

CHAPTER I

INTRODUCTION

INTRODUCTION

Project entitled 'Modulation of Malondialdehyde, Glutathione and Formaldehyde by H₂O₂ and Vitamin C during brain development in chick' is studied with relevant experimental work details of which will appear in Chapter II. Different features of approach towards the working of the project are reasoned and relevant literature is reviewed in forgoing pages of Chapter I.

I.1. Reasons to take the problem:

Free radicals are constantly formed in the body during normal metabolic processes & biological systems have evolved to live with them, control them & even utilize them (Harman, 1956; Cheesman and Slater, 1993). Free radicals are implicated in biologic processes ranging from embryogenesis to aging, from normal tissue homeostasis to many human diseases. Reactive oxygen species (ROS) regulate critical steps in the signal transduction cascades and many important cellular events, including protein phosphorylation, gene expression, transcription factor activation, DNA synthesis, and cellular proliferation (Schreck *et al.*, 1991; Sen and Packer, 1996). However when their formation is greatly increased, or protective mechanisms compromised, a state of oxidative stress will result. Various pathologic processes disrupt this balance by increasing the formation of free radicals in proportion to the available antioxidants (thus, oxidative stress). If oxidative stress is persistent, it will lead to molecular damage & tissue injury. Although oxidative stress is thought to be involved in the pathophysiology of several diseases, differentiation and aging (Allen, 1991, Haorah, 2007), to date, molecular mechanisms remain poorly defined. Cell injury mediated by free radicals was studied here. Increased free radicals generation may occur predominantly (1) intracellular (2) extracellular and (3) both. Injury to fetus is deciding the fate of the new generation and hence to study the effect of different stresses during development is important.

Oxidative stress may play an important role in neuronal degeneration in diseases such as Parkinsons disease, Alzheimers disease, and amyotrophic lateral sclerosis.

Elucidating the pathway important in the production of and defense from free radicals may be important in devising new pharmacologic strategies to slow or halt neuronal degeneration (Simonian and Coyle, 1996). Free radical generation in fetal alcohol syndrome and associated fetal neuronal defects have also been studied in chick (Choudhury, 2006).

The cells have evolved number of counter acting antioxidant defenses. These antioxidant defense mechanisms can be categorized into two types- Free radical scavenging and chain breaking antioxidants. The free radical scavenging mechanisms includes enzymatic antioxidants like superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px), Glutathione reductase (GSH-Rx) and Catalase, which limit the cellular concentration of free radicals and prevent excessive oxidative damage (Scott, 1994). For the free radical management natural free radical scavengers exist in cell that are intracellular enzymes like superoxide dismutase (SOD), glutathione peroxidase and endogenous molecules like glutathione (GSH), sulfhydryl groups, alpha lipoic acid, CoQ 10 and thioredoxin. Many organisms like rat exhibit marked increase in lipid peroxidation or in the susceptibility of their tissues to peroxidation during different developmental stages (Utsumi and Yoshioka *et al.*, 1977) and product of free radicals generated *in vivo* and is taken as indicator of lipid peroxidation (Bird and Draper, 1984).

There is a positive correlation between metabolic rate (VO_2), or ambient oxygen (O_2) tension, and the rate of formation of free radicals from O_2 was shown in chick embryos exposed to 72 h of hyperoxia (60% O_2) late in incubation. Significantly higher concentrations of malondialdehyde i.e. lipid peroxidation product was present in liver than in chorioallantoic membrane, brain, or heart. The studies indicated that the chick embryo adapts to hyperoxia in such a way as to escape additional free radical damage, perhaps by increasing the capacity of its antioxidant defenses to compensate for a potential increase in the rate of free radical generation (Stock *et al.*; 1990). In this system ROS were generated by high levels of O_2 exposure.

Hydrogen peroxide (H_2O_2) is one of the ROS which contributes to *in vivo* oxygen damage in addition to other radicals. The body has a hierarchy of defense strategies to deal with oxidative stress within different cellular compartments, and superimposed on

these are gene-regulated defenses involving the heat-shock and oxidant stress proteins (Gutteridge, 1995).

Glutathione is one of tissue metabolite that acts as an antioxidant (Ault and Lawrence, 2003). Reviews indicate the interrelationship between LPO and antioxidants during development. Thus introduction of H₂O₂ to *in vivo* (developing) system including brain even during development and *in vivo* system is capable of managing them by alterations in antioxidant metabolites in system viz. glutathione. If such system is helped with antioxidant metabolite provided externally the limit of ROS management and associated impact can provide data which will be of use in similar type of disturbed system during normal development. Mortality and associated abnormalities will be of use in assessment of death during development and associated impact on adult animal along with impact of externally provided antioxidants.

In this light present project was designed and accordingly workout.

As stated earlier the project was to study *in vivo* developmental system and for this purpose chick embryo was used as developmental model for the project work.

I.2 Reason to take chick embryo as a animal model:

Ever since Aristotle first followed its 3-week development, the domestic chicken has been a favorite organism for embryological studies. It is accessible all year and is easily raised. Thus, large numbers of embryos can be obtained at the same stage. Moreover, at any particular incubation period, its developmental stage can be accurately predicted. The chick embryo can be surgically manipulated and, since it forms most of its organs in ways very similarly to those of mammals, it has often served as a surrogate for human embryos (Gilbert, 2000). The drug toxicity is of great concern during pregnancy (Mastan *et al.*, 2007). Thus to study drug toxicity in developing fetus chick embryo is used as a model. The use of chick model for toxicological and pharmacological studies is promoted, as the mother does not influence the pharmacokinetics of the drug (Hamilton *et al.*, 1983). Chick embryo is used as an *in ovo* model to study the systemic and localized

teratogenic effects of valproic acid (Amy *et al.*, 2002). Uri *et al.*, (2005) also used chick embryo as a model for neurobehavioral teratogenicity of mustard gas.

Chick embryo is used as a model for differentiation and developmental studies and used to determine the neuroteratogenic effects of nicotine (Wielgus, 2004). The antiepileptic valproic acid (VPA) is a teratogen whose embryopathic mechanism(s) remain uncertain. Elucidating potential cellular and molecular effects of VPA is complicated by systemic application paradigms. Thus chick embryo is used as an *in ovo* model to reproduce the teratogenic effects of VPA and a localized VPA application procedure to determine whether VPA can selectively effect abnormal development in one region of the embryo (Whitsel, 2002). Sulfur mustard (SM), is a powerful mutagen and carcinogen. Due to its similarity to the related chemotherapy agents nitrogen mustard (mechlorethamine), it is expected to act as a developmental neurotoxicant. As revealed the chick model is established for the study of the mechanisms of SM on neurobehavioral teratogenicity, free of confounds related to mammalian maternal effects (Wormser, 2005).

It is also used as a model to study angiogenesis by use of studies of chorioallantoic membrane CAM (Pacini *et al.*, 2002). The studies have shown that the chick embryo is an efficient reliable model for the assessment of teratogenic effects on central nervous system development (Raymon *et al.*, 1970).

For the reasons discussed above the chick embryo model was selected for the study in present project.

Hamburger and Hamilton stages table (Hamburger and Hamilton, 1951) was followed to select the different developmental stages required for the studies in present project work.

I.3 Reasons to select the Brain:

The brain has an extremely high rate of oxygen consumption and neuronal membranes are rich in unsaturated fatty acids which are susceptible to lipid peroxidation and hence it is more vulnerable to oxidative stress (Warner *et al.*, 2004). 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).

Avian embryonic tissues are characterized by the accumulation of polyunsaturated lipids during the last week of *in ovo* development (Noble and Speak, 1997). One consequence of these features is the development of encephalomalacia, a recognized clinical consequence of α -tocopherol deficiency during chick development, characterized by peroxidative damage to the cerebellum (Dror *et al.*, 1976). Surai *et al.*, (1998) investigated susceptibility of chick embryo brain to peroxidation and antioxidant profile of the cerebellum and other brain regions. Brain tissues obtained from embryos at 16 to 22 day of incubation was characterized by low concentrations of malondialdehyde in fresh tissues but very high susceptibility to spontaneous and Fe stimulated lipid peroxidation. The antioxidant profile of the different regions of 1- day old chick shows a significant difference in different brain regions. The cerebellum contained more α -tocopherol but less ascorbic acid as compared to the cerebrum. The cerebellum was also characterized by greater catalytic activity but a lower activity of mitochondrial Mn-Superoxide dismutase (Mn-SOD) than the cerebrum. Because mitochondria are main source of superoxide radical in biological systems, the low Mn-SOD activity might be at the risk factor with regard to encephalomalacia development. The low concentration of vitamin E in the various regions provides effective protection against lipid peroxidation under physiological conditions because the level of malondialdehyde in fresh tissue was negligible. Protection is presumably provided by recycling of α -tocopherol by the action of ascorbic acid, present at high concentration in the brain. Thus the lower ascorbic acid concentration in the cerebellum may predispose to encephalomalacia (Surai *et al.*, 1998). 19

Comparing antioxidant concentrations in the embryo brain with other tissues showed that the brain contained low concentrations of vitamin E, glutathione and low glutathione peroxidase and catalase activities. Thus the highly unsaturated brain tissue will be susceptible to peroxidation in stress conditions. Incubation of chicken or turkey embryo brain homogenates at 37°C produced high concentration of malondialdehyde as a result of lipid peroxidation (Surai *et al.*, 1998). 19

Neurons are more vulnerable to free radical damage than glial cells, but oligodendrocyte progenitors and immature oligodendrocytes in very prematurely born infants are selectively vulnerable to depletion of antioxidants and free radical attack. Reactive oxygen and nitrogen species play important roles in the initiation of apoptotic mechanisms and in mitochondrial permeability transition. Oxidative stress-regulated release of proapoptotic factors from mitochondria appears to play a much more important role in the immature brain. Oxidative stress is an early feature after cerebral ischemia (Blomgren and Hagberg, 2006).

In addition, the brain contains large amount of iron and polyunsaturated fatty acids and is relatively poor in antioxidant enzyme content (Halliwell, 1989). The generation of free radicals increase with age and also the lipid peroxidation in the brain, concomitantly the antioxidant defense mechanism gradually decline during aging (Mo *et al*, 1995). The nonmetabolised lipids get cross linked to other lipids and lipoproteins.

Since above review indicates that brain is high oxygen demanding and hence ROS producing organ besides their management involves Vitamin C and hence it was selected for studied in present project.

I.4 Selection of the different developmental stages of brain:

In late developmental stages free radicals generation and its management by Vitamins have been studied as realized by the above review.

But stress generation in early stages of brain differentiation and brain development remains to be studied. 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 following stages of brain differentiation and development were selected in present project.

The stages of brain development were selected as per the morphological developments of the brain as described below (Hamburger and Hamilton, 1951) i.e. 24, 34, 40, 48, 72, 96 and 120 hrs. So that increased free radical impact affecting the further development and mortality can be studied.

- i) The embryos of 24 hrs incubation (HH-7)- Neural tube closure.
- ii) The embryos of 34 hrs incubation (HH-10)- Formation of three primary brain vesicles.
- iii) The embryos of 40 hrs incubation (HH-11)-Formation of five neuromers of hind brain.
- iv) The embryos of 48 hrs incubation (HH-13)- Flexion and torsion
- v) The embryos of 72 hrs incubation (HH-18) -Differentiation of different brain regions.
- vi) The embryos of 96 hrs incubation (HH-23)- Enlargement of brain regions.
- vii) The embryos of 120 hrs incubation (HH-26)- Enlargement of brain regions.

As described above, the stages are selected for H₂O₂ treatment and study the Vitamin C effect on ROS stress with reference to morphologically distinction of differentiating and developing brain.

Thus to understand the alterations the normal chick brain development needed to be reviewed.

1.5 Brain development in Birds:

Fertilization of the chick egg occurs in the oviduct, before the albumen and the shell are 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. Cleavage occurs only in the blastodisc, a small disc of cytoplasm 2–3 mm in diameter at the animal pole of the egg cell. Peripheral area of blastodisc is called periblast.

The first cleavage furrow appears centrally in the blastodisc gives two blastomeres. Second cleavage arise at right angle to first cleavage and gives 4 blastomeres. Third cleavage variable in number and position and gives 8 blastomeres. Fourth cleavage furrow is irregular and circular it divides the central cells from peripheral cells. Further successive cleavage furrows divide the central cells and other radial furrow divides peripheral cells.

Blastula:

Cleavage planes are irregularly placed and number of cells are considerable. The entire group of blastomeres is termed as Blastoderm. The peripheral margin of cells remains a single cell thick and cells remain unseparated from yolk. As a result of segmentation, a closely packed mass of blastomeres is formed called as Morula.

The rearrangement of cells leads to formation of blastula. Equatorial and vertical cleavages divide the blastoderm into five to six cell layers, which are linked by tight junctions (Bellairs et al. 1975; Eyal-Giladi 1991). The deep cells in the center of the blastoderm are shed, die and a one-cell-thick area pellucida is formed. Remaining part of the blastoderm forms most of the actual embryo. Between the area pellucida and area opaca is a thin layer of cells present called the marginal zone (or marginal belt) (Eyal-Giladi, 1997; Arendt and Nübler-Jung, 1999). Some cells of which become very important in determining cell fate during early chick development.

Gastrulation:

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. This is concomitant with the elongation of the primitive streak which extends 60–75% of the length of the area pellucida.

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. The primitive groove along which cells migrates to blastocoel, is analogous to amphibian blastoderm.

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.

In birds, 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 well underway. The neural folds migrate toward the midline of the embryo, eventually fusing 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 from either side of the notochord and it extends somewhat rostral to the cephalic end of the notochord. This thickened area is resulted from rapid proliferation of 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 on the overlying ectoderm, the process is called induction. 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, longitudinal ridges formed are called neural folds. These two processes are the first indications of the differentiation 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 is much more clearly marked at 24 hrs. By 27 hrs the neural folds in the cephalic region are brought together at the dorsal midline. The mesial components of the two folds fuse with each other and outer layer of unmodified ectoderm also become fused. Thus in the same process the neural tube eventually formed. Although the cells that will become the neural tube originally express E-cadherin, they stop producing this protein as the neural tube forms, and instead synthesize N-cadherin and N-CAM.

Neurulation in the cephalic (head) region is well advanced as compared to caudal region. At about time of closing of the neural tube a phenomena appears called neuromery. It is the process of segmental arrangement of transverse bulges along the neuraxis which defines the various brain regions. But at caudal region, the neural tube remains as a simple tube that tapers off toward the tail. The two open ends of the neural tube are called the anterior neuropore and the posterior neuropore.

Different neural tube defects are caused when various parts of the neural tube fail to close. Failure to close the human *posterior* neural tube regions at day 27 results in a condition called spina bifida. Failure to close the *anterior* neural tube regions results in anencephaly. The failure of the entire neural tube to closure is called craniorachischisis. Neural tube defect in humans are seen in about 1 in every 500 live births.

Secondary neurulation:

Secondary neurulation involves the making of a medullary cord and its subsequent hollowing into a neural tube. Secondary neurulation is usually seen in the neural tube of the lumbar (abdominal) and tail vertebrae. In both cases, it can be seen as a continuation of gastrulation.

Differentiation of the Neural tube:

The differentiation of the neural tube into the various regions of the central nervous system occurs on the gross anatomical level, the neural tube and its lumen bulge

and constrict to form the chambers of the brain and the spinal cord. At the tissue level, 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. Finally, on the cellular level, the neuroepithelial cells themselves differentiate into the numerous types of nerve cells (neurons) and supportive cells (glia) present in the body.

Differentiation of brain regions:

Cephalic part of the brain is markedly enlarged by as compared to caudal part. Its thickened wall and dilated lumen mark the region which will develop 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 mouse and chick (Kallen and Linds, 1953; Kallen, 1955) and observation on living chick embryos maintained *in vitro* (Kallen, 1953) have showed that neuromery develops in an orderly way. The bulges of neuromeres develops in rostral sequence and disappears in a caudorostral sequence.

These bulges can be observed and have been called proneuromery, neuromery and post neuromery, respectively. These are three primary brain vesicles. Most rostral part of the head is a fore brain or prosencephalon. Posterior to the prosencephalon and marked off from it by a constriction is mid brain or mesencephalon. Posterior to the mesencephalon there is a very slight constriction marking the boundary of hind brain or rhombencephalon. It continuous posteriorly with cord region of neural tube.

Divisions of brain and their neuromeric structures:

Embryos of 24 hrs shows series of 11 enlargements the neuromeres. The 3 most rostral neuromeres form the prosencephalon, neuromeres 4 and 5 incorporated in the mesencephalon and neuromeres 6 to 11 in the rhombencephalon. Anteriorly the innerneuromic constriction soon disappears except for two, one between prosencephalon and mesencephalon and one between prosencephalon and rhombencephalon. Rhombencephalic neuromeres however, remain clearly marked for a considerable period.

At (29-30 hrs) the lateral walls of prosencephalon becomes outpocketed to form pair of primary optic vesicles. At this stage there is no constriction between optic vesicles

and lateral wall of prosencephalon and lumen of optic vesicle communicate mesially with the lumen of prosencephalon. By 33 hrs optic vesicles come to lie closely approximated to the superficial ectoderm. A depression is formed in the prosencephalon, known as infundibulum.

The relation of notochord to the division of the brain is of prime importance in lateral developmental process. The notochord extends anteriorly as far as a depression in the floor of the prosencephalon known as infundibulum, therefore the rhombencephalon, mesencephalon and that part of the prosencephalon posterior to the infundibulum lie immediately dorsal to the notochord (are epicardal) while infundibular region and the parts of the prosencephalon cephalic to it project rostral to the notochord are prechordal.

Closure of anterior neuropore:

Closure of neural fold at the extreme rostral end of brain region is delayed and resulted in the formation of anterior neuropore. At 30 hrs neuropore is still open, at 30 hrs it appears much narrowed and almost closed at 33 hrs. The scar left by its closure serves to mark the point originally most anterior in the developing brain.

38 hrs hrs of development:

The prosencephalon becomes divided into the anterior telencephalon and caudal diencephalon. These two bilaterally symmetric telencephalic vesicles contain the rudiments of the cerebral cortex, hippocampus, basal ganglia, basal forebrain nuclei, and olfactory bulb. The diencephalon contains the rudiments of the thalamus and hypothalamus, as well as a pair of lateral (the 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 (anterior neuromeres) and the posterior 4 neuromeres form the myelencephalon which becomes the medulla oblongata. The metencephalon gives rise to the cerebellum. The rhombencephalon shows segmental

pattern specifies the origin of nerves. The rhombencephalon divides into rhombomeres. These rhombomeres maintain their developmental "territories" (Guthrie and Lumsden, 1991). Each of them will form ganglia, the clusters of neuronal cell bodies whose axons form a nerve which generates the cranial nerves.

In the chick embryo of 3-4 days 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 in to 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:

Being about 38 hrs, processes are initiated which eventually change the entire configuration of the embryo and its positional relation to the yolk. These processes involve positional changes of two distinct types, flexion and torsion.

Flexion:

Flexion means bending of the body about the transverse axis. In chick embryo it takes place in the head region and hence known as cranial flexion. The axis of bending in the development of the cranial flexion is a transverse axis passing through the mid brain at the level of the anterior end of the notochord. 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: In the chick, torsion is normally carried out towards a definite side. Telencephalic region of embryo is twisted so that left side lies next to the yolk and right side away from the yolk. At about 38 hrs the cranial flexion and torsion are just becoming evident in the head region of embryo. In chicks of about 43 hrs the further progress of both flexion and torsion is well marked. Finally the entire embryo comes to lie with its left side on the yolk. The progress of torsion, flexion proceeds in cervical, dorsal and caudal regions. Thus spinal axis becomes C shaped and head and tail lie close to each other.

50-55 hrs of development:

The mesencephalon comes to be the most anterior located part of the head and prosencephalon and myelencephalon lie opposite to each other; ventral surface to ventral surface. The original anterior end of the prosencephalon is thus brought close proximity to the heart, optic vesicle and the auditory vesicle are brought opposite to each other at nearly the same antero-posterior level.

At this stage, flexion involves the body further caudally as well as in the brain region. It especially marked at about the level of the heart in the region of transition from myelencephalon to spinal cord. Since this is the future neck region of the embryo the flexion at this levels is known as cervical flexion.

Growth of telencephalon region:

The completion of torsion in the cephalic region causes rapid changes in the configuration of the brain as seen in the entire embryo between 40 and 50 hrs of incubation, rostral part undergo slight enlargement and slight constriction in the dorsal wall indicating the impending division of the prosencephalon into telencephalon and diencephalons. Except for its considerable increase in size more important changes have not been taken in telencephalon.

In mid dorsal wall of the diencephalons region, epiphysis arises as a small invagination which becomes distinct to form pineal body of the adult. In the floor of the diencephalons the infundibular depression has become deepened and lies close to a newly formed ectodermal invagination known as Rathke's pocket. The epithelium of Rathke's pocket is destined to be separated from the superficial ectoderm and to become permanently associated with the infundibular portion of the diencephalons from the hypophysis or pituitary body.

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

The mesencephalon enlarges to separate cephalic diencephalons and caudal metencephalon. The roof of metencephalon begins to show thickening. In

myelencephalon the neuromeric constriction are still evident in the ventral and lateral walls. The dorsal wall becomes much thicker than ventral and lateral wall and shows no trace of division between the neuromeres. In spinal cord region of neural tube lateral walls have become thickened so that the neural canal appears slit like. At this closure of neural tube is completed throughout its entire length. Cephalic and caudal region were the regions to close. In younger stages they remain open, these regions are known as anterior neuropore and the sinus rhomboidalis respectively.

72 and 96 hrs of development:

Formation of the telencephalic vesicles:

The antero-lateral walls of the primary fore brain have been invaginated to form a pair of vesicle lying one on either side of mid line. These lateral vesicles are telencephalic vesicles; their cavities are continuous with lumen of median portion of the brain and known as "foramina of monro." Telencephalic division of brain includes the median portion of the brain along with two lateral vesicles. The lumen of telencephalon has three divisions, a median telocoele, posterior diocoele and two lateral telocoele.

The telencephalic vesicles becomes the cerebral hemisphere, and their cavities becomes the paired lateral ventricles of adult brain. The cerebral hemisphere enlarge enormously, extended dorsally and posteriorly as well as rostrally, eventually covering the entire diencephalons and mesencephalon under their posterior lobes.

Vellum transversum is internal ridge formed by the constriction which was first noted in the chicks of 55 hrs. The recessus opticus is a transeverse furrow in the floor of the brain which in the embryo leads on either side into the lamina of optic stalks. It is located just rostral to the optic chiasma where some of the optic nerve fibers cross and it is often spoken as the preoptic recessus.

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. Except for some elongation it doesn't differ from its condition when first form in embryos at about 55 hrs. Infundibular depression fuses with Rathke's pocket to form hypophysis. Later in development the lateral walls of diencephalons becomes greatly thickened to form the thalami thus reducing the size and changing the shape of the diocoele, which is known in adult anatomy as the third brain ventricle. The anterior part of the roof of the diencephalon lumens and becomes richly vascular. Latter these vessels, invaginate the roof and with them push into the third ventricle to form the anterior choroids plexus.

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 to the tuberculum posterious. The tuberculum posterious is a rounded elevation in the floor of the brain, it is regarded as marking boundary between diencephalons and mesencephalon.

Mesencephalon:

The dorsal wall of the mesencephalon thickens rapidly and becomes the corpora quadrigemina of the adult brain. These are four symmetrically placed elevation. The anterior pair (superior colliculi) constitutes the brain centre for visual reflexes, the posterior part(inferior colloculi) are the centers for auditory reflexes. The floor on mesencephalon also becomes greatly thickened which is known in adult as the crura cerebri. It serves as the main pathway of the fiber tracks which connect the cerebral hemispheres with the posterior part of the brain and the spinal cord. The originally capacious mesocoele is thus reduced by the thickening of the walls to form a narrow canal, the cerebral aqueduct of sylvius.

Metencephalon:

The boundary between the mesencephalon and metencephalon is indicated by the original interneuromeric constriction which separates them at the time of their establishment. The caudal boundary of metencephalon is being located approximately at the point where the brain roof changes from the thickened condition characteristic of the metencephalon to the thin condition characteristic of the myelencephalon. 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 cerebral peduncles. The roof of metencephalon undergoes extensive enlargement and becomes the cerebellum of the adult brain and coordinating centre for the complex muscular movement.

Myelencephalon:

The dorsal myelencephalic wall reduced in thickness, it later receives a rich supply of small blood vessels which carries the roof with them, grow into the myelocoel to form the posterior choroids plexus. The ventral and lateral myelencephalic wall becomes the floor and side walls of the medulla. Functionally the medulla serves both as a conduction path between cord and brain, as a reflex centre for involuntary activities such as breathing.

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

I.6 Brain and brain parts used for bioassay:

As the brain development proceeds different brain parts are not distinct during 0 hrs to 72 hrs of development and hence for bioassay whole embryos was used. From 96 hrs up to 144 hrs brain/brain parts were distinct and therefore brain was used for bioassay.

As the above review on development indicated 120 hrs onwards brain parts show growth in the established compartments and no further morphological alterations are observed. All the exposures were terminated at 120 hrs> Besides 24 hrs exposure on 120 hrs embryo was also studied.

To specify the influenced intermediary stages make out the more sensitive phases both for H₂O₂ induced damage and vitamin C mediated alterations, brain was also studied at intermittent intervals.

As stated earlier most of the physiological processes in organisms produce reactive oxygen species or free radicals which are simultaneously maintained by antioxidants *in vivo* (Patil *et al.*, 2007).

This is also true during chick embryonic development. To elaborate the point following is the review on free radicals during development.

I.7 Free radicals:

Free radicals are generally reactive oxygen or nitrogen species. Examples of free radicals (oxidizing molecules) are hydrogen peroxide, hydroxyl radical, nitric oxide, peroxyxynitrite, singlet oxygen, superoxide anion and peroxy radical. Oxygen is an unusual molecule in that it has two unpaired electrons with parallel spins. It is therefore a biradical. Free radical is any species that has one more unpaired electrons. These include hydrogen atoms (one unpaired electron), most transition metals and the oxygen molecules itself. O₂ has two unpaired electrons, each located in a different π^* anti-bonding orbital. These two electrons have same spin quantum number and so if O₂ attempts to oxidize another atom or molecule by accepting a pair of electrons from it. They must be with parallel spin impose restriction on oxidations by O₂ which tend to make O₂ accepting its electrons are at a time and slows its reaction with non-radical species (Halliwell and Gutteridge, 1984).

To overcome spin restriction, oxygen prefers to accept electrons one at a time, and the sequential addition of electrons leads to the formation of ROS. As a consequence of aerobic metabolism, all aerobic organisms are subjected to a certain level of physiologic oxidative stress as ROS are produced continuously in numerous biological processes within the body.

These free radicals are highly reactive, and unstable molecules that have an unpaired electron in their outer shell. They react with (oxidize) various cellular components including DNA, proteins, lipids / fatty acids and advanced glycation end products (e.g. carbonyls). These reactions between cellular components and free radicals lead to DNA damage, mitochondrial malfunction, cell membrane damage and eventually cell death. These are capable of mediating many kinds of injuries and/therefore are used

by neutrophils and macrophages to kill the microbes. These are generated as a product of normal cellular oxidation-reduction reaction. Since the 1960s, scientists have known that these molecules permeate the environment as reaction by-products of substances such as oxygen, smog and cigarette smoke. Each cell in our body produces billions a day through common reactions such as turning food into energy.

Metabolism of chemicals by liver oxidases and reperfusion injuries are also common causes. Because of generation of free radicals lipid peroxidation is seen to be elevated indicating the loss of membrane integrity. DNA damage is also observed. In addition, the protein cross-linking is occurring which is evident by the loss of enzymatic activities. The irreversible injury due to free radical is evident by different changes at cellular and molecular levels that include intracellular proteases and phospholipases. Nucleus shows pyknosis and karyorrhexis. Lysosomal rupture is evident membrane injury, calcium ion accumulation in cell, triggering the activation by the enzyme studies (Farber, 1994). Free radicals can turn other molecules into radical.

Superoxide is generated via several cellular oxidase systems (enzyme reactions). Once formed, it participates in several reactions yielding various free radicals such as hydrogen peroxide, peroxynitrite, etc. In turn, these can lead to chain reaction byproducts that also act to damage cells. Thus, excess free radical formation is associated with many disease states e.g. neuro-degenerative diseases, heart disease, HIV disease, chronic fatigue syndrome, hepatitis, cancer, autoimmune diseases, etc. Inflammation, poor blood flow, degenerative diseases, and toxin exposures among other mechanisms all lead to oxidative stress. A wide variety of diseases have evidence of excess generation of free radicals, oxidative stress and inadequate antioxidant activity. Some examples are neuro-degenerative diseases are given below.

Free radicals and Brain:

Free radicals are constantly produced in the brain *in vivo*. May be the leakage of electrons from the mitochondrial electron transport chain to generate superoxide radical (O_2^-). Because of its high ATP demand, the brain consumes O_2 rapidly, and is thus susceptible to interference with mitochondrial function, which can in turn lead to increased superoxide radical formation. Similarly Others are generated for useful

purposes, such as the role of nitric oxide in neurotransmission associated production of O₂-by activated microglia. The brain contains multiple antioxidant defences, of which the mitochondrial manganese-containing superoxide dismutase and reduced glutathione seem especially important. Iron is a powerful promoter of free radical damage, able to catalyse generation of highly reactive hydroxyl, alkoxyl and peroxy radicals from hydrogen peroxide and lipid peroxides, respectively (Halliwell, 2001).

Oxidative stress and free radicals:

There is considerable evidence that even 21% O₂ has slowly manifested damaging effects. These effects vary with type of organism, its age, physiological state and diet such as the presence in the diet of varying amount of vitamins A, E and C, transition metals, antioxidants and polyunsaturated lipids. Superoxidation theory of oxygen toxicity states that formation of the superoxide radical's *in vivo* plays a major role in the toxic effect of oxygen (Fridovich, 1975; 1978; 1983).

The cumulative production of reactive oxygen species and reactive nitrogen species through either endogenous or exogenous insults is termed oxidative stress and is common for many types of cancer cell that are linked with altered redox regulation of cellular signaling pathways. Oxidative stress induces a cellular redox imbalance which has been found to be present in various cancer cells compared with normal cells; the redox imbalance thus may be related to oncogenic stimulation. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions (8-OH-G) have been noted in various tumours, strongly implicating such damage in the etiology of cancer. It appears that the DNA damage is predominantly linked with the initiation process. In the review by Rhodes *et al* (2006) examined the evidence for involvement of the oxidative stress in the carcinogenesis process. Attention is focused on structural, chemical and biochemical aspects of free radicals, the endogenous and exogenous sources of their generation, the metal (iron, copper, chromium, cobalt, vanadium, cadmium, arsenic, nickel)-mediated formation of free radicals (e.g. Fenton chemistry), the DNA damage (both mitochondrial and nuclear), the damage to lipids and proteins by free radicals, the phenomenon of oxidative stress, cancer and the redox environment of a cell, the mechanisms of carcinogenesis and the role of signalling cascades by ROS; in particular,

ROS activation of AP-1 (activator protein) and NF-kappaB (nuclear factor kappa B) signal transduction pathways, which in turn lead to the transcription of genes involved in cell growth regulatory pathways. The role of enzymatic (superoxide dismutase (Cu, Zn-SOD, Mn-SOD), catalase, glutathione peroxidase) and non-enzymatic antioxidants (Vitamin C, Vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), flavonoids, selenium and others) in the process of carcinogenesis as well as the antioxidant interactions with various regulatory factors, including Ref-1, NF-kappa B, AP-1 are also reviewed.

I.8 Reasons to use H₂O₂ as free radical inducing agent:

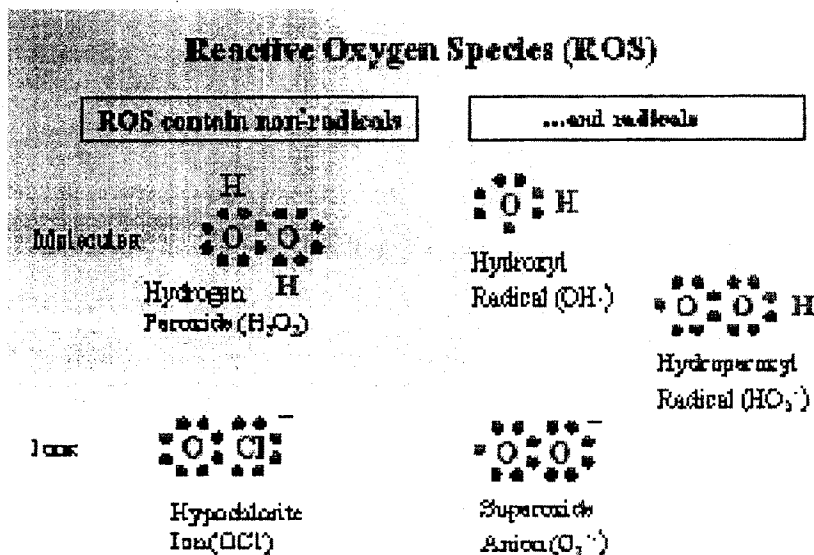
Hydrogen peroxide is known to be generated in the animal body as the consequence of many oxidative metabolisms (Halliwell and Gutteridge, 1984). Since its stability in body is redox state dependent, any alteration in that status may cause the persistence presence of H₂O₂ in animal body (Reth, 2002). It is known to generate free radicals and had shown neurotoxicity in cultures (Desagher *et al.*, 1996).[?]

Therefore H₂O₂ was used to induce free radicals at different stages of chick brain development to induce alterations in brain.

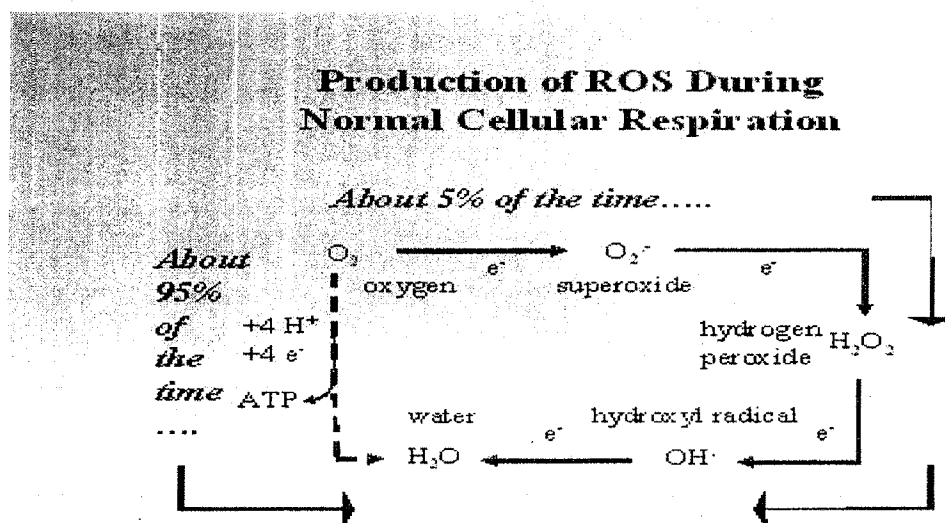
The detailed metabolism of H₂O₂ production, clearance and toxicity with reference to brain and neurons have been reviewed here in the following pages.

H₂O₂:

Hydrogen peroxide appears to be a ubiquitous molecule but not very abundant in nature. We exhale it, excrete it and take it in the form of diet. It is formed mainly by the action of sunlight on water and can be detected in drinking water, snow, rain water and sea water (Fujiwara, 1999; Willey J *et al.*, 1999). H₂O₂ is used as a powerful oxidizing agent (Nindl, 2004). H₂O₂ is used as a bleaching agent in paper and textile industries, for treatment of waste water, and during metallurgical processes. H₂O₂ is weakly reactive, but it forms hydrogen and a hydroperoxyl radical or two hydroxyl radicals by breaking bond between the two oxygen atoms. The later molecules are highly reactive (with hydroxyl radicals being the most reactive of all reactive oxygen species).



Reactive oxygen species (ROS) including H_2O_2 are produced as a by product of oxidative metabolism. Oxidative stress by H_2O_2 has become increasingly recognized to the point that it is discussed as a component of virtually every disease. Just as H_2O_2 has



the ability to harm microorganisms, it also has the ability to kill our body's cells. This damaging power is due to hydroxyl radicals that indiscriminately react with a wide variety of organic substrates causing peroxidation of lipids, cross-linking and inactivation

of proteins, and mutations in DNA (Knievel, 2004). Hydroxyl radicals formed when H_2O_2 is exposed to ultraviolet light or when it comes in contact with transition metal ions such as iron. Organisms have evolved a wide variety of defense mechanisms to counteract stress caused by H_2O_2 probably by sequestering the metal ions that would otherwise act as catalysts into proteins. Most of the obtained data emphasize the importance of metal ion sequestration in preventing the toxicity of H_2O_2 *in vivo* by decreasing the occurrence of Fenton chemistry (Halliwell and Gutteridge, 1999). Ferritin, transferrin, hemosiderin and heme are examples of proteins that enclose iron and thus play a role in protecting the cell against oxidative damage (Nappi & Vass, 2000). Other tools involves the enzymes that are employed to rapidly dismutate H_2O_2 to water e.g. Superoxide dismutases (SOD), catalases, peroxidases (especially glutathione peroxidases), and thioredoxin-linked systems (Droege, 2001; Arnér & Holmgren, 2000). A whole new family of enzymes, peroxiredoxins, has recently been described in addition to the two conventional enzymes, catalase and glutathione peroxidase, peroxiredoxins remove intracellular H_2O_2 by reducing it to water (Seo *et al.*, 2000; Wood *et al.*, 2003).

Excessive production of ROS such as H_2O_2 from inflammatory processes, leads to destruction of healthy body tissue, and development of autodestructive disease. It is almost a central medical dogma that dietary or pharmacological practices that strengthen the body's mechanisms to fight oxidative stress improve health.

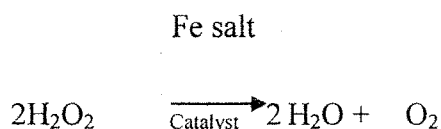
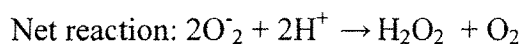
Administered directly (orally or intravenously) or generated indirectly (e.g. by UV irradiation of blood), H_2O_2 is claimed to strengthen the body's immune defense and thus to act effectively against a long list of ailments including some types of cancer, cardiovascular diseases, fractures, and depression.

Generation of H_2O_2 in the body:

Hydrogen peroxide is generated in peroxisomes to aid in the degradation of fatty acids and other molecules, and H_2O_2 is used for detoxification reactions involving the liver cytochrome P-450 system. Some cells, such as phagocytic leukocytes, have evolved the use of H_2O_2 as a bactericidal defense chemical, a phenomenon known as oxidative burst. In these inflammatory cells, NADPH oxidase associated with the plasma membrane

reduces molecular oxygen to generate superoxide. If ground state O_2 molecule accept a single electron it enters one of the π^* antibonding orbital. This resulted in production of superoxide radical, O_2^- . Addition of second electron to O_2^- gives O_2^{2-} it is not radical. At physiological pH O_2^{2-} immediately protonate to give H_2O_2 . The pKa of H_2O_2 is very high. In aqueous solution, O_2^- undergoes the so-called dismutation reaction to form H_2O_2 and O_2 . Homolytic fission of O-O bond in H_2O_2 produces hydroxyl radical, $-OH$. Homolysis can be achieved by heat or ionization radiation. Iron also catalyse the decomposition of H_2O_2 (Halliwell and Gutteridge, 1984).

Copper salts react with H_2O_2 to make $-OH$ radical with a much greater rate constant.



The resultant hydroxyl radicals reacts with almost every type of molecules found in living cells: sugars, amino acids, phospholipids, DNA base and organic acids. The reactivity of $-OH$ radicals is so great that when they react with biological molecules produce second radicals of variable reactivity e.g. the reaction with carbonate ions (CO_3^{2-}) produces the carbonate radical a powerful reducing agent (Anbar and Neta, 1967). Hydrogen peroxide probably mainly via O_2^- has been observed from whole bacterial species, from phagocytic cells, from protozoans (Holland *et al.*, 1982) and from mitochondria, microsomes and chloroplasts *in vitro* (Chance *et al.*, 1979, Halliwell, 1981b). Several enzymes, including glycollate and urate oxidase, produces H_2O_2 without the intermediacy of free O_2^- radical (Chance *et al.*, (1979).

H_2O_2 as Signaling Molecule:

Hydrogen peroxide plays a role as a signaling molecule similar to other ubiquitous signaling molecules such as cAMP, nitric oxide and calcium. Understanding the regulation of cellular H_2O_2 and redox metabolism will aid in developing novel therapeutic tools. Regulation of vascular tone, sensing of oxygen tension, and enhancement of

membrane receptor signal transduction are only a few examples of non-detrimental processes that involve ROS (Droege, 2001; Lander, 1997). Most of the regulatory effects are not directly mediated by the most reactive members of ROS, such as O_2^- , and $\cdot OH$, but rather by their derivatives such as H_2O_2 . In fact, H_2O_2 seems to be the most important ROS member that acts as a signaling molecule. Just as ROS and H_2O_2 can be considered “death molecules”, they also can be called “molecules of life” (Droege, 2001; Rhee *et al.*, 2003).

In contrast to other small messenger molecules such as calcium, H_2O_2 *in vivo* is short lived, in the range of milliseconds. The half life for O_2^- is estimated to be a microsecond and that for $\cdot OH$ a nanosecond. The stability of H_2O_2 is influenced by the pH. While the blood pH in a healthy body is known to be homeostatically regulated within a narrow range, information on the local pH in inflammatory tissues is limited. The stability of H_2O_2 further depends on the cell's redox state. H_2O_2 is more stable in an oxidizing environment (the extracellular space), than in a reducing environment (the cell interior). Therefore, H_2O_2 acts as an inter-cellular messenger (Reth, 2002).

H_2O_2 is well suited to act as cellular messenger since it does not randomly react with all molecules, as most other ROS do, but instead primarily targets cysteine residues. It oxidizes the -SH group of cysteine to -S-OH which is then reduced by cellular reducing agents such as glutathione and thioredoxin. The H_2O_2 -dependent modifications of the target proteins can cause their activation or inactivation. An important group of molecules that are downregulated by H_2O_2 is the protein tyrosine phosphatase (PTP) group, evolutionarily conserved molecules that play a central role for transmitting signals from cell surface receptors to the nucleus (Halliwell *et al.*, 2000).

At sites of inflammation H_2O_2 appears to have the potential to augment or inhibit T cell signal pathways outside of normal receptor control as presented in more detail below. T lymphocytes are especially interesting candidates for redox regulation. T cells are vital in regulating inflammatory responses and they are exposed to exogenous H_2O_2 produced by activated inflammatory phagocytic leukocytes. Studies show that H_2O_2 is able to promote either proliferation or death of inflammatory T cells, depending upon the circumstances. It seems that multiple steps in various T cell signal pathways are redox

sensitive, so that the overall outcome of oxidative stimulation for the cells would be a weighted balance of the individual signaling activities. There is also substantial evidence that T cells can produce H_2O_2 in response to various activating stimuli. One source of H_2O_2 production is the phagocyte-type NADPH oxidase, but other sources such as the T cell receptor itself are likely. An improved understanding of the actions of H_2O_2 in T cells will have enormous therapeutic implications since down-regulation of inflammatory T cells is the treatment of choice for many inflammatory diseases (Halliwell *et al.*, 2000).

Effects of H_2O_2 :

As the generation of free radical seems to be the important alterations occurring during the development its induction by H_2O_2 administration and its associated effects on brain is of importance for the brain development and it may help to decide the defects caused by free radicals during different period of development. Hence, the same was planned to study in present project.

Because of potential importance of hydrogen peroxide in injury during myocardial ischemia and reperfusion, its mechanism of cytotoxicity was assessed in cultured chick embryo cardiac myocytes. Injury was quantitated by release of lactate dehydrogenase (LDH) or ^{51}Cr , both of which correlated with loss of cell viability assessed by trypan blue exclusion. The iron chelator deferoxamine (0.25-2 mM), but not equimolar iron-loaded deferoxamine, markedly reduced LDH and ^{51}Cr release. Injury was also prevented or attenuated by the diffusible reactive oxygen metabolite scavengers dimethylthiourea (10-20 mM) and N-(2-mercaptopropionyl)-glycine (20 mM). The hydroxyl radical scavenger, dimethyl sulfoxide (200-400 mM), also reduced injury. Other scavengers that probably remained extracellular, superoxide dismutase and mannitol, were ineffective. Thus, with exposure of cardiac myocytes to H_2O_2 , cytotoxicity requires reactions catalyzed by intracellular iron (Byler *et al.*, 1994). Chick embryo myocytes were subjected to H_2O_2 to assay the catalase and lactate dehydrogenase release (Horwiz *et al.*, 1995). The acute anti-oxidant and protective effect of American ginseng berry extract (AGBE) has been demonstrated in cultured cardiomyocytes. Chick embryo cardiomyocytes were treated with AGBE (0.5-2.5 mg/ml) for up to 72 h. The treated cells were then exposed to exogenously added H_2O_2 (500 μM). The oxidant-mediated

significant oxidative injury and cell death was observed. Cell death caused by H_2O_2 was significantly attenuated in AGBE-pretreated cells in a concentration- and time-dependent manner ($p < 0.005$). Caffeic acid and chlorogenic acid from AGBE contributed significantly to AGBE's protective effects. Pretreatment with AGBE upregulates peroxide detoxifying mechanisms, which could affect intracellular oxidant dynamics in cardiomyocytes (Mehendale *et al.*, 2006).

Effects on neurons:

It has been reported that neurons are particularly sensitive to hydrogen peroxide (H_2O_2). Investigation of the putative role of astrocytes in the modulation of the neurotoxic effect of H_2O_2 was done by Desagher *et al* (1996). The exposure to H_2O_2 of cultured striatal neurons from mouse embryos induced a concentration-dependent (10-1000 μM) cell death as estimated 24 hr later. Two methods were used to estimate neuronal survival: the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay or an enzyme-linked immunosorbent assay with antibodies directed against an antigen located in neurons (microtubule-associated protein-2). The neurotoxic effect of H_2O_2 on neurons cocultured with astrocytes was strongly attenuated compared with that observed on a pure population of neurons seeded at the same density. Moreover, the protective effect of astrocytes depended on the astrocytes/neurons ratio, a significant neuroprotection being detectable for 1 astrocyte to 20 neurons. Catalase seems to be the main hydrogen peroxidase activity involved in the neuroprotective effect of astrocytes. Indeed, in the culture conditions used, this enzymatic activity was enriched in this cell type compared with neurons; its inhibition, and not that of glutathione peroxidase, reduced the disappearance rate of the oxidant. Therefore, astrocytes could delay neuronal death in pathological situations in which H_2O_2 has been, at least partially, demonstrated to be involved (Desagher *et al.*, 1996).

1.9 Reasons to select doses of hydrogen peroxide:

Hydrogen peroxide treatment was initiated as a single dose at different hrs of development and the animals were observed for mortality and other alteration on successive developmental hours. At designed hrs of incubation hydrogen peroxide was

administered with HBSS such that 1 ml of HBSS contains desired concentrations i.e. 0.05 mM, 0.5 mM and 1.5 mM. Exposure period of doses was as shown in table 2. These doses were given according to Mehendale *et al.*, (2006) as mentioned in Introduction. From the data obtained after preliminary experiment shown in Table 2 (Mortality) from the tested doses 0.5 mM dose was selected. Selection was done on the basis of mortality observed. Lowest dose i.e. 0.05 mM of H₂O₂ showed 20% mortality and the highest dose used i.e. 1.5 mM H₂O₂ showed 100% mortality while 0.5 mM dose showed 50% mortality therefore this dose was selected for the further experimental studies so as to study the teratogenic effects if any with this sub lethal dose.

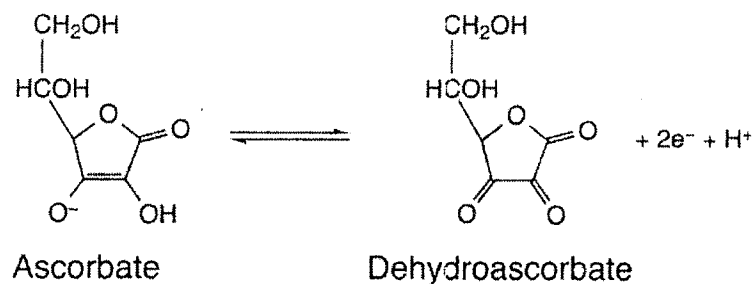
I.10 Reasons to use Vitamin C as free radical scavenger:

In addition to glutathione vitamin C also takes part in free radical scavenging So to study the protection of brain development by vitamin C in H₂O₂ induced free radical impact at different stages of brain development Vitamin C was used to scavenge the free radicals.

Its availability to cells and its concentrations seem to have important influence on free radical concentration and hence on associated metabolism in organism therefore its influence is reviewed in the following section.

Vitamin C as free radical scavenger:

Vitamin C (Ascorbic Acid) was first isolated in 1928, by the Hungarian biochemist and Nobel Prize winner Szent-Gyorgyi. 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).

It is an unstable, easily oxidized acid and can be destroyed by oxygen, alkali and high temperature. 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.

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), Potassium 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). Vitamin 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 dysfunctioning 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:

Antioxidant property of ascorbate is due to its properties as an electron donor. Ascorbate is a broad-spectrum radical scavenger that is effective against peroxy- 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 brain:

The brain, spinal cord and adrenal glands have the highest ascorbate concentrations of all the tissues in the body (Hornig, 1975). Under normal conditions, turnover of ascorbate in brain is about 2% per hour (Spector, 1977). Under conditions of ascorbate deficiency however, brain ascorbate content is retained tenaciously, with decreases of less than 2% per day (Hughes *et al.*, 1971).

Ascorbate enters the central nervous system primarily by active stereospecific Na^+ -dependant transport at the choroid plexus. It diffuses from cerebrospinal fluid to brain extracellular fluid, where its concentration is regulated homeostatically. Extracellular ascorbate levels are also dynamically modulated by glutamate-mediated activity, via glutamate-ascorbate heteroexchange. From the cerebrospinal fluid.

Ascorbate can also enter the ECF by carrier-mediated uptake and by simple diffusion across brain capillaries at the blood–brain barrier (Lam and Daniel, 1986). However, detectable levels of the recently described Na^+ -dependent ascorbate transporters, SVCT1 and SVCT2, are found in the choroid plexus, but not in brain capillaries (Tsukaguchi *et al.*, 1999). *In situ* hybridization in the rat brain indicates that SVCT2 is localized at high levels in neurons, but not in glial cells (Tsukaguchi *et al.*, 1999). An alternative mechanism for ascorbate entry across the blood–brain barrier has been proposed to be transport of its neutral oxidation product, dehydroascorbate, by a facilitative glucose transporter, GLUT1, with subsequent reduction of dehydroascorbate to ascorbate once it is in the brain (Agus *et al.*, 1997).

Brain-tissue content of ascorbate is regionally dependent: higher levels are found in anterior regions, such as the cerebral cortex and hippocampus, with progressively lower levels in more-posterior regions, such as the brainstem and spinal cord (Milby *et al.*, 1982 and Rice *et al.*, 1995). This pattern primarily reflects the increasing white-matter content of the posterior regions of the CNS, because the ascorbate content of white matter is much lower than that of neuron-rich gray matter (Rice *et al.*, 1995). Extracellular ascorbate concentration, $[Asc]_o$, is also maintained homeostatically (Schenk *et al.*, 1982).

Several lines of evidence from whole-tissue studies, however, indicate that ascorbate is localized preferentially in neurons, with much lower concentrations being found in glia. Shimizu and colleagues identified ascorbate in the cytosol of neurons in the locus coeruleus, which use ascorbate as a co-factor for noradrenaline synthesis (Diliberto *et al.*, 1987), with lower levels in surrounding glia. More recently, studies that quantified tissue levels of ascorbate, using HPLC with electrochemical detection, found that the ascorbate content of adult rat cortex was roughly fourfold higher in neuron-rich cerebral cortex than in the essentially neuron-free optic nerve, again consistent with predominant localization of ascorbate in neurons (Rice *et al.*, 1995).

The best quantitative information on neuron versus glial compartmentalization has come from studies of the ascorbate content of brain tissue with known neuron density or neuron-to-glial ratio. For example, the neuron density of the cerebral cortex varies across mammalian species, with an inverse dependence on brain size (Tower and Elliott, 1952). Across species, cortical ascorbate content increases linearly with increasing neuron density human <rabbit<guinea pig<rat<mouse (Rice and Russo-Menna, 1998).

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 has been studied by the acid silver nitrate method by Barnett and Bourne, (1942). In the cells of the central nervous

system ascorbic acid may be localized 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. A reaction is shown at the 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. A reaction is also shown in ganglion cells, the meninges, and the choroid plexuses.

In chick induced hypoxia increases cerebral vitamin C. It may 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 O₂ 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 O₂ concentration decreased ascorbic acid concentration by four-fifths in plasma and by one-half in brain, compared to values in normoxic (21% O₂) or hypoxic (15% O₂) controls. Hyperoxic preincubation of embryos also inhibited ascorbic acid transport, as evidenced by decreased initial rates of saturable and Na⁺-dependent [¹⁴C]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 O₂ 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 (AA) 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). Fertilized eggs were used for each treatment in experiments I and II. In experiment I the live embryos at 13th day of incubation were subjected to the following treatments uninjected (control); eggs injected with 0.5 ml sterile saline solution; and eggs injected with 0.5 ml of saline solution containing 1, 3, 5 or 7 mg of AA per egg. In experiment II, glucose injection at varying concentrations was applied to the eggs before hatching i.e. at 18 days of incubation and grouped as uninjected (control); eggs injected with 0.5 ml deionized sterile water; and eggs injected with 0.5 ml of deionized sterile water containing 5, 10 or 15 mg of glucose. The effect of AA injection on the hatchability of fertile eggs was significant ($P < 0.01$). The highest hatchability was obtained from the group treated with AA at 3 mg concentration. There was no effect of glucose injection on hatchability and chick weight (Ipek *et al.*, 2004).

Ascorbate as an antioxidant in the brain

High levels of ascorbate and of SVCT2 in a variety of neuronal cell types imply a function for neuronal ascorbate beyond its actions as a cell-specific enzyme co-factor. Importantly, the tenfold difference between ascorbate levels in neurons and glia (Rice and Russo-Menna, 1998) is consistent with the estimated tenfold higher rate of oxidative metabolism in neurons compared with glial cells (Siesjö, 1980). Brain ascorbate (but not glutathione) levels in pond turtles are two to three times higher than in mammals this suggests that (Rice *et al.*, 1995), these diving animals have a remarkable tolerance of hypoxia (Lutz, 1992) and Sick *et al.*, 1992). High levels of ascorbate could represent a specific adaptation to prevent oxidative damage during reoxygenation after a hypoxic dive (Rice *et al.*, 1995). Although ascorbate can act as a pro-oxidant *in vitro*, the occurrence of naturally high levels of ascorbate in neuronal cytosol, taken with other data discussed below, argues strongly against normally pro-oxidant actions *in vivo*. This conclusion was also reached by Halliwell in a recent review of the antioxidant versus pro-

oxidant effects of ascorbate (Halliwell, 1996). Indeed, neuroprotection by ascorbate has been demonstrated in several recent studies, both *in vitro* and *in vivo*.

Under normal circumstances, ascorbate is recycled as it is used. During anoxic depolarization, ascorbate is lost from cells and released into extracellular fluid (Hillered *et al.*, 1988). With continued ischemia, tissue levels of ascorbate and other low-molecular-weight antioxidants fall (Lyrer *et al.*, 1991). Consequently, when aerobic metabolism resumes, intracellular stores of these agents are no longer adequate to quench reactive species. Thus, only excessive radical production (although this can occur) is not responsible for ischemic cell death. Rather, loss of antioxidants from the intracellular compartment during ischemia or other injury leaves cells vulnerable to oxidative damage. Increased detection of hydroxyl radical during post-ischemic reperfusion (Cao *et al.*, 1988) is consistent with this hypothesis. Enhanced brain ascorbate content after dietary supplementation can protect cortical mitochondria from *in vivo* ischemia/reperfusion injury in rats (Sciamanna and Lee, 1993). Similarly, ascorbate can prevent mitochondrial hyperoxidation during post-ischemic reoxygenation *in vitro* in brain slices (Pérez-Pinzón *et al.*, 1997).

Ascorbate might prevent redox imbalance resulted from reactive oxygen species (ROS) generated by activation of glutamate receptors (Dykens *et al.*, 1987 ; Coyle and Puttfarcken, 1993; Lafon-Cazal *et al.*, 1993; Prehn, 1998). Ascorbate was also found to buffer glutamate-generated ROS and limit consequent cell death in cultured neurons (Ciani *et al.*, 1996 and Atlante *et al.*, 1997). An intracellular site of action for these effects was suggested by other studies in brain slices, in which both ascorbate and glutamate-receptor antagonists were shown to inhibit edema formation (Brahma *et al.*, 2000). For ascorbate to be effective it had to be accumulated by brain cells; moreover, isoascorbate, which is not a substrate for the stereospecific ascorbate transporter, was not effective in preventing brain-slice edema.

Direct scavenging of ROS is likely to have the greatest role, because ascorbate can protect neurons from oxidative damage (Sato *et al.*, 1993 and Sharma, 1997) and decrease levels of glutamate-generated ROS (Ciani *et al.*, 1996 and Atlante *et al.*, 1997).

Other actions of ascorbate:

In addition to its functions as an antioxidant in the CNS, ascorbate has been shown to be a neuromodulator of both dopamine- and glutamate-mediated neurotransmission, as reviewed by Grunewald (Grünewald, 1993), and Rebec and Pierce (Rebec and Pierce, 1994). Predominant localization of ascorbate in neurons is consistent with such neuromodulatory functions. In addition, ascorbate is an essential co-factor for noradrenaline synthesis (Diliberto, *et al.*, 1987), and is required for the release of noradrenaline and ACh from synaptic vesicles (Kuo *et al.*, 1978). Ascorbate is also an essential co-factor in the synthesis of many neuropeptides (Glembotski, 1987) and at physiological concentrations enhances peptide release (Miller and Cicero, 1987). In addition, ascorbate promotes myelin formation by Schwann cells by enabling these cells to assemble a basal lamina (Eldridge *et al.*, 1987).

A number of other actions of ascorbate have been described in the literature, from alterations in neurotransmitter binding affinity to inhibition of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. Because most of these studies have been conducted *in vitro*, often in isolated cells or cell membranes, the potential for pro-oxidant effects of ascorbate is high, which casts doubt on the physiological relevance of some of the findings. This does not necessarily mean that ascorbate never acts as a pro-oxidant *in vivo*; indeed, under pathological conditions such as ischemia, when ascorbate compartmentalization is disrupted, these actions could occur and contribute to CNS injury. Under normal conditions, however, it is increasingly clear that ascorbate facilitates neuroprotection.

Ascorbic acid plays role in Vitamin-E recycling (Buettner and Jurkiewicz, 1996), and therefore it is used for a counter management of induced free radicals and its effect on brain development in this study. The antigenotoxic effect of some phytoproducts like carotenoid (β -carotene), curcumin, ascorbic acid and flavonoid (genistein) was demonstrated on the genotoxicity induced by hydrocortisone on human lymphocyte culture. This study showed that the ascorbic acid and curcumin were more effective than carotenoid and flavonoid (Ahmed *et al.*, 2004).

The above review indicated production of free radicals during differentiation and development of brain and their management by in situ free radical scavengers including chemicals like glutathione and vitamins like C, A and E.

Therefore, reasons to use H_2O_2 as a free radical inducing agent and use of vitamin C as a free radical scavenger are discussed here.

I.11 Reasons to select doses of vitamin C:

For the reasons described in Introduction, 3 mg/egg ascorbic acid dose which 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. Its efficacy as an antioxidant and associated alterations were if any were studied in developing brain.

I.12 Lipid peroxidation:

Hydrogen peroxide generated free radicals/or other species generate free radicals from the biomolecules *in vivo*. Thus they affect lipid molecules also through process of lipid peroxidation.

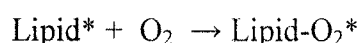
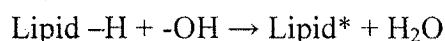
Lipid peroxidation (LPO) is a complex process whereby unsaturated lipid material undergoes reaction with molecular oxygen to yield lipid hydroperoxides. Although attack by singlet oxygen on unsaturated lipid has been shown to give lipid hydroperoxide by a non-radical, non-chain process. The vast majority of situations involving lipid peroxidation proceed through a free radical-mediated chain reaction initiated by the abstraction of a hydrogen atom from the unsaturated lipid by a reactive free radical followed by a complex sequence of propagative reactions.

The peroxidation of polyunsaturated fatty acids proceeds through non-enzymatic auto-oxidative pathway or enzymatic catalysis. Many toxic agents can be metabolically activated within the cell to free radicals intermediate that can initiate lipid peroxidation and result in cell injury (Slater, 1984).

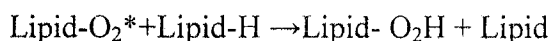
Malondialdehyde is of interest primarily as product of lipid peroxidation *in vivo*. It is identified among the products of the oxidative decomposition of amino acids complex carbohydrates, pentoses and hexoses formed in the presence of a metal catalyst as a product of free radicals generated *in vivo* and is taken as indicator of lipid peroxidation (Bird and Draper, 1984).

Initiation of lipid peroxidation:

Lipid peroxidation is initiated by attack of any species that has sufficient reactivity to abstract a hydrogen atom such as hydroxyl radical. Since hydrogen atom has only one unpaired electron this leaves behind an unpaired electron on the carbon atom. The carbon radical in a polyunsaturated fatty acid tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which rapidly reacts with O₂ to give a hydroperoxy radical. Hydroperoxyradicals abstract hydrogen atoms from other lipid molecules and so continue the chain reaction of lipid peroxidation. The hydroxyl radical combines with the hydrogen atom that it abstracts to give a lipid peroxide (Halliwell and Gutteridge, 1984).



After molecular rearrangement



Illumination of unsaturated fatty acids in the presence of sensitizers of singlet O₂ formation such as chlorophyll, porphyrins, bilirubin or retinal initiates rapid peroxidation and such reactions occurs *in vivo* in the mammalian eye, the illuminated chloroplast and in patients suffering from porphyrrias (Foote, 1902, Krinsky and Denek, 1982).

Rate of peroxidation of purified membrane lipids or microsomal fractions in the presence of Fe (III) chelators can be accelerated by adding ascorbic acid, a reducing agent (Wills, 1966) and sometimes by adding thiol compounds (Tien *et al.*, 1982; Searle and Wilson, 1983).

The unsaturated fatty acyl side chains of membrane phospholipids are easily oxidized to form lipid hydroperoxides. This is an autopropagative process called lipid peroxidation. It is a potential mechanism for amplifying free radical processes. The sequence of chemical reactions involved in the formation of lipid hydroperoxides and the operation of cellular antioxidant mechanism against this form of membrane injury have been well characterized (Tribble *et al.*, 1987; Farber *et al.*, 1990). Lipid peroxidation can be demonstrated in association with cellular injury from a wide variety of toxins. It provides a logical link between free radical initiated processes and resultant dysfunction. Notwithstanding this, it has proved difficult to determine whether lipid peroxidation is a crucial link between oxidative cell injury and the mechanism of cell death (Smith *et al.*, 1982; Sevinan and Hochstein, 1985; Tribble *et al.*, 1987).

The C-H bonds of polyunsaturated fatty acids are weakened at the allylic position by the neighboring double bonds, and therefore hydrogen atoms can be abstracted by free radicals. Following such hydrogen abstractions, conjugated dienes are formed. In the next step, the resulting lipid radical reacts with O₂ to form a lipid peroxy radical. There are also strong oxidizing species which extract another allylic hydrogen (from the same or different fatty acids) to form lipid hydroperoxides, thereby propagating the reaction. Lipid peroxides are then reduced to hydroxylipids. The ultimate products of lipid peroxidation are derived from more complex secondary processes during which fragmentation of fatty acyl chain occurs. The products are indicators of lipid peroxidation.

The radical processes that initiate lipid peroxidation can damage proteins and nucleic acids directly. These direct reactions may be more important in the production of cell injury than peroxidation of lipids. The combined effects of protein and DNA oxidation, which can occur simultaneously with lipid peroxidation, offer a more general mechanism for cell injury by free radical processes (Tribble *et al.*, 1987). It should be noted, however that not all authors share this view (Faber *et al.*, 1990).

There are several ways by which lipid peroxidation can amplify and propagate the effects of oxidative stress. Lipid peroxidation results in a chain reaction. The resultant lipid peroxides can react with metal ions or with metalloproteins generate alkoxy radicals;

these can initiate further free radical reactions. Each initiation step can amplify four to ten fold, thereby markedly increasing the extent of damage to other cellular macromolecules.

The amount of fatty acids present decreases very slightly if at all and the changes in the fatty acid content chiefly concern the unsaturated acids, which decreases rapidly. A synthesis of unsaturated fatty acids only now occurs, reaching a maximum, on further decrease sets in and continues until emergence. The amount of saturated fatty acids present remains constant and the unsaturated fatty acids only being utilized.

The biochemical details of 4-hydroxy malonaldehyde and related aldehydes have been studied (Esterbauer *et al.* 1991) and mechanism associated with the injury is also studied (Slater, 1984).

Cultured neurons from chick embryo telencephalon were protected from cyanide induced neurotoxicity by inhibitors of lipid peroxidation, indicating that lipid peroxidation plays an important role in the genesis of cyanide-induced neuronal injury (Müller and Krieglstein, 1995).

The propagation of lipid peroxidation:

Lipid hydroperoxides are stable at physiological temperature and transition metals catalyse its decomposition. These metal catalysts includes simple complexes of iron salts with phosphate ion or phosphate esters such as ADP, haemoglobin, methhaemoglobin, peroxidase, cytochrome P450 , other cytochromes and non haeme iron proteins (O' Brien, 1969; Kaschinitz and Hateti, 1975; Gutteridge, 1977; Aust and Svingen, 1982). All these should contribute to the propagation of lipid peroxidation in membrane *in vivo*.

Lipid peroxidation and development:

Lipid peroxidation has been implicated in a large number of cellular processes, including prostaglandin synthesis and control of cellular proliferation (Cornwell and Morisaki, 1984 ; Lands *et al.*, 1984). Many organisms exhibit a marked increase in lipid peroxidation or in the susceptibility of their tissues to peroxidation during development

(Utsumi *et al.*, 1977; Yoshioka *et al.*, 1977). Similar observations have been made in various tissues of other organisms (Allen and Baiin, 1989). Antioxidant enzymes known to be lower in neonates than adults, such as GPx, GSH reductase and glucose-6-phosphate dehydrogenase and have no effect on the susceptibility to lipid peroxidation of lung homogenates from young rats. Whereas α -tocopherol inhibits peroxidation, the concentration required to achieve inhibition in neonate tissue is far greater than would ever be found in fetal or adult tissue (Kehler and Autor, 1977). Strains of mice with relatively higher SOD activity in early neonatal development are also less vulnerable to peroxidation than strains that contain low SOD activity in early postpartum life (Novak *et al.*, 1978). The greater susceptibility of mammalian neonatal lipids to peroxidation is probably partly due to differences in lipid composition that exist in adults and newborns. The degree of lipid unsaturation increases in neonatal rats (Kehler and Autor, 1977). The lung tissues of neonatal rats and mice contain large amounts of polyunsaturated lipid and both of these organisms are known to be highly resistant to oxygen. In contrast, the lung tissues of newborn and adult guinea pigs, adult rats and adult mice contain predominantly saturated fat and these animals are highly susceptible to hyperoxia (Kehler and Autor, 1978). Although lipid peroxidation is generally regarded as deleterious to cells, the presence of lipids susceptible to oxidation forms the basis of antioxidant protection in organisms during early stages of development when other antioxidant defenses are at relatively low levels.

Oxygen radicals, or reactive oxygen species (ROS) act as primary or secondary messengers to promote cell growth or death. Many instances demonstrate an important direct role of ROS in development because redox status regulates key transcription factors that influence cell signaling pathways involved in proliferation, differentiation and apoptosis. Therefore, oxidative stress can alter many important reactions that affect embryonic development both positively and negatively. During particular periods in development, the embryo is more or less susceptible to oxidative stress, and teratogens, which can modify redox status, such as thalidomide, phenytoin, and ethanol (Dennery, 2007).

It has been suggested that developmental alcohol induced brain damage is mediated through increase in oxidative stress (Smith *et al.*, 2005). Uncontrolled lipid peroxidation is very much detrimental to the cell. Hence the reduction of lipid peroxides has been claimed to play a key role in the control of lipid peroxidation in living system (Witting, 1980). Lipid peroxidation is causative reaction in cellular deterioration in the ageing process. Lipid peroxidation also play a key role in environmental pollutants and in a variety of pathological conditions (Slater *et al.*, 1987). A possible role of lipid peroxide has been reported in various biological systems exposed to heavy metals such as lead (Somashekhran *et al.*, 1992), mercury, cadmium (Gregus and Verga, 1985) and selenium (Padmaja, 1993). Chvapil *et al.*, (1976) reported that zinc functions as a stabilizer of biomembrane and biostructures.

Lipid peroxidation and Chick development:

Hoffman and Ramm (2005) induced hyperoxia in 10 day chick embryos with injection of 0.1 ml of 1.5% trypan blue in sterile saline in yolk-sac. Some embryos were placed into a hyperoxic environment of 45% oxygen, or they were exposed to trypan blue plus hyperoxia and examined after 5 hrs. Hyperoxia eliminated trypan blue-induced mortality and caused considerable reduction in the appearance of hemorrhages as compare to embryos not subjected to hyperoxia. Embryonic wet weight, how-ever, increased. In this case hyperoxia functioned protectively by either acting against electron transport inhibition or permitting the normal passage of oxygen to lysosomes.

It is known that hyperoxia stimulates growth late in incubation when the chick embryo outgrows the O₂ diffusion capacity. Scientist wondered whether hyperoxia could have an effect in the early period prior to the stage where metabolism exceeds the oxygen diffusion capacity of the eggshell. For this Golde *et al* (1998) studied four groups of chicken eggs: control group (CG; *n*=100) and three test groups (TGs) exposed during 48 h to 60% O₂ on days 10, 14, and 18. In the CG, embryonic and organ mass (brain, heart, lungs, liver and intestine) were measured from day 10 until day 21 of incubation. In the TGs embryonic and organ mass were obtained from 24 h after the start of hyperoxia exposure until the end of incubation. The most striking growth rate acceleration was observed in the liver and intestine, maximum growth rate accelerations were respectively,

19 and 42% in TG1, 43 and 173% in TG2 and 39% and 84 in TG3. In contrast, the brain was little affected by the hyperoxia exposure. These results suggest that also in the middle of the incubation period O₂ availability can be a limiting factor for growth, before metabolism exceeds the oxygen diffusion capacity of the eggshell (Golde *et al.*, 1998).

Padmaja and Ramamurthi (1997) studied the effect of lethal doses of zinc on Lipid peroxidation system in developing chick embryo. Chick embryos were treated with different concentrations (25 and 75 pmoles/kg egg wt.) of zinc on the 14th day of embryonic development. The levels of thiobarbuturic acid reacting substances (TBARS), glutathione (GSH) and activity levels of antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase were measured in both hepatic and brain tissues after different time intervals (24, 72 and 120 hrs) of zinc exposure. Increased levels of TBARS were observed after 24 h hrs of zinc treatment and thereafter (72 h and 120 h) the levels were decreased in both the tissues. Significant induction was observed in antioxidant enzyme activities in both the tissues after 24 h and 72 h when compared to 120 h. However, the GSH levels were increased at 24 h and 72 h and thereafter decreased in both the tissues at 120 h. The elevated levels of antioxidant enzymes at 24 h and 72 h may be responsible for the reduction of TBARS at 72 h and 120 h in developing chick embryos (Padmaja and Ramamurthi, 1997).

Developmental studies of the antioxidant enzymes like superoxide dismutase and glutathione transferase were done in liver, brain, yolk sac membrane, kidney, lung, heart and skeletal muscle in 10-22 days (per day). The results indicated that specific activity in the brain was high (Surai, 1999), which was interpreted as counter activity to interact with free radicals formed during development. But direct free radicals or their metabolic products have not been studied. The immature brain is particularly susceptible to free radical injury because of its poorly developed scavenging systems and high availability of iron for the catalytic formation of free radicals. Hence free radical generation in adult and developing brain and its roles in the pathophysiology after cerebral hypoxia-ischemia was also studied (Blomgren and Hagberg, 2006). To study the effects of free radicals the

chick brain cells in culture were used (Lindner *et al.*, 1989), which showed morphological alteration in brain cells.

Magnesium deficiency and oxidative stress have been identified as correlative factors in many diseases. The origin of free radicals correlated with oxidative damage resulting from Mg-deficiency is unclear, therefore to investigate whether hydrogen peroxide (H_2O_2) is associated in the oxidative stress induced by Mg-deficiency, the effect of Mg^{2+} deficiency (0, 0.4, 0.7 mM) on the metabolism of H_2O_2 was investigated in cultured chick embryo hepatocytes. With various concentrations of Mg^{2+} for 1, 2, 4, 6 and 10 days, parameters of H_2O_2 production, catalase activity, lipid peroxidation, intracellular total Mg and cell viability were analyzed. Results demonstrated that long-term incubation of chick embryo hepatocyte in extracellular Mg^{2+} -deprivative and Mg^{2+} -deficient (0.4 mM) states significantly enhanced the production of H_2O_2 (approximately twofold, respectively) and lipid peroxidation in the cell cultures, while decreasing the cell viability. Readded Mg^{2+} (1.0 mM) and the partial reversing action of dimethylthiourea suggested that (i) $[\text{Mg}^{2+}]_e$ deficiency induced the increase of H_2O_2 production, (ii) $[\text{Mg}^{2+}]_e$ deficiency decreased catalase activity in chick embryo hepatocyte *In Vitro*, subsequently causing oxidative stress and cell peroxidative damage (Ying, 2006).

I.13 Oxidants and antioxidants in development and differentiation:

The development of living organisms occurs as a progression of irreversible changes in gene expression that increasingly are tissue specific. The process that stimulates differentiation at critical junctures of development appear to be regulated both by genetic and epigenetic influences. Apart from the genes regulating the development, components of cellular environment also influence the development. Free radicals and other reactive oxygen species are produced in the metabolic pathways of aerobic cells and affect a number of biological processes. Oxidation reactions have been postulated to play a role in aging, a number of degenerative diseases, differentiation and development as well as serving as subcellular messengers in gene regulatory and signal transduction pathways. The discovery of the activity of superoxide dismutase is a seminal work in free

radical biology, because it established that free radicals were generated by cells (Allen, 2006).

It has been demonstrated that high oxygen tension stimulate morphological and biochemical differentiation of variety of tissues from diverse organisms (Allen and Bali, 1989). Conversely, hypoxia is inhibitory to differentiation (Nations *et al.*, 1986). Flour beetles reared under half the normal atmospheric concentration of O₂ exhibit a high rate of abnormal metamorphosis, however the actual rate of O₂ consumption is not altered by maintenance under hyperoxia (Loudon, 1988). This suggests that the effect of hyperoxia on development are not mediated by effector on aerobic biochemical pathways. Similarly, hyperoxia induces differentiation in neuroblastoma even in the presence of enough cyanide to eliminate aerobic metabolism (Erkell, 1980). Although variations in ambient oxygen concentration can alter gene expression, the effect of oxygen on differentiation appear to be dependent of aerobic metabolism. Many oxygen mediated reactions occur via oxygen free radical intermediates and the rate of free radical generation in cells is influenced by ambient oxygen concentration.

Metabolic gradients are established during early phases of development and their existence influences subsequent developmental events. Variations in oxygen supply and oxygen metabolism associated with the gradation of metabolic rate in embryos appear to form one basis for the influence of metabolic gradients on development. The rate of oxygen metabolism affects the rate of oxidant generation by various cellular biochemical pathways. Cells contain antioxidant defenses that respond to variations in cellular oxidant production. Large changes in the activity of the antioxidant enzyme superoxide dismutase and changes in cellular redox state occur during the differentiation of many types of cells. These changes correspond to an increased rate of oxidant production; the cellular environment becomes more pro-oxidizing during differentiation. Evidence is presented that implicates oxidants as a factor that can stimulate alterations in gene expression (Allen, 1991).

Metabolic gradients are established during early phases of development and their existence influences subsequent developmental events. Variations in oxygen supply and oxygen metabolism associated with the gradation of metabolic rate in embryos appear to

form one basis for the influence of metabolic gradients on development. The rate of oxygen metabolism affects the rate of oxidant generation by various cellular biochemical pathways. Cells contain antioxidant defenses that respond to variations in cellular oxidant production. Large changes in the activity of the antioxidant enzyme superoxide dismutase and changes in cellular redox state occur during the differentiation of many types of cells. These changes correspond to an increased rate of oxidant production; the cellular environment becomes more prooxidizing during differentiation. Evidence is presented that implicates oxidants as a factor that can stimulate alterations in gene expression (Allen, 1991).

Oxidants and antioxidants in birds:

The sensitivity to oxidative stress and antioxidant also differs according to species. The role of oxidative stress and antioxidant defense in 3,3',4,4',5-pentachlorobiphenyl (PCB 126)-induced toxicity and species-specific sensitivity was examined in White Leghorn chicken (*Gallus domesticus*) and Pekin duck (*Anas platyrhynchos*) embryos by Jin *et al* (2001). Eggs were injected into the air cell with 0.4–1.6 µg PCB 126/kg egg in corn oil prior to incubation. Lipid peroxidation measured by thiobarbituric acid reactive substances (TBARS), the GSSG:GSH ratio, and glutathione peroxidase (GPox) activities were determined in liver and adipose tissue of day 19 chicken and day 26 duck embryos. In chicken embryos, PCB 126 increased mortality and the incidence of edema and liver lesions, decreased embryo size, increased eye and head malformations, and markedly reduced fat storage. In contrast, no effects on the endpoints were observed in duck embryos even at the highest dose used in chicken embryos. PCB 126 increased hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity in a dose-dependent manner in chicken but not duck embryos. PCB 126 significantly increased TBARS levels in liver and to a greater degree in adipose tissue of chicken embryos, indicating that adipose tissue is a sensitive target for this compound. Increases in lipid peroxidation by PCB 126 were associated with significant decreases in GPox activity in these tissues. These biochemical changes support oxidative stress playing a role in PCB 126-induced embryo toxicity while antioxidant defenses provided protection against oxidative damage induced by this compound. Ducks, the less-sensitive species, showed higher basal levels of hepatic GPox

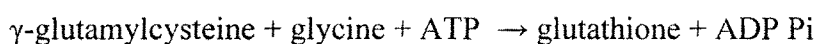
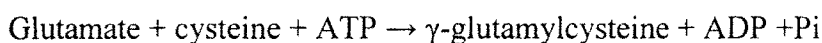
than chickens, suggesting that this antioxidant enzyme may contribute to the differences in sensitivity to this compound between the two species (Jin *et al.*, 2001).

I.14 Glutathione:

One of the antioxidant involved during natural free radical scavenging activity is glutathione, therefore it is reviewed here.

Biosynthesis of Glutathione:

Glutathione is not an essential nutrient since it can be synthesized from the amino acids L-cysteine , L-glutamate and glycine. It is synthesised in two adenosine triphosphate dependent steps. One molecule of ATP is broken down to ADP and phosphate for each peptide bond generated:



First, gamma-glutamylcystein is synthesised from L-glutamate and cystein via the enzyme gamma-glutamylcystein synthetase (a.k.a. glutamate cysteine ligase, GCL). This reaction is rate limiting step in glutathione synthesis. Second , glycine is added to the C-terminal of gamma-glutamylcystein via the enzyme glutathione synthase.

While all cells in the human body are capable of synthesizing glutathione. liver glutathione synthesis has been shown to be essential . Following birth, mice with genetically induced loss of GCLC (i.e. GSH synthesis) only in liver die within 1 month.

It is limited in its production by negative feedback inhibition of its own synthesis through the enzyme cysteine synthetase. Glutathione appears to be synthesized primarily, if not exclusively, in the cytoplasmic compartment in cells, yet is utilized for a variety of detoxification, protection and physiological functions in other compartments, including nucleus, mitochondrial matrix, endoplasmic reticulum and extracellular spaces (Meister, 1991). The availability of glutathione in these compartments is determined by complex interactions of utilization, transport, synthesis and reduction of glutathione disulfide

(GSSG) and GS-SX mixed disulfides. Glutathione is supplied to the mitochondria through an energy dependent transport system that appears to couple glutathione uptake to efflux of metabolic anions. In contrast, supply to the nucleus appears to involve passive diffusion, yet the nuclear pool also appears to remain kinetically distinct in its different sensitivity to depletion of chemotherapeutic agents.

Glutathione occurs mainly intracellularly and major fraction of transpeptidase is one of external surface of the cell membranes. Glutathione transported across cell membranes interact with γ -glutamyl transpeptidase. γ -glutamyl amino acid formed by γ -glutamyl transpeptidase are transported into cells.

Glutathione participates directly in the neutralization of free radicals, reactive oxygen compounds, and maintains exogenous antioxidants such as vitamins C and E in their reduced (active) forms. In addition, through direct conjugation, glutathione plays a role in the detoxification of many xenobiotics (foreign compounds) both organic and inorganic. Glutathione is an essential component of the human immune response. Proposed mechanisms of immune enhancement include optimizing macrophage functions, offsetting oxidative damage associated with lymphocyte monoclonal expansion, and stabilizing the mitochondrial membrane thereby, reducing apoptosis in lymphocytes.

Forms of Glutathione:

Glutathione cycles between a reduced thiol form (GSH) and an oxidized form (GSSG) in which two tripeptides are linked by a disulfide bond. Maintenance of cellular GSH levels is achieved through a complex pathway involving γ -glutamylcysteine synthetase, glutathione *S*-transferase, glutathione peroxidase, and glutathione reductase (Bains and Shaw, 1997).

In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG/GSH ratio is considered indicative of oxidative stress. In the reduced state, the thiol group of cysteine is able to donate an electron (H^+) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide

(GSSG). Such a reaction is possible due to the relatively high concentration of glutathione in cells (up to 5mM in the liver). GSH can be regenerated from GSSG by the enzyme glutathione reductase. GSSG is reduced to GSH by *glutathione reductase*, a flavoprotein that uses NADPH as the electron source. The ratio of GSH to GSSG in most cells is greater than 500.

Intracellular glutathione is converted to GSSG by selenium-containing glutathione peroxidase, which catalyzes the reduction of H_2O_2 and other peroxides; there is evidence that certain glutathione-S-transferases can also catalyze such reactions. Glutathione is also converted to GSSG by trans-hydrogenation; a number of reactions of this type have been studied. Reduction of GSSG to glutathione is mediated by widely distributed enzyme GSSG reductase which uses NADPH. Extracellular conversion of glutathione to GSSG has also been reported; the overall reaction requires O_2 and leads to formation of H_2O_2 . GSSG is also formed by reaction of glutathione with free radicals.

Intracellular glutathione is major substrate transpeptidase. The finding of an enzyme and its substrate on opposite sides of a membrane led to postulate that intracellular glutathione is transported to the membrane bound transpeptidase. Studies on patient with γ -glutamyl transpeptidase deficiency who has marked glutathionuria and glutathionemia, led to suggestion that transport of intracellular glutathione to the plasma and glomerular filtrate in this patient reflects an aspect of the normal processes that provide substrates to the membrane bound enzyme. Thus, in that absence of significant transpeptidase activity substantial amounts of glutathione appear extracellularly. Under conditions of marked toxicity or oxidative stress, intracellular GSSG increases substantially and there may be a mechanism for its export.

Glutathione deficiency contributes to oxidative stress which plays a key role in aging and pathogenesis of many diseases (Wu *et al.*, 2004). The formation of reactive intermediates by cysteine conjugate β -lyase may play a role in the target organ toxicity and the possible renal tumorigenicity of several chlorinated olefins widely used (Wu *et al.*, 2004).

Glutathione is known as a substrate in both conjugation reactions and reduction reactions, catalyzed by glutathione s-transferase enzymes in cytosol, microsomes, and mitochondria. However, it is also capable of participating in non-enzymatic conjugation with some chemicals, as in the case of n-acetyl-*p*-benzoquinone imine (NAPQI), the reactive cytochrome P450 -reactive metabolite formed by paracetamol (or acetaminophen as it is known in the US), that becomes toxic when GSH is depleted by an overdose of acetaminophen. Glutathione in this capacity binds to NAPQI as a suicide inhibitor and in the process detoxifies it, taking the place of cellular protein thiol groups which would otherwise be covalently modified; when all GSH has been spent, NAPQI begins to react with the cellular proteins, killing the cells in the process.

Neurons and glutathione:

Glutathione is a major neuronal antioxidant, with important roles in detoxification of H₂O₂ and prevention and repair of peroxidative damage to lipids, proteins, and nucleic acids (Bains and Shaw, 1997; Du *et al.*, 2008). In neurodegenerative diseases, the neuronal cell loss is unlikely to result from reduced activity of brain glutathione peroxidase that couples the oxidation of reduced glutathione to the detoxification of peroxides (Kish *et al.*, 1986).

Excess glutamate at synapses, which may be released in conditions such as traumatic brain injury, can prevent the uptake of cysteine, a necessary building block of glutathione living the brain without the protection from oxidative injury afforded by glutathione.

Increasing glutathione levels in developing neurons demonstrates a unique role for enhanced redox potential in developing neurons and cells at the cerebrospinal fluid and blood-brain interface (Xiaojian, 2006). Differentiated rat oligodendrocytes contained more glutathione than did progenitors and were less susceptible to decreases in glutathione concentration induced by H₂O₂ stress (Fragoso *et al.*, 2004). Levels of glutathione, total glutathione (i.e., GSH and GSSG), and Glutathione S-transferase activities are much higher in cultured chick astrocytes and neurons which are resistant to

reactive oxygen species (and potentially toxic xenobiotics) and may play a protective role in the brain (Makar, 1994).

Loss of intracellular neuronal glutathione (GSH) is an important feature of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Bains and Shaw, 1997). The consequences of GSH depletion include increased oxidative damage to proteins, lipids, and DNA and subsequent cytotoxic effects. Loss of intracellular neuronal GSH can occur quickly after exposure to peroxide-generating events (Dringen, 2000). Although the pathways involved in neuronal GSH depletion *in vivo* and *in vitro* have yet to be fully elucidated, evidence suggests that increased oxidative stress, (free radical generation) may overwhelm the cell's capacity to regenerate oxidized glutathione (GSSG). The subsequent oxidative damage from H₂O₂ and hydroxyl radicals may play an important role in neuronal dysfunction and/or death in neurodegenerative diseases. Glutathione depleted cortical neuronal cultured cells showed; Cu induced increase in oxidative stress resulting in DNA damage and activation of p53-dependent cell death (Du *et al.*, 2008). Although GSH is important for intracellular Cu transport, the consequences of GSH loss for Cu metabolism have not been adequately investigated.

Redox status and glutathione:

The redox status of cells is the ratio of oxidizing equivalents to reducing equivalents present in cells. It can be regarded as the net balance of interaction of all cellular oxidants and antioxidants. Redox state is modulated by variety of environmental factors, including nutritional status e.g. increased dietary antioxidants tends to depress the synthesis of glutathione as well as the activity of enzymatic antioxidants defenses (Sohal *et al.*, 1985). More than 90% of the reducing equivalents in cells are contained in the sulfhydryl group of cystein in GSH (Chance *et al.*, 1979). Thus any change in the rate of GSH synthesis or utilization can profoundly influenced the redox status of cells.

Both cell cycle and development are associated with enormous change in redox balance (Allen, 1989). Cells loosing their mitotic capacity loose more than half of their GSH (Allen, 1989; Allen and Sohal, 1986) in contrast cells differentiating into tissue that

retain high regenerative capacity such as liver tissue, exhibit no loss of GSH during development (Allen, 1991). GSH concentration increases sharply during the development of meiotic tissue (Allen, 1989). The inductive capacity of postnodal pieces of chick embryo can be modified by soaking the tissue in a solution of GSH or cysteine (Waheed and Mulhekar, 1967; Rao, 1969). Experimentally induced changes in GSH concentration stimulates the expression of heat shock protein (Freeman, 1987). Cause of these effects appears to stem from the influence of redox status on calcium metabolism. Upsurge in the rate of oxidants generation stimulates an increase in SOD, loss of GSH and release of calcium from mitochondrial stores, this free calcium concentration great enough to activate pathways leading to differentiation (Allen and Venkatraj, 1992).

GSH is also central to maintenance of the intra- and extracellular redox environments and modulating intracellular transport of copper (Cu). Glutathione incorporates Cu into metalloproteins immediately after uptake and prevent toxicity from unbound, intracellular redox-active Cu (Freedman, *et al.*, 1989; Ferreira, *et al.*, 1993 and Vulpe and Packman, 1995).

Glutathione in birds:

Comparisons between birds and mammals revealed the developmental patterns that have been conserved during evolution. Hepatic GPx and CAT specific activities increased in birds (Wilson *et al.*, 1992) during the final week before hatching. Also, Wilson *et al.* (1992) found that, aside from hepatic GPx and CAT, however, the expression of antioxidant enzymes differs between chick and mammalian embryos in a number of ways. The changes in SOD and GPx which occur in chick brain during the final 2 weeks of embryonic development may result from the particular timing of neuronal and glial proliferation and differentiation. During the final 2 weeks *in ovo* the specific activity of GPx doubles and that of CAT falls 4-folds (Wilson *et al.*, 1992). The ontogeny of SOD also varies between vertebrate species. In chick embryo MnSOD predominates brain (Wilson *et al.*, 1992). Additionally, the specific activities of SOD enzymes in brain vary markedly during the development of embryonic chick (Wilson *et al.*, 1992).

I.15 Formaldehyde:

Under stressed conditions free radicals generate due to any cause/source or as an effect of toxicant. Some metabolic pathways are activated and HCHO production is the pathway which is known to be activated in early developmental conditions also. Therefore, in present condition HCHO alterations were also studied. Here literature on formaldehyde is reviewed.

Formaldehyde and Development:

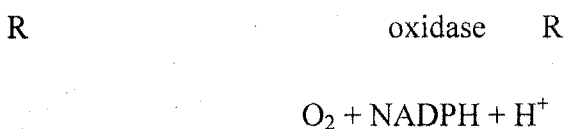
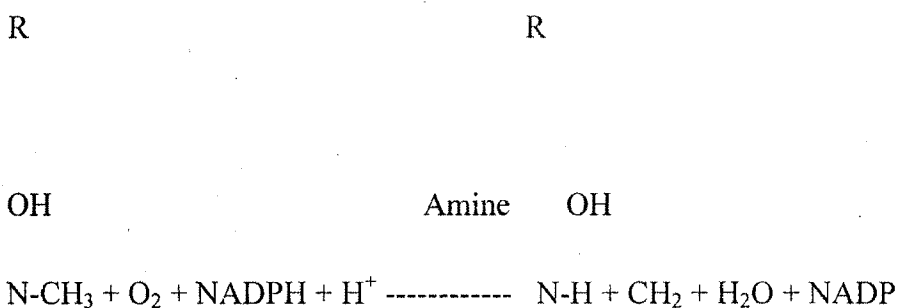
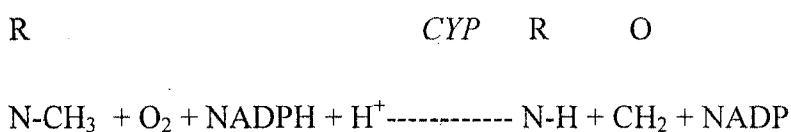
Biotransformation of enzymes of phase I are involved in the synthesis and degradation of many endogenous compounds while phase II reactions are conjugation reactions. Biotransformation of foreign chemical may result in increased or decreased toxicity depending upon chemical nature and function of metabolic pathway by which compound is degraded (Crnic *et al.*, 2003). Many endogenous factors, such as species, age, sex and developmental or hormonal status, regulate the activities of biotransformation enzymes (Ronis and Cunny, 1994). Microsomal enzymes have been extensively studied in mammals but not much in birds. Much is known about metabolism in adult organism, but little information exists on cytochrome P450 dependant enzymes during early development. The developing organisms is remarkably dynamic and many of the enzymes present in adult are not expressed in the foetus (Miller *et al.*, 1996).

Toxic effect of foreign organic chemicals during prenatal life is different in different species (Juchau *et al.*, 1980). There are no references about the activities of aniline hydroxylase and ethylmorphine N-demethylase in turkeys embryos but few studies showed the activities of these two enzymes in the first days and weeks after hatching (Bartlet and Kirinya, 1976; Thabrew *et al.*, 1982; Short *et al.*, 1988). Increased $O^{\cdot -}$ and OH^{\cdot} radicals, at different stages of development ought to induce increased activities of enzymes like N-demethylase; end product of which is formaldehyde. Therefore in present study HCHO content in brain of embryo in early development is studied. Elevated H_2O_2 production by certain P450 isozymes, e.g., P450 IIE1, may contribute to enhanced rates of glycerol oxidation because the H_2O_2 and nonheme iron are required for oxidation of glycerol to formaldehyde (Clejan and Cederbaum, 1991).

Increased level of formaldehyde may be the indication of either patho-physiological process, or environmental contamination, or malnutrition (Kalasz, 2003) and hence in the present work formaldehyde production was assayed as marker of pathophysiological effect of free radicals generated by H_2O_2 .

A variety of NADPH and oxygen dependent reactions catalyzed by the microsomal fraction has been recognized to yield formaldehyde as a product. The most common and frequently studied reaction resulting in the liberation of formaldehyde is the end demethylation of secondary and tertiary amines. These reactions are mediated by Cytochrome P-450 the terminal oxidase of the microsomal mixed function oxygenase system (Cooper *et al.*, 1965; Paulsen *et al.*, 1974).

The reactions are depicted in the following equations:



The reaction is initiated by amine oxidase which catalyses N-oxidation of certain hydroxylamines. The highly unstable hydroxyl amine oxidases formed by rapid dehydration and the resulting nitron intermediates liberate formaldehyde upon non enzymatic hydrolysis (Paulsen *et al.*, 1974). A third reaction sequence resulting in the formation of formaldehyde has been demonstrated to be associated with oxidation (reaction 3).

These reaction may involve the Cytochrome P-450 containing electron transport system as a source of H_2O_2 (Hildebrandt *et al.*, 1975) which in the presence of methanol is reduced peroxidatically by catalase (Oshino *et al.*, 1973) containing many microsomal preparations. Microsomal oxidation reduction reactions are mainly NADPH dependent and Cytochrome P-450 and b5 dependent. Alterations which are directly occurring in these cytochromes give directly the efficiency of the microsomal system and hence drug detoxifying system (Krishnamurthy, 1985).

The reactive oxygen intermediates (including super oxide and hydroxyl radicals as well as hydrogen peroxide) can cause direct cellular injury by lipid and protein peroxidation and damage to nucleic acid (Takeda *et al.*, 1984 and Richard *et al.*, 1990). Formaldehyde is the product of lipid peroxidation and used as a marker of the developmental oxidative stress in tissues and plasma during ischemia and reperfusion syndrome in rat brain (Maboudou *et al.*, 2002). Formaldehyde and ROS are cytotoxic, potentially carcinogenic, appeared to decrease cell viability of Jurkat cells dramatically *in vitro*. Formaldehyde alone can also induce cell death (Saito *et al.*, 2005).

A variety of NADPH and Oxygen dependent reactions mediated by Cytochrome P-450 yield formaldehyde as a product of N-demethylation of secondary and tertiary amines (Cooper *et al.*, 1965; Paulsen *et al.*, 1974). Microsomes and reconstituted systems containing cytochrome P450 can oxidize glycerol to formaldehyde in a reaction catalysed by oxidants produced from interaction of non heme iron with H_2O_2 (Rashba-step *et al.*, 1994). Production of formaldehyde by oxidative demethylation of N-mono-methyle amino acids by demethylase appears to be a general reaction (Ling and Tung, 1948). Tertiary-butyl alcohol can be oxidized to formaldehyde and acetone by hydroxyradicals

generated four different systems, H_2O_2 serves as a precursor of hydroxyl radicals (Cederbaum *et al.*, 1983).

Biochemical pathway of both the formaldehyde production and demethylation/methylation process is connected to the methionine-homocysteine cycles. Another important way of demethylation generated formaldehyde production is given by microsomal cytochrome P450 dependant oxidation of Xenobiotics, such as various drugs prescribed by doctors (Kalasz, 2003). Metabolism of methylamine by semicarbazide sensitive amine oxidase (SSAO) also produce formaldehyde (Boor *et al.*, 1992). Non-heme iron proteins, namely soybean lipoxygenase (SLO) and human term placental lipoxygenase (HTPLO) were also able to mediate N-demethylation of N,N-dimethylaniline (DMA) and related compounds in the presence of hydrogen peroxide. In addition to being hydrogen peroxide dependant, the reaction was also dependent on incubation time, concentration of enzyme and DMA and the pH of the medium (Hover and Kulkarni, 2000). C-14 formaldehyde crosses the placenta and enters fetal tissues. The incorporated radioactivity is higher in fetal organs (brain and liver) than in the maternal tissues. Russian literature on the embryotoxicity of formaldehyde in rodents demonstrate that formaldehyde crosses the placenta to the fetus, causes birth defects and affects the enzyme function in mitochondria, lysosomes and endoplasmic reticulum (Thrasher, 2005). DNA methylation may have an important role in the pathogenesis of certain diseases (Kalasz, 2003).