

# **CHAPTER - I**

## **INTRODUCTION**

## INTRODUCTION

Project entitled " Vitamin C mediated alterations in H<sub>2</sub>O<sub>2</sub> influenced Chorioallantoic membrane angiogenesis of Chick embryo. " is studied with relevant experimental work details of which will appear in chapter II . Different features of approach towards the working project are reasoned and relevant literature is reviewed in fore going pages of chapter – I .

### **Angiogenesis :**

#### **Reasons to take the problem :**

Angiogenesis can be defined as the growth of new blood vessels and capillaries and is controlled by the balance between proangiogenic and angiogenic molecules. Angiogenesis plays important role in many physiological processes like wound healing, corpus luteum formation and embryonic development. It is also important in pathological processes such as cancer, ischemic diseases and chronic inflammation including atherosclerosis (Arjan and Grietje, 2000). Angiogenic growth factors like VEGF and angiopoietin-1 induce Endothelial cells migration or proliferation through increase in ROS ( Fukai, 2002, Harfouche *et al* 2005, Ikeda *et al* 2005 ). Endothelial cells produce Reactive oxygen species like O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> which are important in physiologic and pathologic processes. High concentration of ROS causes apoptosis and cell death and oxidative stress is associated with the cardiovascular diseases including hypertension, heart failure, atherosclerosis (Griendling and Fukai, 2000). Levels of ROS produced by growth factors function as signaling molecules to mediate EC proliferation which may contribute to angiogenesis (Moulik, 2002). Angiogenesis also play key role in healing processes and the graft survival (Agenes *et al.*, 2003). Angiogenesis can be done in experimental animals by observing organs such as kidney by using simple technique (Ciancic, 2000). In bone allotransplantation neo-angiogenesis is an alternative to immunosuppression (Teruyasu, 2007). Angiogenesis has also potential role in chronic renal diseases and transplant setting. In ovary angiogenesis occur during female reproductive cycle and factor responsible for the angiogenesis in ovary is angiogenin, which bring out the morphological changes in ovary (Lee, 1999). Angiogenesis, i.e. the development of new blood vessels from pre-existing ones, represents a crucial step during this process, because similar to tumor metastases, endometritic implants require

neovascularization to guarantee oxygen and essential nutrient supply (Groothuis *et al.*, 2005). Few details about the angiogenesis types and process and factors influencing them are considered below

## **Types of Angiogenesis :**

### **Sprouting angiogenesis**

Sprouting angiogenesis is the first identified form of angiogenesis. Sprouting angiogenesis occurs by . First, biological signals like angiogenic growth factors activate receptors present on endothelial cells of pre-existing veins. Second, endothelial cells then are activated and begin to release enzymes called proteases that degrade the basement membrane in order to allow endothelial cells to escape from the original (parent) vessel walls. The endothelial cells then proliferate into the surrounding matrix and form solid sprouts connecting neighboring vessels. As sprouts extend toward the source of the angiogenic stimulus, endothelial cells migrate in tandem, using adhesion molecules, the equivalent of cellular grappling hooks, called integrins. These sprouts then form loops to become a full-fledged vessel lumen as cells migrate to the site of angiogenesis. Sprouting occurs at a rate of several millimeters per day, and enables new vessels to grow across gaps in the vasculature. It is markedly different from splitting angiogenesis, however, because it forms entirely new vessels as opposed to splitting existing vessels (Burri 2004).

### **Intussusceptive angiogenesis**

Intussusception, is also called splitting angiogenesis, it was first observed in neonatal rats. In this type the capillary wall extends into the lumen to split a single vessel in two. Intussusceptive angiogenesis includes four phases . First, the two opposing capillary walls establish a zone of contact. Second, the endothelial cell junctions are reorganized and the vessel bilayer is perforated to allow growth factors and cells to penetrate into the lumen. Third, a core is formed between the two new vessels at the zone of contact that is filled with pericytes and myofibroblasts. These cells begin laying collagen fibers into the core to provide an extracellular matrix for growth of the vessel lumen. Finally, the core is fleshed out with no alterations to the basic structure. Intussusception is important because it is a reorganization of existing cells. It allows a

vast increase in the number of capillaries without a corresponding increase in the number of endothelial cells. This is especially important in embryonic development as there are not enough resources to create a rich microvasculature with new cells every time a new vessel develops.

### **Therapeutic angiogenesis**

In therapeutic angiogenesis the formation of new blood vessels take place during combat disease . The presence of blood vessels where there should be none may affect the mechanical properties of a tissue, increasing the likelihood of failure. The absence of blood vessels in a repairing or otherwise metabolically active tissue may retard repair or some other function. Several diseases (eg. ischemic chronic wounds) are the result of failure or insufficient blood vessel formation and may be treated by a local expansion of blood vessels, thus bringing new nutrients to the site, facilitating repair. Other diseases, such as age-related macular degeneration, may be created by a local expansion of blood vessels, interfering with normal physiological processes.

### **The Angiogenesis Process: How Do New Blood Vessels Grow?**

The process of angiogenesis occurs as an orderly series of events:

1. Diseased or injured tissues produce and release angiogenic growth factors (proteins) that diffuse into the nearby tissues
2. The angiogenic growth factors bind to specific receptors located on the endothelial cells (EC) of nearby preexisting blood vessels
3. Once growth factors bind to their receptors, the endothelial cells become activated. Signals are sent from the cell's surface to the nucleus. The endothelial cell's machinery begins to produce new molecules including enzymes
4. Enzymes dissolve tiny holes in the sheath-like covering (basement membrane) surrounding all existing blood vessels
5. The endothelial cells begin to divide (proliferate), and they migrate out through the dissolved holes of the existing vessel towards the diseased tissue (tumor)

6. Specialized molecules called adhesion molecules, or integrins (avb3, avb5) serve as grappling hooks to help pull the sprouting new blood vessel sprout forward
7. Additional enzymes (matrix metalloproteinase's, or MMP) are produced to dissolve the tissue in front of the sprouting vessel tip in order to accommodate it. As the vessel extends, the tissue is remolded around the vessel
8. Sprouting endothelial cells roll up to form a blood vessel tube
9. Individual blood vessel tubes connect to form blood vessel loops that can circulate blood
10. Finally, newly formed blood vessel tubes are stabilized by specialized muscle cells (smooth muscle cells, pericytes) that provide structural support. Blood flow then begins.

### **Growth factors and angiogenesis :**

For regulation of angiogenesis growth factors are required the known growth factors which regulate angiogenesis and which inhibit process of angiogenesis are

#### **Angiogenic Growth Factors**

Angiogenin

Angiopoietin-1

Del-1

Fibroblast growth factors: acidic (aFGF) and basic (bFGF)

Follistatin

Granulocyte colony-stimulating factor (G-CSF)

Hepatocyte growth factor (HGF) /scatter factor (SF)

Interleukin-8 (IL-8)

Leptin

Midkine

Placental growth factor

Platelet-derived endothelial cell growth factor (PD-ECGF)

Platelet-derived growth factor-BB (PDGF-BB)

Pleiotrophin (PTN)

Progranulin

Proliferin  
Transforming growth factor-alpha (TGF-alpha)  
Transforming growth factor-beta (TGF-beta)  
Tumor necrosis factor-alpha (TNF-alpha)  
Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF)

### **Angiogenesis Inhibitors**

Angioarrestin  
Angiostatin (plasminogen fragment)  
Antiangiogenic antithrombin III  
Cartilage-derived inhibitor (CDI)  
CD59 complement fragment  
Endostatin (collagen XVIII fragment)  
Fibronectin fragment  
Gro-beta  
Heparinases  
Heparin hexasaccharide fragment  
Human chorionic gonadotropin (hCG)  
Interferon alpha/beta/gamma  
Interferon inducible protein (IP-10)  
Interleukin-12  
Kringle 5 (plasminogen fragment)  
Metalloproteinase inhibitors (TIMPs)  
2-Methoxyestradiol  
Placental ribonuclease inhibitor  
Plasminogen activator inhibitor  
Platelet factor-4 (PF4)  
Prolactin 16kD fragment  
Proliferin-related protein (PRP)  
Retinoids  
Tetrahydrocortisol-S  
Thrombospondin-1 (TSP-1)

Transforming growth factor-beta (TGF- $\beta$ )

Vasculostatin

Vasostatin (calreticulin fragment)

Because of the importance of angiogenesis in various physiological and pathological conditions considered above it is necessary to study the process and its modifiers in animal models to evaluate proangiogenic and antiangiogenic drugs (Herbert *et al*; 1996 ). In experimental animals angiogenesis can be studied e.g. Kidney (Ciancio;2000), Bone (Aliano *et al*,2004/Teruyasu,2007), Ovary (Lee,1999), endometrial implants (Groothuis *et al*; 2005). These models have been studied in the laboratory rodents.

But in recent years chick embryo is being used as an animal model to study differentiation, development and embryonic toxicity studies. In present project Chick embryo was used as an animal model and its chorioallantoic membrane was used as system of angiogenesis. The reasons for these selections are given below.

#### **Reasons to take chick embryo as an animal model:**

To use chick embryo as an animal model its advantages include easy access to the vascular network of the CAM, lack of immunocompetence, low costs, and avoidance of animal experiments. ( Joerges *et. al* ;2003 ). The early chick embryo lacks a mature immune system and was therefore used to study tumor-induced angiogenesis (Folkman *et al* ;1975).

#### **Reasons to select chorioallantoic membrane:**

Chorioallantoic membrane lies beneath the egg shell and perform functions as gas exchange and waste elimination between embryo and outside. The egg shell is porous and thus allows oxygen and CO<sub>2</sub> to pass freely back and forth from the environment to inside the egg. The chorioallantoic membrane is also responsible for drawing calcium from egg shell. Calcium is needed for the carrying general metabolism and to bone (Patten, 1977).

#### **CAM as a angiogenesis model in vivo :**

The chick chorioallantoic membrane assay is perhaps the most widely used assay for screening purposes (Ribatti 1996). CAM of chick is also used for grafting experiments around the graft it surrounds the blood vessels within the four days ( Brooks *et al* ; 1994). CAM is a promising model to study the role of angiogenesis in

both normal human endometrium and diseases involving the endometrium ( Maas *et al*; 1999 ). CAM is useful to study *in vivo* angiogenesis (Kilarski and Bikfalvi, 2007). In tissue engineering CAM is used because of its advantage like easy assessment of vascular network, lack of immunocompetence low cost and avoidance of animal experiments (Borges *et al*; 2003). CAM is also used as a model for testing biosensors (Valdes *et al.*, 2003).

CAM is useful to study human endometritis by transplanting endometrium on the CAM of chick (Nap *et al.*, 2004). The well vascularised chorioallantoic membrane is useful to study *in vivo* cancer research as an animal substitute model (Kunzi-Rapp *et al.*, 2003). The CAM contains the chorioallantoic fluid in which waste products are delivered because of its extensive vascularisation CAM is used as *in vivo* model for evaluation of angiogenic and antiangiogenic molecules (Wilting *et al.*, 1991; Ribatti *et al.*, 1999). On selection of CAM as an *in vivo* model of angiogenesis the developmental hrs to perform the angiogenesis influencing experiments were selected for the reasons given below. To study the details of development of CAM McEwen(1969) Patten(1977) and Carlson (2007) were used as guide lines. CAM also provides new approach to study human ovarian tissue transplantation in its first eschismic stages yielding information on the timing of tissue changes before the establishment of neovascularisation (Madrid *et al.*, 2008).

#### **The extraembryonic Blood vessels :**

By the end of the second day the two anterior wings of the area vasculosa and the extraembryonic mesoderm and entoderm which accompany them, have bent toward one another

#### **Reasons to select the developmental stages**

Selected hours to study of angiogenesis were 48, 55, 66, 72, 88 and 96 hrs. The hours are according to development of CAM and vitelline veins of CAM.

**A] Development 48 hrs** -At the end of second day area vasculosa surrounded by sinus terminalis. Meanwhile certain veins and arteries have extended from embryo in to the area vasculosa.

1) From the posterior end of the ductous venosus union of vessels passes outward in to the area pelucida called as omphalomesentric veins. This vein in area vasculosa gives extensions called right and left vitelline veins.

**B] Development at 55 hrs -** No allantois is formed and transitory vein develop toward the intestine.

**C] Development 66 hrs-** Before the end of the third day one other new extra embryonic vessel start to appear the posterior vitelline vein

**D] Development at 72 hrs**

The vitelline arteries reach further out into the area vasculosa than during the second day terminating near its border in network of capillaries which empty into sinus terminalis

**E] Development at 96 hrs-** By the end of the fourth day vitelline veins, such as anterior, posterior and lateral vitelline veins are well developed and are more defined.

To know details of CAM development and hence angiogenesis the literature is reviewed under following points.

### **Development of chorioallantoic membrane in birds:**

#### **The folding –off of the body of the embryo:**

In early chick embryos the somatopleure and splanchnopleure extend over the yolk peripherally, beyond the region where the body of the embryo is being formed.

From the embryological point of view all the vertebrates belong to two classes i.e. an-amniota or the amniota. The amniota as the name implies are those which possess an-amnion while the an-amniota are those which lack it. The chick as an example of the former or amniotic group. The amnion begins to form the second day of chick's incubation but, it is not completed until about the fourth day.

#### **Amnion in process of development:**

##### **Development during second day:**

During the second day a fold in the blastoderm occurs just in front of the head of the embryo in the region of proamnion since there is as yet no mesoderm in this region. The fold at first contains only ectoderm and endoderm. The mesoderm extends in this vicinity and plits in to the embryonic extension of the somatic and splanchnic layers with the extraembryonic coelomic space between them. Both these folds become involved in the fold. The splanchnic layer together with the endoderm however is withdrawn to the surface of the yolk, while the somatic layer and the extraembryonic ectoderm which covers it constitute the two permanent layers of the

amniotic head fold. The embryo now has begun to sink somewhat into the surface of the yolk, and it does so the amniotic fold gradually grows back over it. This backward growth is also accompanied by the development of lateral amniotic folds extending posteriorly on the either side. By the end of the second day the embryo has been covered in this manner almost as far back as the vitelline arteries.

#### **Development during the third day:**

At the end of the second day or at the beginning of the third day another fold appears at the posterior end of the embryo and grows forward towards the head fold. This is the amniotic tail fold which soon becomes coextensive upon either side with the posterior end of the amniotic fold. It is similar to the corresponding head fold except that from the first it contains only ectoderm and somatic mesoderm since the anterior portion of the amnion starts earlier and grows rapidly, the point at which folds finally meet and fuse is quite near the posterior end of the animal. The oval opening existing above the chick previous to the closure is amniotic umbilicus. The embryo has by this time turned upon its left side throughout the greater of its length, and inasmuch as the folds do not turn with it, the closure occurs not above its back, but above its right side. It also follows from this that the fold of the left side covers the back of the embryo as well as a part of the right side. The amnion now said to be complete.

#### **The completed amnion and related parts:**

##### **The amnion and the amniotic cavity:**

It is obvious that the amniotic fold like other folds must be composed of two many parts each part being continuous with other at the crest of the fold. It is also obvious that one of these parts that is the inner or lower one lies everywhere next to the embryo. When fusion occurs therefore this inner part will become continuous completely bounding a new cavity which surrounds the embryo at every point except for a restricted region. This continuous inner membrane is amnion and the cavity thus formed is the amniotic cavity. Moreover inasmuch as folds involved both ectoderm and mesoderm the inner membrane or amnion must likewise consist of ectoderm and mesoderm, the former lining the amniotic cavity and later forming the coat outside the lining.

##### **The Chorion :**

At the fusion of folds the outer part, like the inner necessarily becomes continuous. Likewise it consists of both ectoderm and mesoderm, but in this case, the

ectoderm lie out side and the mesoderm inside, i.e. toward the amnion . The outer membrane thus formed is called the chorion, serosa or false amnion. An important function of the mature chorion is the transport of  $Ca^{++}$  from the egg shell from the embryonic circulation, where it is distributed to the developing beak and skeleton. A direct interaction with the shell membrane is required for the maximal levels of  $Ca^{++}$  transport by the chorion (Dunn *et al*;1981). Between it and the inner membrane or true amnion; there the naturally same space which separated the inner and outer parts of the amniotic folds, i.e. the extraembryonic coelome or exocoelome .This exocoelomic space eventually becomes filled by an important sac like organ allantoises. Whose origin and structure will be described as below.

#### **The sero-amniotic connections:**

It has been implied that the extra-embryonic coelom with whatever may occupy it every where separates the amniotic membrane from the chorionic membrane. This is true except at one point. At the point of final fusion at the amniotic folds that is the amniotic umbilicus the coelomic space is interrupted by a small area of mesoderm which persists and serves to unite the above membranes. It is called the sero-amniotic connection.

#### **The amniotic fluid:**

Shortly after the completion of the amniotic cavity fluid begins to accumulate within it. Thus the embryo is soon practically surrounded a liquid cushion which protects it from pressure by its membranes and rigid shell. This is amniotic fluid. Presently about the fifth day muscle fibers develops in the mesoderm of the amnion and begin to send waves of contraction over it.

#### **The somatic umbilicus, the yolk stalk, and the yolk sac:**

##### **The somatic umbilicus:**

During the formation of amnion the gradual separation of the embryo from the yolk has been progressing. This has been accomplished by steady in-pushing of the ventral portion of the head, tail and lateral folds beneath body of the growing chick. The result is that by the time amnion is completed, these folds have approached one another quite closely though with out coming in to contact. In this manner they give rise to a short, thick, hollow stalk which connects the embryo with the yolk sac and its extra-embryonic membranes. The outermost wall of this stalk is continuous

with that of amnion and is therefore composed of ectoderm and somatic mesoderm for this reason this outer wall is referred as the somatic umbilicus.

**The yolk stalk:**

Within this wall and surrounding the inner wall of the stalk is a space continuous externally with the extra-embryonic coelom and internally with the coelom embryo itself. Finally, the inner wall of the stalk consists of splanchnic mesoderm and endoderm. It is known as yolk stalk, but it is really merely an inner tube of the somatic umbilicus separated from it by coelomic space.

**The yolk-sac:**

The wall of the yolk stalk is coextensive within the embryo with the wall of the gut, and externally with the layer of the endoderm and the splanchnic mesoderm which overlies the yolk. This layer is continually growing out around the yolk, and at its outermost border i.e. the region of the zone of junction the endodermal portion of it becomes continuous with the chorion which overlies it. Thus by means of the extension of these layers the yolk is gradually enclosed a covering, whose inner layer of splanchnic mesoderm and endoderm constitutes the yolk sac, attached to embryo by means of yolk stalk. In addition to its nutritive function the endoderm of the yolk sac is the site of the synthesis of the serum proteins (transferrin, alpha globulins and prealbumin) in the early embryo (Young, *et al* 1980) Upon the ninth day of incubation this sac has become virtually complete, save at a point on the side of the yolk postero-ventral to the body of the chick, where an opening remains known as the yolk sac umbilicus.

**The Allantois :**

Another extremely important extra-embryonic organ possessed in some degree by all amniota is the allantois .

**Early Development:**

The allantois starts in the form of an out pushing from the ventral wall of the hind-gut .This is visible before the beginning of the third day. This out pushing naturally involves the endoderm and the mesodermal ventral mesentery which occurs in this region. Thus the sac which is formed has an inner endodermal and outer mesodermal layer. By the end of fourth day the allantois has pushed out through the coelomic space between the somatic umbilicus and the yolk stalk and is beginning to spread out in the extra-embryonic coelome. The narrow neck of the organ which then connects

the outer sac like portion with the gut is known as allontoic stalk or neck. Along this stalk pass the two allontoic arteries and the single allontoic vein to end in abundant ramification over the surface of the sac. The allontoise now grows rapidly, and within a couple of days has entirely covered the amnion , occupying the space between that organ and the chorion .**Presently the amniotic and chorionic mesoderm fuse forming the chorioallontoic membrane.** Chorioallontoic membrane has been used effectively as site for grafting for the small explants from unger embryos for testing their developmental potencies. Through the chorioallontoic membrane and the shell the chick embryo takes about 5 liters of oxygen and gives of about 4 liters of carbon dioxide during the 21 day period before hatching (Wangensteen, 1972). In this manner , the above ramifications of blood vessels are brought near the shell , through which exchange of gases is possible. Thus allontoic serves as an organ of respiration in Chick during embryonic life. Its cavity also acts as a receptacle for the waste product of metabolism which are conveyed through the allontoic stalk from the region of cloaca.

#### **Selection of the problem:**

In any organism mitochondrial electron transport system also generates toxic metabolites (Philips *etal* 2003 ) and reactive oxygen species oxy and hydroxyl (Przekwas *etal* 2003) which leaks from mitochondria to cause cellular damage to all molecules to lead pathogenesis of various diseases (Kohen and Nyska, 2002 ) in developing animals also. For the management of free radicals organisms use antioxidant defense systems to protect against free radicals (Sies 1991). Thus pro-oxidants and antioxidants balance and detoxification potentially damaging reactive oxygen species is crucial for cellular homeostasis causing stress which can lead to stress leading to pathogenesis. Which also true incase of development of organism. In this light Ahmed (2005) had suggested balancing of these systems to get rid of stress and continued with normal development. Many of the pathogenic studies reviewed further also direct the same but to verify the homeostasis it needed to be shifted in free radical stress direction and compensating it with the antioxidant system so that the homeostatic process can be understood.

In this direction in present project naturally producible free radicals generating hydrogen peroxide was used to create free radicals stress and to scavenger the free radicals naturally used Vitamin C was used as an antioxidant and its influence was

studied on CAM angiogenesis ( CAM is directly related to the to the nourishment of embryo).The oxidative stress was measured by studying lipid peroxidation and HCHO and antioxidant activity was measured by glutathione and protein content in addition to mortality and abnormality in development studies.

Thus to understand the different parameters and their metabolisms in detail they are received in following pages.

#### **Free radicals :**

ROS can be formed in the heart by a variety of mechanisms, including generation during oxidative phosphorylation in the mitochondria as a byproduct of normal cellular aerobic metabolism (Davis 1995, Idet, *et al.* 1999). Thus, the major process from which the heart derives sufficient energy can also result in the production of ROS (Ide *et al* 1999).ROS can be formed in the heart, and other tissues, by several mechanisms; they can be produced by xanthine oxidase (XO), NAD(P)H oxidases, cytochrome P450; by autooxidation of catecholamines; and by uncoupling of NO synthase (NOS) (Seshiah *et. al* 2002, Sawyer *et al* 2002). NO contains an unpaired electron, and under certain conditions can react with  $O_2^{\cdot-}$  to form peroxynitrite ( $ONOO^{\cdot-}$ ), a powerful oxidant. ROS formation in the heart can be induced by the action of cytokines and growth factors as well. Angiotensin II (ATII), PDGF, and TNF- $\alpha$ , for example, can induce  $H_2O_2$  and  $O_2^{\cdot-}$  formation via activation of the NAD(P)H oxidases ( Seshiah *et. al* 2002,Thannickal *et al* 2000). This NAD(P)H-dependent pathway is best described in vascular smooth muscle cells but has also been documented in other cell types, including cardiomyocytes (Bendall *et al* 2002 Sabri *et al* 2003 ). A number of additional ligands have been associated with the induction of ROS, including several with particular relevance to the cardiovascular system ( Thannickal *et al.*2000 ).

There are several cellular mechanisms that counterbalance the production of ROS, including enzymatic and nonenzymatic pathways (Nordberg *et al* 2001). Among the best-characterized enzymatic pathways are catalase and glutathione peroxidase, which coordinate the catalysis of  $H_2O_2$  to water, and the superoxide dismutases (SODs), which facilitate the formation of  $H_2O_2$  from  $O_2^{\cdot-}$  (Kirkman *et al* 1984). Thioredoxin and thioredoxin reductase together form an additional enzymatic antioxidant and

redox regulatory system that has been implicated in a wide variety of ROS-related processes (Nordberg *et al* 2001). Thioredoxin and thioredoxin reductase can catalyze the regeneration of many antioxidant molecules, including ubiquinone (Q10), lipoic acid, and ascorbic acid, and as such constitute an important antioxidant defense against ROS. (Cornad *et al* 2004). It is observed that massive oxygen supply results in increased formation in mitochondrial ROS production. However, it also seen that hypoxia can lead to reductive stress, which also results in increased ROS production by the mitochondrial electron transport system (Mohanraj *etal*; 1998). This is believed that ROS is generated at complex I and complex III of the electron transport chain. During hypoxia, less O<sub>2</sub> is available to be reduced to H<sub>2</sub>O at cytochrome oxidase, causing accumulation of reducing equivalents within the mitochondrial respiratory sequence. This called as reductive stress, which leads to ROS formation by the auto-oxidation of one or more mitochondrial complexes such as the ubiquinone-ubiquinol redox couple. (Khan and Brian 1995)

**Biological significance of Reactive oxygen species :**

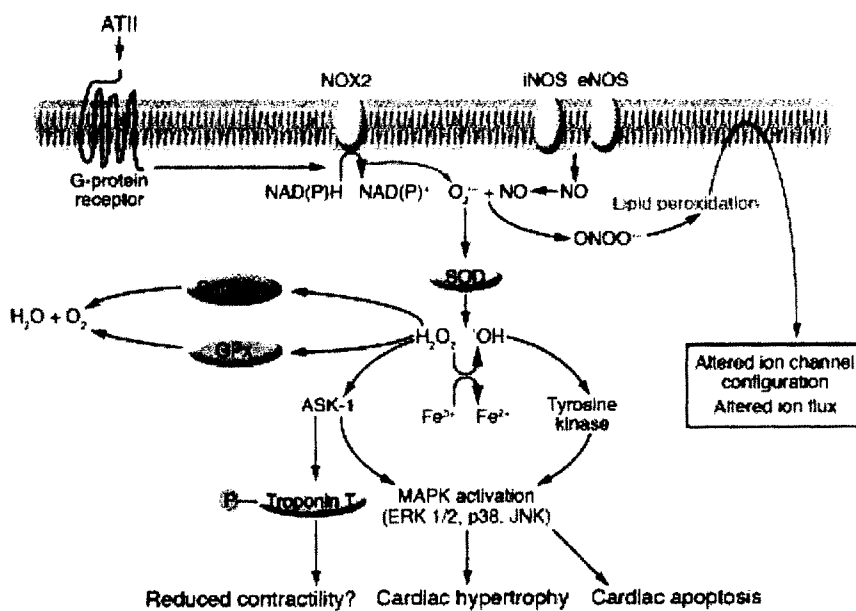


Fig several pathways by which ROS can mediate biological effects germane to the cardiovascular system

ROS can alter the structure and function of cardiac muscle. The mechanism indicating effect of ROS on cardiac muscle is first ATII binds a G-protein-associated receptor, initiating a cascade of events that involves activation of  $O_2^-$  production by the NAD(P)H oxidase NOX2.  $O_2^-$  is converted by SOD into  $H_2O_2$  and  $^{\cdot}OH$  that mediates activation of MAPKs via a tyrosine kinase. MAPK activation can lead to cardiac hypertrophy or to apoptosis. The ROS that is generated can also signal through ASK-1 to induce cardiac hypertrophy, apoptosis, or phosphorylate troponin T, an event that reduces myofilament sensitivity and cardiac contractility. NO production by the NO synthases iNOS and eNOS can interact with  $O_2^-$  to form  $ONOO^-$ .  $ONOO^-$  can cause lipid peroxidation, an event that can alter ion channel and ion pump function. Catalase and glutathione reductase (GPx) are shown as enzymatic pathways to produce water and oxygen from  $H_2O_2$ .

#### **Oxidative Stress :**

Reactive oxygen / nitrogen species (ROS/RNS) and free radicals are constantly formed in the human body and removed by an antioxidant defense system. A certain amount of ROS / RNS production is, in fact, necessary for proper health; for example ROS / RNS help the immune system to eliminate microorganisms. The generation of ROS / RNS must be approximately in balance with antioxidant defense. An imbalance between ROS / RNS and antioxidant defenses in favor of the former via excessive production of ROS / NOS, loss of antioxidant defenses, or both, has been described as oxidative / nitrosative stress (Halliwell 1993, Dalle 2006). ROS are generated continuously within the vasculature of air-breathing organisms, and this process may accelerate as a consequence of ischemia, reperfusion, arterial balloon injury, angiotensin II binding to receptors on vascular endothelial and smooth muscle cells, and leukocyte-mediated inflammation of atherosclerotic plaques (Halliwell 1991). Similarly production of ROS / RNS exceeds beyond the antioxidant defenses, then excess species react with all classes of biological molecules, including lipids, proteins, and nucleic acid bases which can severely affect cell structure and viability. Especially cellular targets such as thiols, proteins and lipids, many of them have special roles for cellular signaling, are affected by increased ROS / RNS. Consequently, when ROS / RNS production is increased a variety of cellular

responses through generation of secondary reactive products result in cell death by necrosis or by Apoptosis (Yucel *et al* 2006). Reactive oxygen species (ROS) leak from mitochondria into the cytoplasm where they cause cellular damage by oxidizing a variety of biologically important molecules, including DNA, proteins, lipids, and carbohydrates( Prekwas *etal* 2003).

### **Free radicals and antioxidants:**

Free radicals are generally reactive oxygen species or nitrogen species e.g. hydrogen peroxide, hydroxyl radical, nitric oxide, peroxinitrite, singlet oxygen, superoxide anion and peroxy radical. Free radicals such as reactive oxygen species are formed during variety of biochemical reactions and cellular functions (such as mitochondrial metabolism). Once formed they lead to various reaction byproducts that can damage the cells (e.g. Lipid peroxidation). Excess free radical formation is associated with many diseases like inflammation, poor blood flow, degenerative diseases in toxin exposures among other mechanisms all lead to oxidative stress. Oxidative stress results from an imbalance between formation and neutralisation of prooxidants. The steady state formation of prooxidants is normally balanced by similar rate of consumption by antioxidants.

Free radical is any species that has one or more unpaired electrons these includes hydrogen atom (one unpaired electron) most transition metals and the oxygen molecules itself. O<sub>2</sub> has two unpaired electrons each located in different  $\pi^*$  anti bonding orbital. These two electrons have opposite spin quantum number and so if O<sub>2</sub> attempts to oxidize another atom or molecule by accepting a pair of electrons from it. They must be with parallel spin impose restriction on oxidation by O<sub>2</sub> which tend to make O<sub>2</sub> accepting electron are at a time and shows its reaction with the non- radical species. ( Halliwell and Gutteridge, 1984 ). Antioxidants are molecules or compounds that act as free radical scavengers. Most antioxidants are electron donors and react with the free radicals to form innocuous end products such as water. These antioxidants protect against oxidative stress and prevent damage of cells.

### **Free radicals, Antioxidants and angiogenesis :**

Free radicals are constantly formed in the body (Halliwell and Gutteridge 1984). Exogenous ROS stimulates induction of VEGF by various cell types including

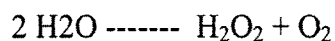
vascular smooth muscle cells ( VSMCS) (Ruef *et al* 1997) and ES (Chua *et al* 1998 ) and promote cell proliferation and migration ( Stone and Collins 2002,Luckzak *et al* 2004,Yasuda *et al* 1999 ) . The ROS and NADPH oxidase inhibits angiogenesis in chick chorioallantoic membrane(84)

The effect of ROS derived from ECS are highly regulated and dependant on the amount and and site of production as well as the balance of prooxidant and antioxidant enzyme activity. It is likely that low level of ROS play an important role in reparative angiogenesis in response to ischemia and wound healing ( Tojo *et al* 2005, Roy *et al* 2006 ) Nitric oxide play an important role in VEGF signaling and postnatal angiogenesis ( Murohara and Asahara 2002 ) .

lipoic acid, and ascorbic acid, and as such constitute are an important antioxidant defense against ROS(Conrad *et al* 2004)

#### **Reasons to use H<sub>2</sub>O<sub>2</sub> as free radical inducing agent:**

Hydrogen peroxide is generated in the animal body in many oxidative metabolisms ( Halliwell and Guttridge 1984 ).Hydrogen peroxide is generated in the body from superoxide dismutase from O<sub>2</sub> ( Ahmed 2005).Super oxide dismutase catalyses the conversion of super oxide anion radical to H<sub>2</sub>O<sub>2</sub>



ROS generates free radicals and and cause damage to lipid , protein , and nucleic acid in cell (Prekwas *et al* 2003 )There fore H<sub>2</sub>O<sub>2</sub> was used to as a generater of free radicals which induce oxidative stress .Which also use full to study ROS mediated effect on angiogenesis of chick during different developmental stages of the CAM .

#### **Generation of H<sub>2</sub>O<sub>2</sub> in the body:**

Each oxygen atom contains 2 unpaired electrons in its outermost shell. Atoms or molecules with unpaired electrons are designated free radicals and are highly reactive entities that can readily participate in a variety of chemical/biochemical reactions. Molecular oxygen, O<sub>2</sub>, is characterized as diradical, a property that allows liquid oxygen to be attracted to the poles of a magnet. This property also dictates that full reduction of oxygen to water as a terminal event in the electron transport chain requires 4 electrons. The sequential donation of electrons to oxygen during this process can generate ROS as intermediates, and “electron leakage” can also contribute to the formation of ROS (Davis 1995,Miwa *et .al* 2003 Genova *et .al* 2003 ) . Donation of a single electron to molecular oxygen results in the formation of the

superoxide radical ( $O_2^{\cdot-}$ ). Donation of a second electron yields peroxide, which then undergoes protonation to yield hydrogen peroxide ( $H_2O_2$ ). Donation of a third electron, such as occurs in the Fenton reaction ( $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$ ), results in production of the highly reactive hydroxyl radical ( $\cdot OH$ ). Finally, donation of a fourth electron yields water. Singlet oxygen ( $^1O_2$ ), a very short-lived and reactive form of molecular oxygen in which the outer electrons are raised to a higher energy state, can be formed by a variety of mechanisms, including the Haber-Weiss reaction ( $H_2O_2 + O_2^{\cdot-} \rightarrow \cdot OH + OH^- + ^1O_2$ ) (Toufkstain *et al* 2001).

#### **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 was shown in table 2. these doses were given according to Mehendel *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  $H_2O_2$  showed 20% mortality and highest dose used i.e. 1.5m M  $H_2O_2$  showed 100% mortality while 0.5 mM dose showed 50% mortality there fore this dose was selected for the further experimental studies so as to study the teratogenic effects if any with this sub lethal dose.

#### **Vitamin C as free radical scavenger:**

Vitamin C (Ascorbic acid) was first isolated by Hungarian biochemist (1928) and Nobel prize winner Szent-Gyorgyi. Vitamin C is water soluble and serves as an key immune system nutrient and potent free radical scavenger. This double duty nutrient many illness like common cold to cancer.

Vitamin C protects the DNA of the cells from the damage caused by free radicals and the mutagens. It prevents the genetic alterations within the cell and protects the lymphocytes from mutations to chromosomes. Vitamin C is important in this day and age because it protects body from environmental pollution such as toxins including Ozone, Carbon Monoxide, Hydrocarbons pesticides and heavy metals (Gaby and Singh 1991). Vitamin C protects the body by preventing the development of nitrosamines, the cancer causing chemicals that stem from nitrates

contained in many foods ( Gaby, p) Free radicals damage in lungs and in even in central nervous system is prevented by Vitamin ( Kronhausen p.104 ).In guinea pigs ascorbic acid treatment effectively diminished the acute lung damage caused by the introduction of super oxide anion free oxygen radicals to the trachea ( Becher and winsel 1989 ) . As ascorbic acid is water soluble in can work both inside and out side the cells to combat free radical damage. Free radical will seek out an electron to regain their stability. Vitamin C is an excellent source of electron it donate an electron to free radicals such as hydroxyl and sueproxide radicals and quench their reactivity ( Bendich 1990 ) .

While antioxidants play role in protecting cells from the action of reactive oxygen species by reducing chemical radicals and preventing the process of lipid peroxidation ( Nishigaki, 1992 . Yu 1994 ) Supplement of Vitamin C and E Zinc and chromium in feed can attenuate the side effects of inflicted at the extreme environmental stress ( Kafri and Cherry 1984 . Mc dowell .1989). vitamins C, E, and *beta*-carotene, proanthocyanidins, anthocyanins, and/or protease inhibitors, and also compounds that might protect the organism against free radical induced injury and diseases (, Harborne *et al* 2000 ,Tapiero *et al* 2002).Similarly antioxidants function as inhibitors at both initiation and promotion/propagation/transformation stages of tumor promotion/carcinogenesis, and protect cells against oxidative damage. Antioxidants are potent scavengers of free radicals and serve as inhibitors of neoplastic processes ( Bagachi *et al*, 1996).

#### **Reasons to select doses of vitamin C:**

For the management of oxidative stress induced by free radicals generated due to Hydrogen peroxide (0.5m M) during development of CAM 3 mg Vitamin C dose was selected .Which is known to improve hatchability in broiler egg ( Ipeck *etal* 2004).Besides these dose 4 mg and 5 mg doses of Vitamin C were also used but were ineffective to scavenge the free radicals .

#### **Lipid peroxidation :**

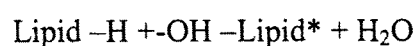
Lipid peroxidation is the oxidative degradation of lipids of cell membranes and that of the cell organelle membrane resulting in cell damage. Free radicals or ROS can damage the cell membrane and the membrane of cell organelles eg. Lipid peroxidation (Rehaman 2003 Konat 2003). Oxidative damage of molecules, e.g. by

lipid peroxidation, is mainly caused by the highly toxic hydroxyl radical that is generated by the hyperhomocysteinemia or by the iron-catalyzed Fenton and Haber-Weiss reactions (Linpisarn *et al.* 1991,).

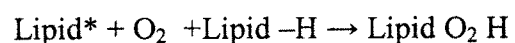
Lipid peroxidation is process where unsaturated lipid material undergoes reaction with molecular oxygen to yield lipid hydroperoxide . Reaction of singlet oxygen can also give lipid hydroperoxide by non radical non chain process .Lipid peroxidation is a potential mechanism for the amplifying the free radical process .The reaction involved in the formation of the lipid hydroperoxides and operation of cellular antioxidant mechanism against this form of membrane injury have been well characterized ( Tribble *etal* 1987 Farber *etal* 1990 ).Malonaldehyde is product of lipid peroxidation *in vivo*. It is identified during the products of oxidative decomposition of the amino acids complex carbohydrates , pentoses and hexoses formed in the presence of metal catalyst as a product of free radical generated *in vivo*. and is taken as indicator of lipid peroxidation .( Bird and Draper 1984 ).

#### **Initiation of lipid peroxidation :**

Lipid peroxidation is initiated by any species having specific reactivity to abstract hydrogen atom such as hydroxy radical .Since hydrogen atom has has one unpaired electron this leaves behind an unpaired electron on the carbon atom .The carbon radical in poly unsaturated fatty acid tends to stabilize by a molecular rearrangement to produce a conjugated diene, which rapidly reacts with O<sub>2</sub> to give hydroperoxyradicals. Hydroperoxyradical abstract hydrogen atoms from other lipid molecules and so continue the chain reaction of lipid peroxidation .The hydroxyl radicals combine with the hydrogen atom to give lipid peroxide (Haliwell and Gutteridge ,1984 ).



After molecular rearrangement



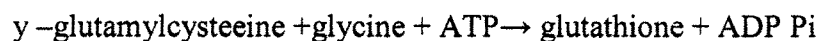
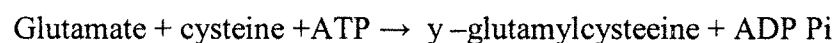
Reactive oxygen species (ROS) leakage from mitochondria into cytoplasm cause cellular damage by oxidizing variety of molecules including DNA, Proteins, Lipids and carbohydrates. Lipid and protein oxidation also play an important role in pathogenesis of variety of diseases (Przekwas 2003). The radicals generated which initiates lipid peroxidation can damage protein or lipid directly. These reactions are more important in the production of cell injury than peroxidation of lipids. The combined effect of protein and DNA oxidation, with lipid peroxidation after a more general mechanism for cell injury by free radical processes (Trible *et al* 1987).

### **Glutathione :**

Glutathione is an endogenous antioxidant produced by the cell. Glutathione participates directly in the neutralization of free radicals, reactive oxygen species and maintains endogenous antioxidants such as Vitamin C and E in their reduced forms. Glutathione is also important component of the immune system.

### **Synthesis of Glutathione:**

The simplest tripeptide Glutathione of animal tissue which serves as component of an amino acid transport system. It is an activator of certain enzymes and protection of lipids against auto oxidation. Glutathione is non essential nutrient which is synthesized from L-cysteine, L-glutamate and glycine. It is synthesized in two adenocine triphosphate dependent steps.



First, gamma-glutamylcysteine is synthesized from L-glutamate and cysteine via the enzyme gamma-glutamyl cysteine synthetase. This reaction is rate limiting step in glutathione synthesis. Second, glycine is added to the C-terminal of gamma-glutamylcysteine via the enzyme glutathione synthetase.

Glutathione occurs intracellular and is major fraction of transpeptidase which is on external surface of cell membranes. Glutathione is transported across the cell membranes interact with gamma-transpeptidase and gamma-amino acids are formed

which are transported in to the cell .Glutathione participates in the neutralization of free radicals and maintains exogenous antioxidants such as Vitamin C and E in their reduced form .In direct conjugation glutathione plays arole in detoxification of many xenobiotics both organic and inorganic .Glutathione is an important of human immune response. Mechanism of process of immune enhancement include optimizing macrophage function ,offsetting oxidative damage associated with lymphocyte monoclonal expansion ,and stabilizing the mitochondrial membrane there by ,reducing apoptosis in lymphocyte . Glutathione depletion increases the oxidative damage to proteins lipids and DNA and causes cell death (Du *et al* 2008 ).Glutathione is major neuronal antioxidant which is important for detoxification of  $H_2O_2$  and prevention and repair of peroxidative damage to lipids proteins and nucleic acids (Du *et al* 2008 ).

#### **Formaldehyde :**

Generation of free radicals in the body causes toxic effects which causes then generation of formaldehyde in the body .There for in present condition HCHO alterations were studied .Here literature on formaldehyde is reviewed.

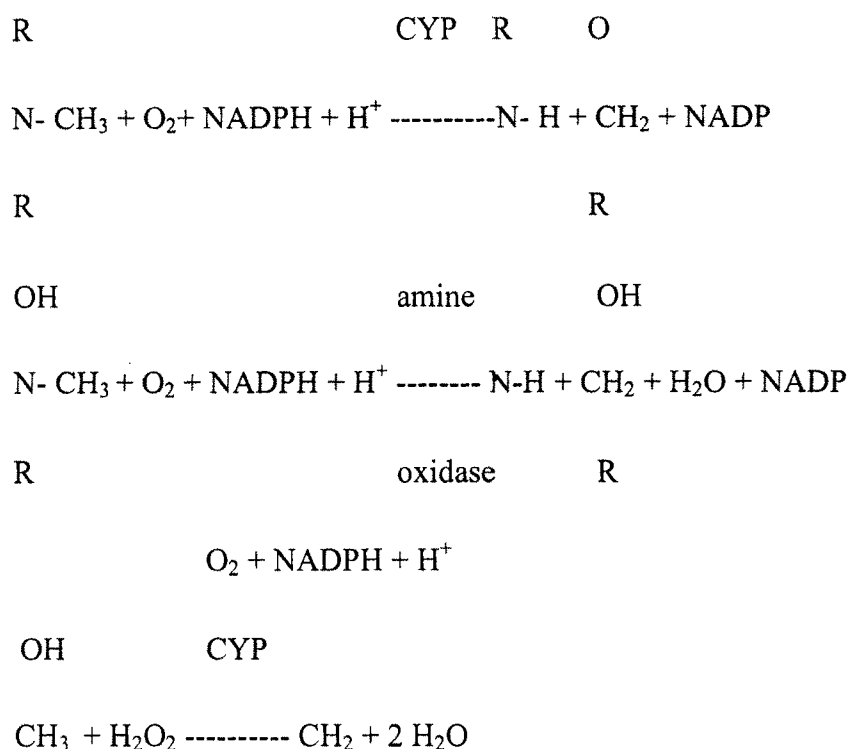
#### **Formaldehyde and development :**

Formaldehyde is a chemical compound with the formula HCHO. It is the simplest aldehyde—an organic compound containing a terminal carbonyl group.Stimulationof adrenaline secretion in the chronic stress causes increase in the level of circulatory formaldehydeand endothelial cells are sensitive to the formaldehyde and resistant to  $H_2O_2$  (Peter *et al* 2004 ).Elevated  $H_2O_2$  rproduction by certain p 450 isozymes , e.g ., p 450 IIE 1, may contribute to enhanced rate of glycerol oxidation because the  $H_2O_2$  nonheme iron are required for oxidation of glycerol to formaldehyde (Clejan and Cederbaum, 1991 ).Increased in the level of formaldehyde is the indicates pathophysiological process (Kalasz ,2003 )

A variety of NADPH and oxygen dependant reactions catalysed by the microsomal fraction produces formaldehyde .The most common and frequently studied reaction resulting in liberation of formaldehyde is the end demethylation of secondary and tertiary amines .These reactions are mediated byCytochrome p 450 the

terminal oxidase of the microsomal mixed function of oxygenase system (Cooper *et al* ., 1965., Paulson *et al*, 1974).

The reaction is depicted in the following equation .



Formaldehyde and ROS are cytotoxic ,potentially carcinogenic , which decreases the cell viability of jurkat cells dramatically *in vitro* .Formaldehyde alone can induce cell death (Saito *et al* ., 2005 ).The reactive oxygen species intermediates can cause direct cell injury by lipid peroxidation and protein peroxidation and damage to nucleic acids (Takeda *et al* , 1984 ).formaldehyde is the product of lipid peroxidation and used as the marker of the developmental oxidative stress in tissue and and plasma during ischemia and reperfusion syndrome in rat brain (Maboudou *et al* ., 2002 ).

Thus using the experimental protocol defined in Chapter II for the reasons given in this Chapter the experimental work of the project was conducted in the departmental laboratory.

The results obtained were analyzed using statistical analysis given under chapter II and the project is presented as following Chapters.

## **Chapter I – Introduction**

Under this Chapter the problem is defined with the theoretical approach behind it. Besides selection of animal model of angiogenesis doses of H<sub>2</sub>O<sub>2</sub> and Vitamin C, experimental schedule and parameter studies are reasoned. Additionally reviews of relevant parameters and associated influences and processes have been provided.

## **Chapter II – Material and method**

The Chick model is described. The experimental schedule that includes CAM exposure to H<sub>2</sub>O<sub>2</sub> and Vitamin C was performed by window method. Experimental protocol is provided in detail along with dose administration along with details of each of the experimental group. The angiogenesis studies associated quantitative studies are provided along with methods used for the bioassay of the parameters studied viz, methods of bioassay of proteins, lipid peroxidation, HCHO and Glutathione are provided.

Statistical methods used to valuate the observations have been provided.

## **Chapter III – Observation and Discussion**

In Chapter III observations and discussion results of the experimental studies conducted are presented under following sections each section is provided with the discussion of the respective parameter.

### **Section I – Mortality**

Different doses of H<sub>2</sub>O<sub>2</sub> and Vitamin C with relation to mortality and abnormality were studied and analysed 0.5 mM H<sub>2</sub>O<sub>2</sub> dose and 3 mg Vitamin C dose were selected for study of remaining parameters.

Section II – CAM - Angiogenesis – The alterations under experimental conditions are evaluated quantitatively under following categories.

- i) Primary vitelline veins and area covered

- ii) Secondary vitelline veins and area covered
- iii) Tertiary vitelline veins and area covered

The alterations are related with the oxidative stress of H<sub>2</sub>O<sub>2</sub> and antioxidant influence of Vitamin C and discussed with the relevant literature.

#### Section 3- CAM - Protein

protein were assayed from CAM and the results are discussed with relevant literature.

#### Section 4 – Lipid peroxidation

Malonaldehyde the TCA-TBA reactive species produced on lipid peroxidation assayed. Results are discussed with relevant literature.

#### Section 5-CAM- Glutathione

Glutathione bioassay provided status of *in vivo* antioxidant system. Its relevance is valued with antioxidant Vitamin C which was introduced experimentally. Results are discussed with other parameters and relevant available literature.

#### Section 6-CAM- Formaldehyde

Formaldehyde is the product of the microsomal metabolism. The alterations in present project are evaluated in the relevant Lipid peroxidation, glutathione and mortality change.

The results are discussed with the relevant literature.

### **Chapter IV – General Discussion**

Alterations in all the parameters studied are considered together to evaluate the Vitamin C mediated impact on H<sub>2</sub>O<sub>2</sub> treated Chick embryo CAM.