polar molecule with partial positive charges on both hydrogen atoms, it is attracted to cell surfaces and membranes. This also contributes to cellular fluid uptake. Cell-cell recognition occurs by means of local cell surface modulation of a small number of proteins rather than by expression of large numbers of different cell surface markers. Several different cell adhesion molecules (CAM's) have now been found in a number of vertebrate species in different tissues such as liver and striated muscle and even in a single complex structure such as the brain, where different molecules specific for neurons and glia have been identified. The neuron-specific molecule is involved in early embryonic events but also mediates neurite fasciculation, neuromuscular interaction, and orderly layering of neural tissue. It undergoes local surface modulation with loss of sialic acid during development. The data on this and other CAM's favor modulation theories rather than strict chemoaffinity theories of cell-cell recognition (G M Edelman, 1983)

Disturbances in sialic acid metabolism:

In schizophrenia, found the more serious the psychosis, the lower the sialic acid content in the glycoproteins of the cerebrospinal fluid. If schizophrenic state was treated successfully, the sialic acid content rose to normal values (Campbell *et al* 1967). The increased content of sialic acid in both glycoprotein and gangliosides in the synaptic membrane may alter electric properties, leading to improvements in schizophrenic patients (Edelfors, 1981). Synthesis and function of gangliosides and sialoglycoproteins during development appears to contribute to brain dysfunction in

phenylketonuria (PKU) (Loo *et al*, 1985). In the offspring of untreated PKU mothers, there is a noticeable disruption of the normal ganglioside pattern (delayed drop in GQlb and GD3 and slower rise in GM1 and GD1 a) and a significant reduction of sialoglycoproteins. Alzheimer's disease and older Down's syndrome typically occurs after 65 years of age, but can also affect people in their 40s and 50s (Warren, 1994). Degenerative and progressive, this disease involves the brain, resulting in loss of memory and cognitive function. Alzheimer's patients have been found to have decreased sialyltransferase activity in serum that affects the α 2,3-linked sialic acid in serum glycoproteins. Ganglioside sialic acid content in cerebral cortex was also decreased (Sorbi *et al*, 1987). It can only be assumed that the defects also occur in neuronal tissue and contribute to the disease process. Serum sialyltransferase may thus be an early biochemical marker of neurodegeneration (Schauer & Kamerling, 1997). These details revealed importance of sisalic acid in brain development and regular maintainance.

Sialic acid in chick embryo brain:

Chick embryo precursor neurons introduce SA biosynthetically into three specific gangliosides GM_3 (monosialosylacetosylceramide), GD_5 (Disialosyl lactosyl ceramide), and GD_2 (disialosylgangliotrihexosyle ceramide) (Ahvaham Rosenberg, 1991). Neural cell adhesion molecules were also with glycan chains. Two types of sturctures were present multiantennary N-linked glycans i.e. tri and tetra antennay to proximal N-acetyl glucose of the di-N-acetylchibiosyl unit. The core glycan is also sulfated (Finne, 1982; McCoy *et al.,* 1985). N-CAM molecules also showed polysialylation which was present at all the developmental stages with distinct changes (Inoue *et al.,* 2000). In isolated molecular structures studies the polysialic acid an α-2-8 linked polymer of N-acetylneuraminic acid represents an essntial regulator of neural cell adhesion molecules N-CAM functions (Galuska *et al.,* 2008). In *in vitro* studies N-CAM N-terminus is extended on cell surfaces and bulk of sialic acid remains in mid of this extended peptide. Sialic acid though not directely involved in binding, it influences the binding. Another protein γ –glutamy transepeptidase is a membrane bound sialoglycoprotein. Developmental changes in it's activity and sialic acid content increased in late period of development.

Three different kinds of sialyltransferases are involved in the biosynthesis of the non-NCAM PSA (Kitazume *et. al.;* 1994). But it is not known how many

sialyltransferases(s) are involved in the biosynthesis of PSA on NCAM. Developmental changes in the level of PST activity were closely correlated with changes in the degree of polysialylation of newly synthesized NCAM, suggesting that the developmental changes observed in PSA expression are at least in part regulated by the level of PST activity (Brusés and Rutishauser, unpublished)

At early stages of embryonic development (stage 30-37), more than 70% of the [$_{14}$C] sialic acid enzymatically incorporated into exogenous NCAM. If the first sialic acid residue of a potential polysialylation site, which is transferred to a galactose residue, is substituted by an α2, 6-linkage instead of the α2, 3-linkage, then the PST would not be able to elongate that chain. Glucuronyltransferase, enzymes in the biosynthesis of the HNK-1 carbohydrates that are commonly expressed on cell adhesion molecules including NCAM (Chou *et. al.* 1986; Das, *et. al.* ,1991; Kawashima et. al. 1992; Oka et. al. 1992), could be considered as a potential regulator of PSA synthesis. Glucuronyltransferase can catalyze the transfer of glucuronic acid to a galactose residue of NCAM, substituting the first sialic acid residue by a glucuronic acid residue and thus blocking polysialylation. This enzymatic argument is bolstered by the fact that in brain the up-regulation of glucuronyltransferase is developmentally correlated with the decrease in PSA expression. In addition to the initiation and elongation of PSA chains, it is also possible that the chains are terminated by an additional enzymatic step (Iwasaki et. al. 1990).

The sialic acid molecule are covalently linked to the N-CAM proteins at three site on the middle of the molecule, within the extracellular domain (Cunningham *et. al.* 1983). Cells with a relatively low amount of sialiç acid on their N-CAM aggregates four times more readily than those with the high level of sialic acid (Hoffman and Edelman, 1983). As the embryo gets older, most of the N-CAM protein progress from the high sialic acid content to the low sialic acid forms (Chuong and Edelman, 1985; Reiger *et al.,* 1985). Polysialc acid on NCAM has a regulatory effect on adhesion between living cells and that the amount of this carbohydrate is critical for the normal morphogenesis of nerve tissue (Rutishauser *et. al.,* 1985). Changes in sialic acid are fundamental aspect of the function of NCAM in development. Decreased sialic acid content enhances the adhesion molecule properties of NCAM (Sunshine *et. al.*, 1987). Chemical analysis and binding studies have been correlated to clarify the relationship of structure to

function in the neural cell adhesion molecule (N-CAM) from embryonic chicken brain. N-CAM isolated from the cell surface appears to include two closely related polypeptide chains. N-CAM appears to be oriented with the amino terminus extending away from the cell surface and with the bulk of the sialic acid near the middle of the peptide chain.

A cDNA clone encoding chick Gal β1,3GalNAc *2,3-sialyltransferase (ST3Gal I) from a chick embryo brain cDNA library. The cDNA sequence included an open reading frame coding for 342 amino acids, and the deduced amino acid sequence showed 64% identity with that of the mouse enzyme. Northern blot analysis of chick embryos revealed that the ST3Gal I gene was expressed in early embryonic stages. The identity of the enzyme was confirmed by construction of a recombinant sialyltransferase in which the N-terminal part including the cytoplasmic tail and signal anchor domain was replaced with an immunoglobulin signal peptide sequence. This enzyme expressed in COS-7 cells (Kurosawa *el al.,* 1995). In the study a primary culture system of chicken embryo brain neurons was used in the early period of chicken brain development from day 6 until day 8, which was shown to be a suitable model of neuritogenesis, cell migration and reaggregation. Dissociated chicken optic tectum cells from embryonic stage 31 were cultured on polylysine-coated dishes under serum-free conditions up to 3 days. GD3 represents the predominant ganglioside of embryonic neurons before neuritogenesis *in vitro* and *in vivo*. Its biosynthesis is followed during day 6 until day 8 of embryonic brain development. Incubation of dissociated neurons blocked neurite outgrowth. Accordingly, neither cell migration nor reaggregation could be found, indicating the disialoganglioside, GD3 plays a central role in neuronal differentiation and development in the embryonic chicken brain.

Glycosphingolipids, in particular gangliosides, play a crucial role in neuronal development and are known to change dramatically in total content and distribution in different brain areas during embryogenesis (Angela *et. al.*, 2002).

Sialic acid and hydrogen peroxide:

The role of antioxidant play by mucin, a typical sialic acid containing high-molecular weight glycoprotein. The function of mucin as a hydroxyl radical (OH) scavenger was characterized using bovine submaxillary gland mucin (BSM). Non-treated BSM effectively protected DNA from the attack of OH; however,

desialylated BSM lost this potential. Moreover, the scavenging effects of BSM against OH generated by UV irradiation of hydrogen peroxide using ESR analysis. The results indicated that BSM has OH scavenging ability the and sialic acid in mucin is an essential moiety to scavenge OH (Ogasawara et. *al.*, 2007). The cytotoxicity of hydrogen peroxide (H_2O_2), and the antitoxicity is a result of a direct chemical reaction, whereby NANA reduces H_2O_2 in the culture media. The influence of the potential of hydrogen (pH) and temperature in this reaction was investigated. The reaction velocity is remarkably less at low pH and/or low temperature, but it increases with these parameters. Furthermore, the reaction product generated in the slow reaction under acidic conditions (pH 3.1) was analyzed. They detected 4-(acetylamino)-2,4-dideoxy-D-glycero-D-galacto-octonic acid (ADOA) as the decarboxylation product of NANA; this is the same product that was previously obtained in a faster reaction at neutral pH (pH 7.5). Furthermore, ADOA was generated not only from the reaction with the NANA monomer but also from that with ALPHA (2.RAR.8) homodimer of NANA (DP2). Thus, it can be considered that the reaction between NANA and H_2O_2 can occur under various pH conditions and for NANA residues in a glycochain (Ryosuke, 2007). N-acetylneuraminic acid (NANA) consumes toxic hydrogen peroxide (H_2O_2) under physiological conditions. Close investigation of this finding revealed that NANA was oxidized by an equimolar amount of H_2O_2 to provide its decarboxylated product, 4-(acetylamino)-2,4-di-deoxy-D-glycero-D-galacto-octonic acid (ADOA). To date, there have been little data on this reaction, and its physiological significance has not been discussed. Examining the detoxification of H_2O_2 in cultured cells with NANA, we were able to confirm that the cell death caused by H_2O_2 was suppressed by NANA in a dose-dependent manner. These results revealed a novel role for NANA as a reactive oxygen scavenger. It is known that terminal NANA residues are removed by neuraminidase and that free NANA molecules are recycled or degraded by enzymes. It is proposed that released monomeric NANA is the potent defense molecule against oxidative damage (Feblal, 2004)

Following is the architecture of the Dissertation.

Chapter I: Introduction:

The chapter includes reasons to take the problem, chick embryo as a developmental animal model, basis of selection of brain. Basis of selection of developmental intervals and treatment durations. Doses of H_2O_2 and vitamin C.

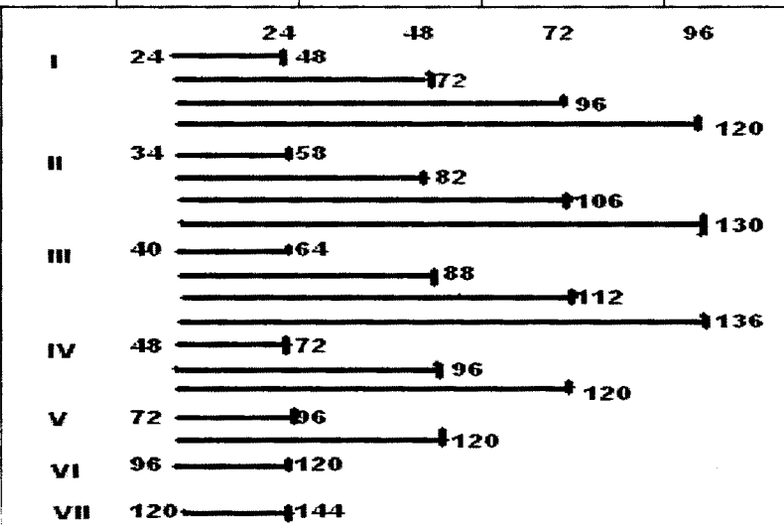
Introduction chapter also contains review of literature on chick brain development, free radicals, hydrogen peroxide, vitamin C, Glycosaminoglycans, Sialic acid and glycan-Hyaluronan.

Chapter II : Material and Methods:

Experimental protocol is presented. The methodology used fp paraffin sections preparation of brain is provided. More than 27 staining techniques used to demonstrate and analyse glycosaminoglycans and sialic acid are provided in Table no. 3

Experimental Protocol :

Table 1: Exposure time to H_2O_2 and vitamin C to different developmental stages of chick embryo (in hrs).

Groups According to Developmental stages (in hrs)	**Groups according to Time of exposure to the Treatment (in hrs)**				
	A	**B**	**C**	**D**	
Initiation of treatment (in hrs)					**Final development (in hrs)**

Group I, II and III each contains 4 sub groups(A, B, C and D) = 12 groups

Group IV contain 3 subgroups (A, B and C) = 3 groups

Group V contains 2 sub groups (A and B) = 2 groups

Group VI and VII contains 1 sub group (A) = 2 groups

Total = 19 groups

Chapter III: Observations and Discussion:

Chapter is divided into following sections -

Section I: Mortality and Abnormalities:

Dose of 3 mg vitamin C prevents mortality induced by 0.5 mM H_2O_2 leading to survival of embryos as observed in normal embryos at all the hrs of development for all the exposure. The results showed that concentrations of both Vitamin C and free radical generated (naturally and by H_2O_2 cumulatively) both modulate the mortality at different levels of chick development.

Section II: Histology:

Treatment of H_2O_2 (0.5 mM) decreased the protein content at all the initiation and exposure intervals and histological alterations in brain and their modulation by vitamin C is presented in seven microphotographic plates and these results are discussed with relevant literature.

Section III: Glycosaminoglycans:

Section III is further divided into sub sections.

A- Neutral glycosaminoglycans (NGAGs).

B- Sulfated glycosaminoglycans (SGAGs).

C- Hyaluronan (HA).

H_2O_2 induced alterations and changes in NGAGs, SGAGs and HA in different brain regions and zones and vitamin C mediated alterations in above parameters in H_2O_2 induced embryos are presented in 15 microphotographic plates. The results are discussed with relevant literature.

Section IV: Sialic acid (SA):

Alterations in SA content induced by 0.5mM H_2O_2 and vitamin C independently and together studied. The changes in different brain regions and zones are presented in seven microphotographic plates and results are discussed with relevant literature.

Section V: General Discussion:

All the results obtained are discussed together integrating all the observations to point out important finding in the project.

The thesis ends with the list of the references.