EFFECTS OF STRESS ON TESTICULAR STEROIDOGENESIS
AND ROLE OF ANTIOXIDANT SUPPLEMENTATION

By

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MBBS, M.Phil

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In

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(2012)
Dedicated to my beloved parents
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Dr Ghulam Mustafa Lodhi
August 08, 2012
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<td>Adult Leydig cells</td>
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<tr>
<td>3β-HSD</td>
<td>3β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
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<tr>
<td>AA</td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>AA-CoA</td>
<td>Arachidonyl-CoA</td>
</tr>
<tr>
<td>AAS</td>
<td>ascorbic acid supplementation</td>
</tr>
<tr>
<td>AChE</td>
<td>acetyl cholinesterase</td>
</tr>
<tr>
<td>ARTISISt</td>
<td>Arachidonic acid -related thioesterase</td>
</tr>
<tr>
<td>AT</td>
<td>Alpha tocopherol</td>
</tr>
<tr>
<td>ATS</td>
<td>alpha tocopherol supplementation</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine – vasopressin</td>
</tr>
<tr>
<td>B₀</td>
<td>Absorb of Blank</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine mono phosphate</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CREAM</td>
<td>centre for research in experimental and applied medicine</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response-element binding protein</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>Dibutryl cyclic adenosine mono phosphate</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
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<td>Abbreviation</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagles Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOPA</td>
<td>dihydroxyphenylalanine</td>
</tr>
<tr>
<td>E</td>
<td>Epinephrine</td>
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<tr>
<td>EIA</td>
<td>Enzyme immune assay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immune sorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>Eagles medium</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FLCs</td>
<td>Fetal Leydig cells</td>
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<tr>
<td>GnRH</td>
<td>Gonadotrophic releasing hormone</td>
</tr>
<tr>
<td>GREs</td>
<td>glucocorticoid responsive elements</td>
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<tr>
<td>hCG</td>
<td>Human Chorionic gonadotropin</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>HEPES</td>
<td>Hydroxy ethyl piperazine ethane sulphonic acid.</td>
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<tr>
<td>HPA axis</td>
<td>hypothalomo-pituitary-adrenal axis</td>
</tr>
<tr>
<td>HPT</td>
<td>Hypothalomo-pituitary testicular axis</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IBMX</td>
<td>Iso butryl methyl Xanthine</td>
</tr>
<tr>
<td>LC</td>
<td>locus ceruleus</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
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<tr>
<td>MDA</td>
<td>malondialdehyde</td>
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<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NUST</td>
<td>National University of Sciences and Technology</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Solution</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC pathway</td>
<td>Protein kinase C pathway</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase A2</td>
</tr>
<tr>
<td>PUFAs</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PVN</td>
<td>Para ventricular nuclei</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended daily allowance</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>SER</td>
<td>Smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone-binding globulin</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>StAR protein</td>
<td>Steroid acute regulatory protein</td>
</tr>
<tr>
<td>TBA</td>
<td>Thio Barbituric Acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid reacting substance</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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<td>TMB</td>
<td>Tetra methyl benzidin</td>
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Summary

Stress is a widespread problem nowadays. Stress of various origins suppresses male reproductive functions through release of stress hormones. Various aspects of hormonal mechanisms affecting testicular steroidogenesis are unclear. Catecholamines increase steroidogenesis and corticosterone decreases it, but net effect of stress is considered to be decreased testosterone. How these two hormones affect the testosterone production is investigated in this study. Stress is thought to disrupt the balance between prooxidants and antioxidants by generating the reactive oxygen species. Thus stress damages the testicular tissue and decreases steroidogenesis. Antioxidants, ascorbic acid and alpha tocopherol are thought to protect the body against stress induced damages. Whether these antioxidants confer protection against stress is investigated in this study.

The present study, comprised of in vivo and in vitro components, was designed to investigate the effects of stress on testicular steroidogenesis, lipid peroxidation and antioxidant enzyme activity. Also the preventive role of antioxidants, ascorbic acid and alpha tocopherol on stress induced derangements was investigated.

The study was carried out at department of Physiology, Army Medical College Rawalpindi, Pakistan in collaboration with National Institute of Health Islamabad. In vivo part of study comprised 80 male Sprague Dawley rats, which were divided into five groups with each group comprising of 16 rats. Group I was control group, fed non supplemented normal standard diet. Group II to Group V were exposed to acute immobilization stress in a mesh wire restrainer for 6 hours. Group II rats were given normal standard rat diet without any supplementation. Group III was fed normal standard diet. Ascorbic acid supplementation was given as 500 mg
ascarbic acid per liter drinking water. Group IV was fed alpha tocopherol supplemented diet with 300 mg alpha tocopherol/kg chow + 2% soyabean oil. Group V was fed alpha tocopherol supplemented diet as well as ascorbic acid supplementation in drinking water. The levels of testosterone, corticosterone, norepinephrine, luteinizing hormone, malondialdehyde and superoxide dismutase were measured.

Also the in vitro model of isolated and cultured Leydig cells of male Sprague Dawley rats was set to investigate the effects and mechanism of action of stress hormones corticosterone and nor epinephrine and the effects of antioxidants, ascorbic acid and alpha tocopherol separately and combined in the presence of stress hormones at Leydig cell level. Purified Leydig cells were incubated with corticosterone, nor epinephrine and antioxidants ascorbic acid and alpha tocopherol in the presence of LH. The testosterone production and extent of lipid peroxidation and superoxide dismutase activity were evaluated at cell level. Data were analyzed by SPSS version 17.

The data of this study indicate that acute restraint stress decreased testosterone and increased corticosterone and norepinephrine levels. LH remained unchanged in all the study groups. Lipid peroxidation increased while super oxide dismutase decreased by stress.

In vitro findings suggest that corticosterone in acute stress decreases the Leydig cell steroidogenesis through non genomic rapid pathways. It may decrease the production of cAMP or may inhibit the enzymes like 17 α hydroxylase or C-17-20 lyase. However, nor epinephrine increases the testosterone synthesis at Leydig cell
level and it may affect the cAMP or the enzymes involved in testosterone synthesis.

*In vivo*, the stress induced increase in nor epinephrine may contribute towards decline in testosterone synthesis probably by causing vasoconstriction and inhibiting the LH access to Leydig cell or by activating the direct hypothalamo-testicular axis which upon activation is thought to inhibit testosterone synthesis by Leydig cells.

Among anti-oxidants, ascorbic acid has shown favorable preventive effects on stress only in *in vitro* part of our studies, while alpha tocopherol prevented fall in testosterone and rise in malondialdehyde levels, both *in vivo* and *in vitro*. Combination of antioxidants, ascorbic acid and alpha tocopherol shown synergistic effect in reducing oxidative stress and prevented stress-induced derangements in testicular steroidogenesis both *in vivo* and *in vitro*.

Therefore, it is concluded from the findings of this study that the antioxidants ascorbic acid and alpha tocopherol decrease the stress induced lipid peroxidation and the resultant membrane stabilization prevented stress induced decline in testicular steroidogenesis.

**Key words**: Acute restraint stress, testosterone, corticosterone, nor epinephrine, ascorbic acid, alpha tocopherol.
INTRODUCTION AND REVIEW OF LITERATURE

In order to maintain homeostasis, along with other systems of body, the reproductive system also contributes by generating new individuals to replace the dying individuals and thus ensures continuity of life, as narrated by great physiologist Arthur C Guyton (Guyton and Hall, 2011). It is known centuries ago that fertility and sexuality are related to the functions of gonads. Significant knowledge about anatomy and physiology of gonads was established about 1500 years ago while about 100 years ago the relationship between gonads and pituitary was recognized (Lindholm and Nielsen, 2009).

1.1 Testis and Leydig cells

The word testis means, ‘witness’ and it was named by De Graaf, because testis provide evidence of virility, as men with testis are thought to be capable of generating offspring’s

Two important cells of testis are Leydig cells and Sertoli cells. In 1850 Franz Leydig described that cells are present in testicular interstitium. Those cells were named as Leydig cells while Enrico Sertoli found cells in the tubules which were named after his name (Lindholm and Nielsen, 2009).

In males, the interstitial cells of Leydig, which are the major sources of sexual steroid hormone testosterone (Wang et al., 2009). Leydig cells arise from interstitial mesenchymal tissue between the tubules during the eighth week of intrauterine life. They are located in the connective tissue between the seminiferous tubules (Sikka and Wang, 2008).

In rat’s testis, two different types of Leydig cells have been differentiated, which are fetal Leydig cells (FLCs), and adult Leydig cells (ALCs). ALCs are
abundant in smooth endoplasmic reticulum and have few rough endoplasmic reticulum. Mitochondria are present as tubulovesicular cristae, while Golgi apparatus is large and well differentiated. Also there are present few lipid droplets with a diameter of about 0.5 \text{um}. Nuclei are elliptical or round and cell surface has few small protrusions, while basal laminae usually absent in ALCs (Haider \textit{et al} 1995; Kuopio \textit{et al} 1989 a, b).

In Leydig cells of rat, the typical mitochondrion is 0.35 \text{um} in diameter while length is 2.4 \text{um}. The average Leydig cell has about 622 mitochondria. The inner mitochondrial membrane which is the site of enzymes that regulate the testicular steroid synthesis has a surface area 1.8 times larger than the outer membrane of the mitochondrion, but its surface area is less than smooth endoplasmic reticulum (SER) that has the majority of steroidogenic enzymes by a factor of 3.6 (Mori and Christensen, 1980). At least 30 or more enzymes that may control the cholesterol synthesis are located on the SER of Leydig cells (Christensen, 1975).

An important organelle in Leydig cells is the smooth endoplasmic reticulum (SER), because most of the steriodogenic enzymes in Leydig cells are bound to membranes of the SER. It has a surface area, which is about 6.9 times the plasma membrane which is about 60\% of the total membrane area of the cell (Mori and Christensen, 1980).

Majority of the enzymes that regulate the testicular steroidogenesis are in bound form with SER of Leydig cells. Some enzymes are also positioned on the inner membrane and cristae of mitochondria. Acetyl-CoA, an enzyme present within mitochondria, testosterone synthesis is catalyzed by those enzymes which
Figure 1.1: Overview of percentage of cell organelles and cell composition in Leydig cells.
(Adopted from Mori and Christensen, 1980)
are bound to SER. Therefore SER and inner mitochondrial membranes are of primary importance (Mori and Christensen, 1980). Important requirements for adequate steroid biosynthesis include adequate membrane potential within the mitochondria, sustained ATP production by mitochondria and maintenance of suitable pH within mitochondria. It means optimally functioning mitochondria are required for acute Leydig cell steroidogenesis (Allen et al., 2006).

The ability of testosterone production by Leydig cells is proportionate to amount of the smooth endoplasmic reticulum within these cells (Zirkin et al., 1982). Blockage of hypothalamo-pituitary testicular axis (HPT) axis with gonadotropin releasing hormone (GnRH) antagonist not only results in Leydig cells atrophy, but also decline in quantity and distribution of SER in Leydig cells along with reduction in number of mitochondria and lipid droplets (Prince et al., 1998). Leydig cells are present in interstitium of testis in such a way that they are usually widely separated from blood vessels, therefore the steroids secreted from Leydig cells diffuse through surrounding loose connective tissue before reaching the blood vessels (De Kretser and Kerr, 1994).

Regulation of Leydig cells functional activity is accomplished by luteinizing hormone (LH), which binds to LH receptors which are located on the Leydig cell membrane (Catt and Dufau, 1976). It is reported that amount of LH reaching Leydig cells is about one tenth that of circulation. This reduction is due to impediment of hormone by endothelial cells. It means Leydig cells are extremely sensitive to actions of LH or endothelial cells might modulate the actions of LH (Satchell, 2003). It has been estimated that there are about 20,000 LH receptors on each Leydig cell (Conn et al., 1977).
1.2 Steroidogenesis:

In order to maintain homeostasis and continue reproductive function in males, steroid hormones are synthesized and released by steroid producing cells of testis, adrenals and brain. Trophic hormones control the regulation of steroidogenesis in minutes to hours (Stocco et al., 2005).

Leydig cells are stimulated by luteinizing hormone that is secreted in a pulsatile fashion into circulation by pituitary gland in response to gonadotropin releasing hormone from hypothalamus. A negative feedback mechanism operates here in hypothalamo pituitary gonadal axis, by which increased levels of testosterone in circulation inhibit release of GnRH from hypothalamus and LH from pituitary (Bremner et al., 1993; Ellis and Desjardins, 1983). The process of steroidogenesis in Leydig cells is regulated by various endocrine, autocrine and paracrine factors (Rommerts et al., 2001). Along with above factors, microcirculation in the testis is an important determinant for the regulation of both steroidogenesis and gametogenesis. The microvasculature permits exchange of oxygen, nourishment and steroid hormones between the steroidogenic and gametogenic sections of the testis freely (Ergun et al., 1994).

1.2.1 Role of cholesterol in steroid synthesis

Cholesterol is principal precursor for synthesis of all steroid hormones. The serum is the chief source of cellular cholesterol. Transport of cholesterol into cell occurs by protein carriers, which may include high or low-density lipoprotein (HDL or LDL). On entry into the cell, cholesterol is either utilized or it is stored in the form of lipid droplets. Upon LH-induced stimulation, mobilization of newly synthesized and stored cholesterol occurs in lipid droplets. Cholesterol is
transported out of the cytoplasm into the mitochondria. It is transported from the outer to the inner membrane. This cholesterol transfer is considered as rate limiting step of steroidogenesis (Stocco, 1999).

Cholesterol entry from the outer to the inner mitochondrial membrane needs a transport protein. Stimulation of Leydig cells by LH stimulates the synthesis of the cholesterol transport protein. As this protein is essential for synthesis of steroid hormones and it regulates the rate limiting step of steroid hormone production, it is named as the steroid acute regulatory (StAR) protein (Stocco, 1999). Another protein is involved in cholesterol transport is peripheral benzodiazepine receptor and may play role in cholesterol transport from outer to inner mitochondrial membrane. It is suggested that it may act as a channel for cholesterol entry (Haider, 2004).

1.2.2 Role of cAMP in steroidogenesis

Leydig cells stimulation results in activation of G proteins which in turn stimulate the adenylate cyclase with consequent increase in cAMP inside Leydig cell. Also there is activation of protein kinase A (PKA) (Selstam, Rosberg, 1976; Zhu and Birnbaumer, 1996). Action of PKA results in the phosphorylation of various proteins like cholesteryl ester hydrolase and also there is phosphorylation of transcription factors that may include cAMP response-element binding protein (CREB) and the cAMP response element modulator. This may result in activation of genetic machinery involved in steriodogenesis, like StAR protein synthesis and activation (Stocco et al., 2001; Manna et al., 2003; Tremblay et al., 2002). Various factors that do not need cAMP and/or protein synthesis are also thought to potently stimulate steroidogenesis (Cooke, 1999).
1.2.3 Role of calcium in steroidogenesis

The increase in intracellular calcium, whether it is released from intracellular stores or mobilized from extracellular spaces, is believed to have considerable role in steroidogenesis. As a result of hormonal stimulation, there is change in intracellular cAMP and calcium levels. All stimulants of steroidogenesis like LH, hCG and GnRH rapidly increase intracellular calcium within 1-5 minutes (Sullivan and Cooke, 1986; Manna et al., 1999). Mitochondria in the Leydig cells store calcium and play important role in regulation of calcium levels within the Leydig cell (Nicholls, 2005). If there is inhibition of the electron transport chain, it results in intracellular hypercalcemia. The process of steroidogenesis is stimulated by increased calcium levels, but if intracellular calcium in Leydig cells falls, as induced through chelation in experimental models, it will lead to suppression of steroidogenesis (Sullivan and Cooke, 1986; Kumar et al., 1994, Tomic et al., 1995).

Calcium is known to potentiate the process of steroidogenesis. Literature shows that in calcium free media, despite stimulation by LH/hCG (100ng/ml), synthesis of steroids was 50% after 06 hours (Ramnath et al., 1997; Sullivan and Cooke, 1986; Manna et al., 1999). Also, there is experimental evidence that removal of calcium decrease steroid synthesis and StAR protein expression while addition of calcium to calcium free media increases steroidogenic capacity at cell level. Also role of calcium after hCG stimulation were studied with calcium channels blockers, which decreased steroidogenic capacity of steroid producing cells. It has been narrated that if calmodulin, (a protein which binds calcium) is blocked, steroidogenesis declines (Capponi et al., 1988; Irusta et al., 2003).
Figure 1.2: Overview of intracellular pathways of steroidogenesis.

(Adopted from Stocco et al., 2005)
1.2.4 Protein Kinase pathway in steroidogenesis

After binding of LH to Leydig cells and formation of cAMP, there is activation of phospholipase, which activates the protein kinase C (PKC) pathway. Also cAMP can activate the protein kinase A (PKA) pathway (Nikula and Huhtaniemi, 1989; Kuhn and Gudermann, 1999; Nishimura et al., 2004). Stimulation of Protein kinase C pathway results in signal transduction that can lead to transcription and translation of StAR and phosphorylation of StAR protein must occur by the activation of PKA, which is a mandatory requirement for its cholesterol-transferring function (Stocco et al., 2005).

1.2.5 Role of arachidonic acid in steroidogenesis

Stimulation of Leydig cells causes the release of arachidonic acid (AA) from intracellular stores. It is also proposed that cAMP also activates arachidonic acid -related thioesterase (ARTISt), an enzyme that catalyzes AA release from arachidonyl-CoA (AA-CoA). Release of AA occurs within one min of LH stimulation (Cooke et al., 1991; Stocco et al., 2005). It is also advocated that, hormone-receptor interaction at Leydig cell level cause the activation of G proteins that will result in activation of phospholipase A2 (PLA2) which as a result catalyzes the release of AA from phospholipids (Ronco et al., 2002). Various other pathways for arachidonic acid release are also cited in literature.

AA is thought to act at the rate limiting step of steroidogenesis, which is the transfer of the substrate cholesterol from outer to the inner mitochondrial membrane (Abayasekara et al., 1990). AA after release may be metabolized through one of three enzymatic pathways, the cyclooxygenase (COX), the lipoxygenase, or the epoxygenase pathways. Inhibition of either lipoxygenase or
epoxygenase activity as shown in some experiments inhibits StAR protein expression and steroid synthesis. It is also shown via experimental work that if AA release is blocked, it will result in inhibition of LH- or dbcAMP-stimulated StAR expression and steroidogenesis. However, the level of PKA activity remained high in above experimental models of Leydig cells, but such high levels of PKA were unable to affect the steroidogenesis significantly (Wang et al., 2000).

Only one pathway is not working for steroidogenesis at Leydig cell level, but various signaling pathways are operating which results in the stimulation of steroid biosynthesis and StAR protein expression (Stocco et al., 2005).

**1.2.6 Overview of enzymatic steps of testosterone synthesis**

LH binds to receptors on plasma membrane of Leydig cells. This results in various events which include activation of adenylate cyclase and increased formation of cyclic adenosine monophosphate (cAMP) inside the cell. There is translocation of cholesterol into the mitochondria, connection of cholesterol with the P450 side-chain cleavage enzyme (P450scc) and formation of pregnenolone from cholesterol in the mitochondria. From mitochondria, pregnenolone is translocated to the smooth endoplasmic reticulum. Cholesterol transfer from the outer to the inner mitochondrial membrane is considered as rate limiting step in the process of steriodogenesis. The exact process of translocation is unclear but the steroidogenic acute regulatory protein (StAR protein), a cycloheximide-sensitive 30-kDa mitochondrial protein which is synthesized in response to LH or cAMP, is involved (Stocco and Clark, 1996; Stocco, 1996; Stocco, 1999).

The next step is the transformation of pregnenolone to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). Now the steroidogenesis
Figure 1.3  Steps of testosterone synthesis

(Adopted from: Zirkin and Chen, 2000)
operates via either hydroxysteroid or ketosteroid pathways. In hydroxy steroid pathway, pregnenolone is converted to 17 α hydroxy pregnenolone, which is converted to dehydroepiandrosterone (DHEA), and then androstenedione is formed. In ketosteroid pathway, progesterone is converted to 17 α hydroxyprogesterone and then androstenedione that will lead to formation of testosterone. Testosterone is converted to dihydrotestosterone (DHT) by 5 alpha reductase which is present in cell membrane, nuclear envelope and endoplasmic reticulum. DHT is the end product hormone and more potent androgen than testosterone (Stocco and Clark, 1996; Stocco, 1996; Stocco, 1999).

1.2.7: Testosterone release and transport in circulation

Testosterone is the principal androgen in males. It was first isolated and discovered in 1935 (Ruzicka and Wettstein, 1935). Testosterone levels in testicular lymph and testicular venous blood are same, but the main route for steroid secretion into circulation is through venous blood (Morse et al., 1973). Testosterone in bound form with plasma proteins is incapable to produce the androgenic effects. More than 50% of testosterone circulating in blood is in state of binding with sex hormone-binding globulin (SHBG) while main part of remaining testosterone is in albumin bound state (Wheeler, 1995). The binding affinity of albumin for testosterone is low but it binds a substantial percentage of the total testosterone because the plasma concentration of albumin is much higher than SHBG. The free and albumin-bound testosterone togetherly is called bioavailable testosterone (Manni et al., 1985). It is also documented that diminished serum testosterone levels are responsible for stimulation of the SHBG production by the liver (Petak et al., 1996).
1.2.8: Regulation of testosterone production

Regulation of testosterone production occurs via a negative feedback loop system involving the anterior pituitary, hypothalamus and testis called the hypothalamo-pituitary-testicular axis. The hypothalamus releases pulses of gonadotropin-releasing hormone (GnRH) into the hypophyseal circulation. The GnRH stimulates the release of luteinizing hormone (LH) by anterior pituitary. The pulsatile release of GnRH results in LH release in an analogous pulsatile manner (Ismail et al., 1986). Circulating testosterone then feedback to hypothalamus and pituitary to suppress LH and consequently testosterone production. This negative feedback cycle results in pulsatile secretion of LH followed by pulsatile production of testosterone (Ellis et al., 1983). Besides luteinizing hormone, the follicle stimulating hormone and growth hormone play roles in the regulation of testosterone (Celec and Ostatnikova, 2003).

There are also various paracrine factors that may control the testosterone production. These include

a) Testicular peptides e.g., Inhibin and Activin.

b) Growth factors like Transforming growth factor α and β, Fibroblast growth factor and Insulin like growth factor I.

c) Cytokines that may include interleukin and tumor necrosis factor α.

d) Vasoactive peptide like angiotensin II, atrial natriuretic peptide and endothelin. (Saez, 1994; Gnessi et al., 1997; Schlatt et al., 1997).

1.2.9: Mechanism of action of testosterone. Testosterone acts via cytoplasmic receptors within the cells of the target tissues. The steroid-receptor complex then
reach nucleus where it enhances the gene transcription and may result in formation of new proteins.

Experimental evidence has also displayed a membrane-bound testosterone channel. Testosterone is also thought to change the functional status of ionic channels. In prostate gland the predominant receptor type is a Na\(^+\)-K\(^+\)-ATPase (Leung et al., 2001). In neurons and macrophages, a G protein- linked androgen receptor increases cAMP or inositol triphosphate (IP3) and diacyglycerol (DAG) through the activation of either adenylate cyclase or phospholipase C. In case of red blood cells, even the cytoskeleton functions as a steroid receptor and effector (Ramirez and Zheng, 1996). Androgen receptors are present on platelets, which are thought to play some role in the gender differences in platelet function and the associated risks of thrombotic diseases (Khetawat et al., 2000).

### 1.2.10: Physiological actions of testosterone

In tissues, testosterone is converted to dihydrotestosterone (DHT) in the presence of enzyme 5α-reductase. The physiological actions of testosterone on scalp, prostate and beard are performed by dihydrotestosterone. Testosterone plays a key role in the growth and development of the male genitalia as well as secondary sex characteristics (Gronowski and Landau-Levine, 1999). Testosterone causes increase in the growth of muscle and larynx that may lead to physical maturation as well as change in voice that occurs in boys at puberty. Development of acne is also related with testosterone levels. DHT not only stimulates the growth of axillary and pubic hair, but may also be responsible for their maintenance. Increased levels of DHT have been associated with the loss of scalp hair with resultant male pattern balding which is also called as androgenic alopecia (Randall., 2001). Testosterone
Fig. 1.4. Some of the interactions and feedback mechanisms of testosterone (Adopted from Zitzmann and Nieschlag, 2001).
causes increase in red blood cell count and hemoglobin concentration by increasing body metabolism and stimulating production of erythropoietin (Gooren, 2003). It is due to fact that testosterone increases erythropoiesis and also has important effects on behavior. Testosterone enhances the libido, competitiveness, and aggression (Ismail et al., 1986).

Testosterone is an anabolic hormone and it has important effects on metabolism in body (Mauras et al., 2000). Many studies indicate the relationship between levels of testosterone and blood pressure. Various authors are in favor of the opinion that testosterone has blood pressure lowering effects, which are probably mediated through atrial natriuretic peptide, vascular endothelial growth factor or could be due to the induction of nitric oxide synthase (Pandey et al., 1999; Sordello et al., 1998).

1.2.11: Testosterone levels in different phases of life in males

Testosterone levels are different in different during different phases of the life cycle (Gronowski and Landau-Levine, 1999). At puberty, the levels of testosterone are increased with resultant changes in male secondary sexual characteristics (Harries et al., 1997).

Significant physiological changes occur in male as age advances, most of them are due to decline in testosterone levels. The prime cause of this decrease in testosterone production is testicular failure, although reduced gonadotropin release can also be a contributing factor (Basaria and Dobs, 2001).

1.2.12 Factors affecting testosterone levels

Restraint stress in rats leads to decrease in testosterone levels (Dong et al., 2004). The levels of testosterone are affected by various endogenous as well as
environmental factors (Zitzmann and Nieschlag, 2001), as shown in figure 1.4. The Leydig cells activity is suppressed by stress and accordingly the levels of testosterone decrease (Haddy and Pankhurst, 1999). Similarly in humans, the stressful conditions may range from exams to sports and result in decline in testosterone levels. On the other hand stress release can result in rise of androgen levels (MacLean et al., 1997). The same effect may appear after loss of jobs or even change in work places (Grossi et al., 1999). The increased glucocorticoid secretion is observed in stressful situations that are due to increased corticotropin-releasing hormone production which may be responsible for down regulated testosterone biosynthesis in the Leydig cell (Hardy and Ganjam, 1997). There is feedback mechanism between testosterone and aggression, which is further influenced by various other factors like education, culture and socio economic status (Adler et al., 2000).

Supraphysiological levels of testosterone are suspected as a cause for carcinoma prostate (Shaneyfelt et al., 2000). Life threatening illnesses and traumas may also cause decline in testosterone levels which may also be accompanied by decrease in LH (Dong et al., 1992). Hyperthyroidism, autoimmune diseases (Handelsman, 2000), and various systemic diseases like Hodgkin’s disease and malignancies may also cause low testosterone levels thereby affecting testes as well as the hypothalamo pituitary axis (Baker, 1998). Vegetarians have significantly higher levels of SHBG, therefore the bioavailable testosterone levels are reduced in them (Belanger, et al., 1989).

Various factors affect reproductive system in males also include environmental disrupters like organic chemicals and pesticides, heavy metals like
lead, mercury, cadmium and ionizing radiations. Pharmacological threats include various agents that range from X rays to drugs like steroids and nicotine like agents. Among biological threats to reproductive system, oxidative stress is one of important factors (Sikka and Wang, 2008).

1.2.13 Effects of testosterone deficiency

Diminished testosterone in adult males may result in loss of libido and that may or may not be accompanied by erectile dysfunction (Petak et al., 2002). Older men with decreased plasma testosterone may show irritability, loss of concentration, lessened libido, erectile dysfunction, and may experience decreased sense of well-being (Tenover, 1998). Depression and behavioral symptoms like irritability and fatigue can also occur. Some secondary sexual characteristics may show regression such as reduced axillary and pubic hair, but quality of voice, length of penis and prostate size often remain unchanged (Petak et al., 2002).

Decreased in testosterone production is an important cause of infertility among men (Gronowski and Landau-Levine., 1999). Decrease in testosterone concentrations can result by a problem at Leydig cell level called testicular failure which may lead to primary hypogonadism. It can also occur when gonadotropins are insufficient to stimulate testis which is termed as secondary hypogonadism (Petak et al., 1996., Gronowski and Landau-Levine., 1999).

Measurement of testosterone and gonadotropin levels are useful parameters that may help in the differential diagnosis of cases of infertility (Petak et al., 1996). Therefore, we used testosterone levels as hormonal marker for well-being of male reproductive system in our study species, i.e., male Sprague Dawley rats. Our
research is focused on effects of stress on testicular steroidogenesis, which will be investigated.

**1.3: Stress**

Rapid industrialization, environmental contamination, changed life styles and stressful working conditions may result in stress with resultant adverse effects (Turek, 2000). Stress is imbalance between production of reactive oxygen species and antioxidant defense (Halliwell and Whiteman, 2004). Cannon was one of the first investigators to recognize the role of emotional reaction in the development of diseases (Cannon, 1909).

Homeostasis is facing continuous threats from various internal and external challenges which are called stressors (Miller and O’Callaghan, 2002). Selye was among the initial researchers to identify that stressors may disrupt homeostasis and have pathophysiological consequences (Selye, 1936). Stress is defined as “a general body response to initially threatening external or internal demands, involving the mobilization of physiological and psychological resources to deal with them” (Montoro et al., 2009). A large variety of homeostatic challenges can activate the hypothalamo-pituitary-adrenal (HPA) axis, including cold, infection, emotional distress, electric shock, sleep deprivation, social stress (Blanchard et al., 2001; Taylor et al., 1997). To overcome these stressors, hormonal, autonomic and behavioral adjustments are required. Activation of HPA and sympathetic nervous system with resultant release of glucocorticoids and catecholamines play important roles in stress induced diseases (Miller and O’Callaghan, 2002). Stress hormones are adaptive if their action remains for shorter durations, but if these hormones are released in excess or they remain in circulation for a longer time when they are no
Figure 1.5: Effects of stress on hypothalamo pituitary adrenal axis. (Modified from Miller and O’Callaghan, 2002)
more required, may become harmful. Consequently, they may contribute to disturbances in homeostasis (McEwen, 2000). Better understanding of pathophysiology of stress and the specific adjustments associated with an anomalous handling of glucocorticoids can help in better elucidation of the mechanisms by which stress may generate a disease (Bjorntorp, 1991).

1.3.1: Generation of reactive oxygen species

In order to maintain life, oxygen is mandatory requirement because the physiological levels of reactive oxygen species (ROS) are essential for the preservation of normal functions of the cell. However, breakdown products of oxygen with resultant excessive liberation of ROS can be injurious to cell function and survival (De Lamirande and Gagnon, 1995). Reactive oxygen species as reviewed by Makker et al., are present in the form of free radicals. ROS include a extensive range of molecules, including collection of radical (hydroxyl ion, peroxyl, superoxide) and non-radical (lipid peroxide, hydrogen peroxide) oxygen derivatives (Makker et al., 2009; Agarwal and Prabakaran, 2005). Environmental pollution and radiation exposure can also generate various ROS such as hydrogen peroxide, super oxides and hydroxyl radicals (Gate et al., 1999). ROS take part in various chemical reactions that liberate free radicals which can damage organic substrates (Agarwal and Prabakaran, 2005)

1.3.2: Stress: Imbalance between pro oxidants and anti-oxidants: Oxidative stress may occur due to imbalance between prooxidants and antioxidants. This imbalance may result from either a decrease in level of antioxidants or an increased production of prooxidants.
1. Decline in the levels of antioxidants that may occur due to mutations affecting the activities of antioxidant defense enzymes like glutathione peroxidase. Also deficiency of some minerals like zinc, magnesium, copper and selenium and various other antioxidants can also result in oxidative stress.

2. Increased production of reactive species that may result from exposure of cells or organisms to high levels of oxidative stress or excessive activation of intrinsic ‘natural’ systems that can produce such reactive oxygen species e.g., excessive phagocytic activity in chronic inflammation (Halliwell and Whiteman, 2004).

1.3.3: Effects of stress at cell level

Oxidative stress affects various components of cell that may be the lipids or the protein components of cell or the nucleic acids. The magnitude of the detrimental effects is affected by duration of ROS exposure, amount of ROS present in vicinity of that cell and some extracellular factors are also important like oxygen tension, temperature and amount of antioxidants present in area surrounding the cell (Makker et al., 2009; Agarwal and Prabakaran, 2005).

ROS most commonly target polyunsaturated fatty acids of cell membrane with resultant oxidation of lipids called Lipid peroxidation. ROS generate chain reactions and steps of a chain reaction include initiation, propagation and termination (Makker et al., 2009). Initiation involves reaction of free radicals with fatty acids and result in release of lipid free radicals. Lipid free radicals on reaction with the molecular oxygen may result in generation of lipid peroxyl radical, which again reacts with fatty acids to propagate the reaction with resultant more free radicals production (Makker et al., 2009). To assess the degree of per oxidative
damage, one of the byproducts of lipid peroxidation i.e., malondialdehyde is estimated, the levels of which are indicative of the extent of damage by oxidative stress (Aitken and Fisher, 1994).

1.3.4: Consequences of stress at cell level

Oxidative damage is the bimolecular damage that can be caused by direct attack of reactive species during oxidative stress. Consequences of oxidative stress can include:

1.3.4.1. Adaptation of the cell: can occur by up regulation of defense systems. This could have various effects:

(a) Cell is completely protected against damage.

(b) There is protection of cell against oxidative damage to some extent but complete protection is not there.

(c) Overprotection of cell to the extent that the cell may become resistant to oxidative stress.

1.3.4.2. Cell injury: Various components of cell are damaged as a result of oxidative stress. Consequently, cellular proteins, lipids and even DNA can be damaged. Oxidative stress may change the various ion levels like that of calcium, with drastic effects on cell functions. Similarly activation of proteases can result in cell injury.

1.3.4.3. Cell death: Oxidative stress induced injury to cell will end up in three ways

(a) Cell may repair or replace the damaged molecules and recuperate, or

(b) Oxidative damage persists and cell may stay alive with that damage, or
(c) Oxidative injury involving genetic machinery of cell may not be repaired and it can result in cell death, by apoptosis or necrosis (Halliwell and Whiteman, 2004).

1.3.5: Effects of stress on organs and systems:

Oxidative stress is thought to be involved in the pathogenesis of many diseases such as malignancies, Parkinson’s disease, diabetes mellitus, atherosclerosis, AIDS, inflammatory bowel disease, nervous system disorders, motor neuron disease and even related with premature child birth (Agarwal and Prabakaran, 2005), therefore stress may affect multiple organs and systems of body.

The stressors involving psychological factors stimulate various neural circuits mediating neuroendocrine responses involving cortical activation of the basolateral amygdala, which in turn activates the central nucleus of the amygdala. The central amygdala then activates hypothalamic neurons, either directly by central amygdala or indirectly through the stria terminalis. The stimulation of hypothalamus can also result via circuits involving brainstem serotonergic and catecholaminergic neurons (Van de Kar and Blair, 1999).

In order to control the stress response, neurons are located in the hypothalamus and brain stem. These include the parvocellular corticotropin releasing hormone (CRH), arginine – vasopressin (AVP) neurons of the paraventricular nuclei (PVN) of the hypothalamus, and the locus ceruleus (LC) – nor epinephrine system which is also called the central sympathetic system (Chrousos, 1992; Tsigos and Chrousos, 1994). The hypothalamo pituitary adrenal (HPA) axis, along with the sympathetic/adreno medullary systems are the effectors which are activated in response to stress with resultant effects of stress on different
organs of body (Gold et al., 1988a; Gold et al., 1988b). Enteric nervous system of gastrointestinal tract may also respond to stress via activation of Vagus nerve and sacral parasympathetic outflow is enhanced (Habib et al., 2001).

Neurons of the hypothalamic Para ventricular nucleus secrete the corticotrophic releasing hormone (CRH) into the portal circulation of the pituitary gland (Van de Kar and Blair, 1999). CRH is a peptide containing 41 amino acids and was first isolated in 1981 by W. Vale (Vale et al., 1981). CRH reaches the anterior pituitary, where it stimulates adreno corticotrophic hormone (ACTH) secreting cells causing the release ACTH into the blood (Van de Kar and Blair, 1999). AVP stimulate the ACTH release in synergism with CRH, however AVP alone do not increase the release of ACTH significantly (Lamberts et al., 1984). ACTH acts on the adrenal cortex, and result in amplified release of cortisol into the blood. Also there is enhanced release of prolactin from the anterior pituitary in stress, that’s why prolactin is also considered as a stress hormone (Van de Kar and Blair, 1999).

When there is no stress, both CRH and AVP are secreted in a pulsatile manner with a circadian rhythm (Engler et al., 1989). Under resting conditions, early morning hours are associated with increased pulses of CRH and AVP as a result of which ACTH and Cortisol levels increase in circulation in early morning hours (Horrocks et al., 1990; Chrousos and Gold, 1998). The diurnal patterns of variations can be disturbed by changes in the duration of dark and light cycles and are disrupted by stress. Number of pulsations in the hypophyseal portal system relevant to CRH increase during acute stressful conditions with consequent increase in ACTH and cortisol secretory episodes (Tsigos and Chrousos, 1994).
The high levels of cortisol as well as nor epinephrine and gamma-amino butyric acid inhibit the release of CRH (Miller and O’Callaghan, 2002).

Repeated stress influences the functions of various parts of brain. Hippocampus is affected significantly due to the presence of receptors for stress hormones like cortisol (McEwen et al., 1986). It is also thought that hippocampus is also important in regulation of stress response by inhibiting the stress induced hyper activity of HPA axis (Jacobson and Sapolsky, 1991).

1.3.6: Stress hormones

1.3.6.1: Glucocorticoids

Glucocorticoids are produced by adrenals from cholesterol in response to adrenocorticotropic hormones (ACTH) (Pizarro and Troster, 2007). They are produced with a circadian rhythm, but stress disrupts their circadian rhythm. Glucocorticoids inhibit the hypothalamo-pituitary-adrenal axis via negative feedback mechanism (Pizarro and Troster, 2007).

The ultimate effectors of the HPA axis are glucocorticoids and they play role in the control of stress response and maintenance of homeostasis. They not only regulate basal activity of HPA axis but also control the termination of stress response (de Kloet, 1991). Negative feedback inhibition by glucocorticoids decreases the exposure time of body tissues and cells to glucocorticoids and in this way decreases the catabolic, immunosuppressive and anti-reproductive effects of stress hormones. The mechanism of action of glucocorticoids involves cytoplasmic receptors which are present everywhere on cell (Pratt, 1990; Smith and Toft, 1993). On ligand binding, the glucocorticoid receptors translocate into the nucleus, where specific glucocorticoid responsive elements (GREs) are activated within the DNA
to activate appropriate hormone-responsive genes. Glucocorticoids thus induce
translation of messenger RNAs with resultant activity of several glucocorticoid-
responsive proteins (Pratt, 1990).

If the HPA axis remains hyper active for prolonged periods, the resultant
increase in the glucocorticoids may suppress the secretion of growth hormone.
Glucocorticoids also inhibit the action of Somatomedin C with resultant decrement
in effects of Growth hormone on target tissues (Burguera et al., 1990). Similarly, if
there is hyperactive HPA axis, it may lead to decrease in production of thyroid-
stimulating hormone and it may also block the conversion of thyroxine to tri-
oidothyronine with decline in thyroid function (Benker et al., 1990).

1.3.6.1.1: Corticosterone: Major glucocorticoid in rats

Predominant glucocorticoid in rodents like rats is corticosterone (Miller and
O’Callaghan, 2002). Therefore, plasma corticosterone level is an indicator of
activity of HPA axis in rats. Corticosterone levels are increased in psychological
and physical stress, e.g., experimentally induced restraint stress in rats will increase
the plasma levels of corticosterone (Bauer et al., 2001), and so the levels of
corticosterone may serve as a good indicator of stress in rats.

Corticosterone has many metabolic effects that may include the glucose
production from protein and diverse effects on fat metabolism. Various other
important effects include inflammatory responses, vascular responsiveness and the
effects on the central nervous and immune systems.

1.3.6.2: Catecholamines

Stress activates sympathetic adrenal system with release of catecholamines
like epinephrine and nor epinephrine. Usually cortisol release is also in
The sympathoadrenal system activation is dependent not only on the impulse activity in nerves or amounts of NE and E released, but the sensitivity and responsiveness of the tissues to catecholamines is also very important, which is changed in various physiological and pathophysiological states by up regulation or down regulation of receptors and change in affinity of receptors (Christensen and Jensen, 1994).

Norepinephrine (NE) as summarized by Weinshenker and Schroeder plays an important role in attention, arousal, and stress reactions in challenging environments. NE is also thought to play role in learning and memory, neuronal excitability, pain and affective disorders (Weinshenker and Schroeder, 2007). The proportionate to release of catecholamines in response to stress. Therefore, greater the cortisol, more will be the norepinephrine (Cacioppo et al., 1995). As narrated by Fernstrom & Fernstrom, norepinephrine (NE) is synthesized in sympathetic neurons and in the adrenal glands. The initial step involves hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) which is catalyzed by the enzyme tyrosine hydroxylase (TH), DOPA is promptly decarboxylated to dopamine (DA) by aromatic L-amino acid decarboxylase. In neurons that use DA as a transmitter, no further change occurs and dopamine is used as such. Neurons that use NE as a transmitter, NE is produced from the dopamine by the action of the enzyme dopamine β-hydroxylase. Neurons using epinephrine as a transmitter contain another enzyme, phenylethanolamine-N-methyl transferase, which catalyze the conversion of NE to epinephrine. The rate limiting is the initial step in the pathway i.e., Tyrosine hydroxylation, which controls the rate of synthesis of both nor epinephrine and epinephrine (Fernstrom and Fernstrom, 2007; Nemeroff, 1998).
levels of epinephrine and nor epinephrine rise in males during physical and mental stress (Gustafson and Kalkhoff, 1982; Barnes et al., 1982), and in rats during immobilization stress (Kvetnansky et al., 1979). Catecholamines are shown to decrease the plasma testosterone levels in human and animal studies (Damber and Janson, 1978).

As mentioned before, CRF is increased in stress and if CRF is injected into the locus ceruleus of rats, it stimulates tyrosine hydroxylase (TH), an enzyme which regulates the NE synthesis, which results in increased synthesis of NE. Similarly activation of TH is seen in experimentally induced stress, in animals (Melia and Duman, 1991), therefore to assess the stress; it is worth to measure the levels of norepinephrine.

Role of catecholamines during stress at Leydig cell level is less known (Hardy et al., 2005). Catecholamines are generally thought to increase steroidogenesis in vitro (Mayerhofer et al., 1993). Beta blockers which decrease sympathetic activity decrease testicular steroidogenesis (Khan et al., 2004). So nor epinephrine increase levels of testosterone, while stress decreases the testicular steroidogenesis. But in stress, along with other hormones like corticosterone, the levels of nor epinephrine increased. How stress induced increase in nor epinephrine affect Leydig steroidogenesis, will be evaluated in vivo as well as in vitro on isolated and cultured Leydig cells.

1.3.7: Malondialdehyde and stress

Reactive oxygen species are known to cause oxidative damage to macromolecules (Cross et al., 1987). Reactive oxygen species usually cause lipid peroxidation which is a multifaceted phenomenon that occurs in biological
membranes made up of polyunsaturated fatty acids and may lead to the formation of lipid hydroperoxides and their metabolites. Most cases concerning lipid peroxidation start from a chain reaction which is initiated by free radicals. Lipid hydroperoxides accumulate in the membrane which may cause its receptors and enzymes to become inactive thereby affecting its functions, causing instability and making the membranes permeable to ions (Kazez et al., 1997). In order to determine the levels of ROS, we can use the levels of lipid peroxidation products in the body fluids as a diagnostic tool (Kiarostami et al., 2006).

The degradation of lipid peroxides of the biological membranes may result in formation of a number of aldehydes such as malondialdehyde (MDA), which may cause damage to cells by reacting with proteins, lipids and nucleic acids (Sim et al., 2003). A simple method of high sensitivity, frequently used as a lipid peroxidation marker, involves thiobarbituric acid-reactive substances. Therefore, MDA which is a thiobarbituric acid reacting substance (TBARS) is considered as a suitable indicator of lipid peroxidation triggered by free radicals (Kazez et al., 1997). Polyunsaturated fatty acids after oxidation may result in increased levels of MDA, which can be used as an in vivo marker to assess lipid peroxidation in diseases like atherosclerosis and diabetes (Haberland et al., 1990; Slatter et al., 2000). Therefore in order to assess the amount of lipid peroxidation and to estimate whether the antioxidants confer production against stress induced lipid peroxidation, we estimated the levels of MDA in our study.

1.3.8: **Role of superoxide dismutase as natural antioxidant in stress**

Halliwell & Gutteridge defined an antioxidant as “any substance that when present at low concentrations compared with those of an oxidizable substrate
significantly delays or prevents oxidation of that substrate” (Halliwell and Whiteman, 2004).

Antioxidants are the most important defense mechanism against oxidative stress that results from generation of free radicals. Antioxidants may play a role to prevent oxidants induced injury and may also act as free radicals scavenger. Prevention antioxidants include the metal chelators and metal-binding proteins which play a role in blocking the formation of new ROS. On the other hand, scavenger antioxidants eradicate the ROS that have previously been generated as a result of oxidative stress process (Agarwal and Prabakaran, 2005).

Endogenous antioxidant systems that may act as free radical scavengers may have important role to combat against stress induced lipid peroxidation. One of important endogenous antioxidant defense system is an enzyme, superoxide dismutase (Ferrari et al., 1991). Superoxide dismutase was discovered by Irwin Fridovich and Joe McCord and initially it was considered that these metalloproteins have no function (McCord and Fridovich, 1988).

The importance of superoxide dismutase (SOD) was investigated in genetically engineered mice lacking these enzymes. Various diseases were found in these mice and excessive oxidative stress caused even death of these SOD deficient mice (Li et al., 1995). SOD deficient mice are more prone to develop diseases like hepatocellular carcinoma (Elchuri et al., 2005). If SOD is given, it will decrease reactive oxygen species generation and oxidative stress in clinical conditions like inflammatory bowel disease (Segui et al., 2004). The mechanism by which SOD exerts its effect is that, it prevents oxidative stress induced damage to cells caused by reactive oxygen species. SOD converts superoxide to $\text{H}_2\text{O}_2$ and oxygen.
In mammalian cells, including humans and rats, super oxide dismutase is present in three forms, which include the Mn SOD, Cu-Zn SOD, and extracellular Cu-Zn SOD. Different genes encode each of these super oxide dismutases and these differ in amino acid sequence and localization (Keller et al., 1991). In humans mental stress is reported to increase antioxidant enzymes, super oxide dismutase (SOD) of seminal plasma with abnormal sperms (Eskiocak et al., 2005). Restraint stress on rats caused increased circulating levels of SOD, catalase. Testicular tissue is highly susceptible to stress because testicular membranes are rich in polyunsaturated fatty acids (Chainy et al., 1997).

Therefore, lipid peroxidation and spontaneous oxygen toxicity is prevented by Superoxide dismutase (Fridovich, 1985). The levels of SOD may thus indicate the activity of natural intrinsic anti-oxidant defense system of body. Therefore in order to assess the natural antioxidant activity in acute restraint stress to rats and to estimate whether the antioxidants confer production against stress induced derangements in antioxidant activity, we estimated the levels of SOD in our study.

1.4: Antioxidant supplements and stress

Antioxidants obtained from dietary sources constitute an essential component of human antioxidant defense system. Various fruits and vegetables and diet supplements are the possible sources of various antioxidants (Agarwal and Prabakaran, 2005). Oxidative stress can be controlled by use of chain breaking antioxidants like ascorbic acid and alpha tocopherol (Agarwal et al., 2005). Many
epidemiologic and clinical trials are in favor that if the diet is supplemented with antioxidant vitamins it may result in a decrease in the occurrence of chronic disease morbidity and mortality (Weber et al., 1996; Enstrom, 1997; Gey, 1998). Among various dietary antioxidants, ascorbic acid and alpha tocopherol are antioxidants which are recognized by US Food and Drug Administration (FDA) as dietary antioxidants (Monsen, 1996).

1.4.1: Ascorbic acid

Ascorbic acid is one of the important and essential micronutrients and it is required for various metabolic functions of the body (Jaffe, 1984). It is thought that human beings have lost the capability to produce ascorbic acid within their body due to mutation in gene coding for L-gulonolactone oxidase, which is an enzyme required for the biosynthesis of ascorbic acid via the glucuronic acid pathway (Woodall and Ames, 1997). So, ascorbic acid forms an essential constituent of diet. Ascorbic acid is found abundantly in fresh citrus fruit and in various vegetables (Bendich, 1997). Deficiency of ascorbic acid in the diet results in the disease called Scurvy (Levine, 1986). Consumption of 10 mg ascorbic acid per day is sufficient to prevent scurvy (Weber et al., 1996), and it can be gained through intake of fresh fruit and vegetables.

Ascorbic acid is a cofactor for numerous enzymes concerned with biosynthesis of carnitine, collagen, and various neurotransmitters (Burri and Jacob, 1997; Tsao, 1997). Procollagen-proline dioxygenase (proline hydroxylase) and procollagen-lysine 5-dioxygenase (lysine hydroxylase), which are the two enzymes concerned with procollagen biosynthesis, need ascorbic acid for optimum activity (Phillips and Yeowell, 1997).
The deficiency of ascorbic acid may lead to the weakening of collagenous structures, the consequences of which may include the tooth loss, joint pains, bone and connective tissue disorders and poor wound healing which are important features of scurvy (Burri and Jacob, 1997). Hypochondria, depression, and mood variations frequently take place during scurvy and these could be correlated with incomplete dopamine hydroxylation. The actions of a variety of other enzymes may also need ascorbic acid. These enzymes include mono and dioxygenases, involved in peptide amidation and tyrosine metabolism (Burri and Jacob, 1997; Tsao, 1997).

Data from many various studies have narrated that ascorbic acid helps to reduce the incidence of various diseases which may include cancer, cataract and cardiovascular disease (Weber et al., 1996; Enstrom, 1997; Gey, 1998). It is also proposed that endogenous and exogenous ascorbic acid decreased lipid oxidation (Carr and Frei, 1999). Generally ascorbic acid has beneficial effects but it is also claimed to play prooxidant role in some situations (Podmore et al., 1998).

1.4.2: Alpha tocopherol

Alpha tocopherol was discovered in 1922 by Evans and Bishop as an essential dietary factor for reproduction in rats (Evans and Bishop, 1922). It was later named as ‘factor 2’ in 1950’s by Klaus Schwarz and placed in the group of cellular antioxidant systems, along with sulfur amino acids and selenium (Schwarz, 1965).

Like ascorbic acid, alpha tocopherol must be obtained from the diet (Bertinato et al., 2007). Recommended daily allowance (RDA) for alpha tocopherol is 8 mg (12 IU) for females and 10 mg (15 IU) for males. Alpha tocopherol is a vital nutrient which is required for normal growth and development.
It is considered as a major lipid-soluble antioxidant in animals (Burton, 1994). Major sources of alpha tocopherol include whole grains, nuts and vegetable oils. Dietary vitamin present in the form of tocopherol esters are hydrolyzed in the intestinal lumen by pancreatic esterases (Debier and Larondelle, 2005; Hacquebard, and Carpentier, 2005; Rigotti, 2007).

Free radical chain reactions are broken up by alpha tocopherol molecules by capturing the free radicals. This action is responsible to make alpha tocopherol an ideal antioxidant. The antioxidant properties are due to free hydroxyl group on the aromatic ring. The hydrogen of the hydroxyl group is given to the free radical, which may result in formation of relatively stable free radical form of alpha tocopherol (Sies and Murphy, 1991). Chain reaction of lipid peroxidation in biomembranes and lipoproteins is blocked by alpha tocopherol (Dieber-Rotheneder et al., 1991).

The antioxidant activity of alpha tocopherol has been proven by various studies and its ability to prevent chronic diseases, especially caused by oxidative stress component such as cardiovascular diseases, atherosclerosis, and cancers have been extensively investigated. Epidemiological studies have reported that high alpha tocopherol intakes are correlated with a decreased risk of coronary heart disease (Rimm et al., 1993). Diabetes mellitus also induces stress and alpha tocopherol prevents diabetic rat testis against oxidative stress (Naziroglu, 2003).

Therefore, alpha tocopherol has antioxidant properties and it is useful in preventing various diseases. We can say that alpha tocopherol protects various biological systems of our body (Packer, 1991), by preventing lipid peroxidation (Evtigneeva et al., 1998). Alpha tocopherol, if present in an adequate amount at
site of oxidative stress where there is free radical generation, it may reduce the toxic effects of ROS (John et al., 2001). Due to these antioxidant properties of alpha tocopherol, we used this antioxidant in our research to evaluate its preventive role as an antioxidant against stress.

The rationale of this study was to investigate the effects of acute restraint stress on testicular steroidogenesis. Catecholamines are thought to increase steroidogenesis and corticosteroids decrease steroidogenesis, but net effect of stress is considered to be decreased testosterone. So the separate and combined effects of these hormones on testicular steroidogenesis will be evaluated at the level of Leydig cell. Also the mechanism of action of stress hormones, corticosterone and nor epinephrine on cultured Leydig cells is also investigated.

Testicular tissue is highly susceptible to stress because testicular membranes are rich in polyunsaturated fatty acids (Chainy et al., 1997). So by giving acute restraint immobilization stress to rats, oxidant and antioxidant status will be seen by MDA and SOD respectively. As explained earlier in detail that antioxidant ascorbic acid and alpha tocopherol are thought to protect the body against stress induced damages (Zaidi et al., 2003). So the role of these antioxidants on stress induced decline of testosterone and the effects of these antioxidant vitamins on stress induced lipid peroxidation and SOD was evaluated, first in vivo study on rats and then in vitro on isolated and cultured Leydig cells of rats.
OBJECTIVES OF STUDY

*In vivo studies*

1. To determine the effects of six hours acute restraint stress on male Sprague Dawley rats on the levels of serum testosterone, serum corticosterone, plasma nor epinephrine and serum LH.

2. To determine the effects of six hours acute restraint stress on male Sprague Dawley rats on lipid peroxidation (in terms of malondialdehyde) and antioxidant enzyme, superoxide dismutase.

3. To determine the preventive effects of antioxidants, ascorbic acid and alpha tocopherol separately and combined on derangements induced by stress hormones on testicular steroidogenesis in male Sprague Dawley rats.

*In vitro studies*

1. To develop an *in vitro* model of isolated and cultured Leydig cells of male Sprague Dawley rats to study the effects of stress hormones at Leydig cell level.

2. To explore the mechanism of action of corticosterone and nor epinephrine on testicular steroidogenesis at Leydig cell level.

3. To ascertain the role of antioxidants like ascorbic acid and alpha tocopherol to overcome the stress hormones induced effect on testicular steroidogenesis.

**Hypothesis**

Stress hormones adversely affect testicular steroidogenesis which can be prevented by the use of antioxidants like ascorbic acid and alpha tocopherol.
MATERIALS AND METHODS

2.1 In vivo study

2.1.1 Setting

The study was conducted in the department of Physiology, Army Medical College, Rawalpindi, Pakistan, in collaboration with National Institute of Health Islamabad. Laboratory work was done primarily in Centre for Research in Experimental and Applied Medicine, (CREAM) which is a multi-disciplinary research laboratory at Army Medical College, established under auspices of National University of Sciences and Technology (NUST), Islamabad, Pakistan.

2.1.2 Duration of study

Two years as full time resident student.

2.1.3 Study design

Quasi experimental studies.

2.1.4 Chemicals used in in vivo part of study

Following chemicals were purchased from local supplier:

2.1.4.1 Ascorbic acid.

MERCK, Research grade. Cat No: 500074.

2.1.4.2 Alpha tocopherol

MERCK, Research grade. Cat No: 500854.

Chemicals were weighed with the help of Sartorius Balance. After cleaning balance top, leveling was done by adjusting the bubble in the centre of ring. First of all, balance was zeroed by pushing the tare button. Then a clean weighing boat was placed on the balance and again tarred to account for its weight. Then chemical was placed into weighing boat and adding continued with the help of spatula till the
desired weight achieved. When the displayed reading for desired weight steadied, it was then shifted to storage container which was then sealed and labeled.

2.1.5 Animals of study: Rats

Male Sprague Dawley healthy rats 90 days old, bred at National Institute of Health, Islamabad were used in this experiment. Average weight of rats was 275±50 grams. Animals were handled two to three days prior to start of experiments to make them acclimatized to new handler.

2.1.6 Animal house

Animal house facility of National Institute of Health (NIH), Islamabad was used. This animal house has a setup according to international standards for breeding and housing of research animals. A separate room was allocated for study groups of this research. Metallic rack was used to place different cages of the rats. Room was well ventilated and 12 hour light and 12 hour dark cycle was maintained. Temperature of that room was maintained at 22 ± 3 °C, with the help of central temperature regulating systems with ducts opening in each room (Dong et al, 2004).

2.1.6.1 Cages and Water Bottles

Animals were placed in cages of 2 x 3 feet size, ten animals per cage. Clean water bottles specific to fit over these cages for continuous supply of water were used.

2.1.6.2 Water for rats

Rats were provided with water in clean bottles that are specific for the rat cages and have valve at their opening from which water comes out when the rat sucks it. Bottle was fitted inverted over the cages with one bottle per cage. Bottles
were cleaned every alternate day as per protocol of the animal house. Water bottles were filled at least twice a day.

2.1.6.3 Rats feed.

It was provided by animal house of NIH. Food was supplemented with antioxidants as described in grouping. Food composition given to rats is attached as annexure A and B.

2.1.7 Sample size: Eighty male Sprague Dawley rats.

2.1.8 Sample selection

2.1.8.1 Inclusion criteria

1 Male Sprague Dawley rats
2 Healthy rats with weight 275 ± 50 grams.
3 90 days old

2.1.8.2 Exclusion criteria

1 Rats with disease
2 Rats who develop disease during course of study.

2.1.9 Grouping (N=80)

16 rats were taken per group and they were fed on standard and supplemented diets for one month. Each cage labeled both for group numbers and type of diet.

Group I (n=16): This was control group. Rats were fed normal standard rat diet (see Annex A & B) without any supplementation. These rats were given plain tap water.

Group II (n=16): Rats were fed normal standard rat diet (see Annex A and B) without any supplementation. These rats were given plain tap water. These rats
were exposed to acute immobilization stress in a mesh wire restrainer for 6 hours. (Dong et al., 2004)

**Group III (n=16):**

This group was fed normal standard diet. Ascorbic acid supplementation was given as 500 mg ascorbic acid/L drinking water (Hsu et al., 1998). These rats were exposed to acute immobilization stress in a mesh wire restrainer for 6 hours, after one month of antioxidant supplemented diet (Dong et al., 2004).

**Group IV (n=16)**

This group was fed alpha tocopherol supplemented diet with 300 mg alpha tocopherol/kg chow + 2% soyabean oil (Hsu et al., 1998). These rats were given plain tap water. These rats were exposed to acute immobilization stress in a mesh wire restrainer for 6 hours, after one month of antioxidant supplemented diet (Dong et al., 2004).

**Group V (n=16)**

This group was fed alpha tocopherol supplemented diet with 300 mg alpha tocopherol/kg chow + 2% soyabean oil. Also, ascorbic acid supplementation was given as 500 mg ascorbic acid/L drinking water. These rats were also exposed to acute immobilization stress in a mesh wire restrainer for 6 hours, after one month of antioxidant supplemented diet (Dong et al., 2004).

**2.1.10 SAMPLING TECHNIQUE**

To avoid bias among different values among cortisol and testosterone because of diurnal variations, all samples were taken at same timings of the day between 11 am and 12 noon (Debigare et al., 2003).
2.1.10.1 Procedure for sampling

First of all, rats were placed one by one in a closed chamber with ether soaked in cotton in that chamber in the main laboratory of animal house. Rats were thus anesthetized (which took approximately 3-5 minutes per rat). After this, rat was placed on dissection board and after ensuring proper anesthesia, intracardiac blood sample was drawn with the help of BD 5cc disposable syringe (Hsu et al., 1998).

2.1.10.2 Intra cardiac sampling:

For intra cardiac sampling, each rat was placed at its back. After palpation of lower rib cage and sternal margin, syringe needle was inserted into heart taking care that it may not pierce the heart up to its posterior wall. Then blood was withdrawn in syringe to ensure that needle is in heart. If no blood came in syringe, then needle was gently pulled out but not to the extent that it comes out of body of rat. After this, needle of syringe was reinserted into the heart and checked for proper insertion.

2.1.10.3 Sampling tubes- 5 ml CP tubes

After this, blood was drawn slowly up to 5 ml in the syringe. Then 3.5 ml from this blood was immediately shifted into BD vacutainer. Blood was allowed to clot. Another 1.5 ml was placed in BD vacutainer containing potassium oxalate for obtaining plasma

2.1.11 Processing of samples:

2.1.11.1 Cold centrifugation.

Samples were first centrifuged at 4000 rpm at 4°C in the cold centrifuge. Here eppendorf centrifuge was used. First of all, samples were adjusted in the
centrifuge machine taking care of balance on opposite site. First temperature was set (to ensure that temperature inside eppendorf chamber is 4°C) and then speed adjusted to 4000 rpm for 15 minutes.

2.1.11.2 Storage of samples

After cold centrifugation, serum was pipetted out and 1.5 ml of serum was taken per sample and placed in 1.5 ml eppendorf storing tubes (which were labeled according to grouping protocol).

Also about 0.5 ml of plasma was obtained from all samples in which preservative (EDTA) added and were placed in separate labeled eppendorf storage tube.

Samples were stored at – 80 °C temperature refrigerator till analysis.

2.2 IN VITRO EXPERIMENTATION

2.2.1 Chemicals used for in vitro experimentation

- Eagles medium M 199 (sigma-Aldrich)
- Hydroxy ethyl piperazine ethane sulphonic acid. (HEPES) (sigma-aldrich)
- Theophylline (sigma-Aldrich)
- Isobutryl methyl Xanthine (IBMX)
- Bovine serum albumin (sigma-Aldrich)
- Gentamycin (sigma-Aldrich)
- Collagenase (sigma-Aldrich)
- Bovine serum Albumin (sigma-aldrich)
- Dulbecco’s modified Eagles Medium (DMEM) (sigma-aldrich)
- Percoll (sigma-Aldrich)
2.2.2 Chemicals used for staining

a. Trypan-blue stain 0.4% (sigma-aldrich)

b. 3-beta-HSD stain (5 ml)
   - Nitro-blue-tetrazolium (Sigma-Aldrich) = 1 mg
   - Beta-NAD (Sigma-Aldrich) = 5 mg
   - Dehydroandrosterone (Sigma-Aldrich) = 0.2 ml
   - PBS = 4.8 ml

2.2.3 Equipment used

- Analytical balance (Sartorius)
- Parafilm (Chicago, USA)
- Micropipettes
- Stirring plate Thermix® -310T (Allied Fisher Scientific, USA)
- pH meter (Selectra)
- Ice flaker (Ilshin)
- Centrifuge (temperature controlled), Eppendorff-5810 R (Hamburg, Germany)
• Shaking Incubator JSSI-100 C (JS Research Inc. Korea)
• Shaking water bath JSSB-30 T (JS Research Inc. Korea)
• Neubauer Chamber 0.100 mm, 0.0025 mm² (Bright Line, Assistant, Germany)
• Microscope (Nikon)
• Glassware Pyrex® Corning (USA)
• Culture tubes 12 x 75 mm, Pyrex ® (USA)
• Conical centrifuge tubes 15 and 50 ml (Falcon)
• Timer (Sanyo)
• Racks
• Glass beakers and bottles (Autoclaved)
• Nylon mesh
• Funnel
• Spinal needle
• Pipettes (all sizes)
• Ethanol washed magnets as stirrers
• Eppendorf tubes and Pasteur pipettes
• Discarding beaker
• Surgical set, kidney tray10 cc syringes
• Ice bucket
2.2.4 Reconstitution of Solutions

2.2.4.1 Eagle’s Medium

In 100 ml M-199, HEPES (0.595 g), Theophylline (0.009 g) and Gentamycin (100 µl) were mixed. Later 0.1 g of BSA was added and allowed to mix without stirring. The pH was maintained at 7.4.

2.2.4.2 Biophosphate Buffer

100 ml bio phosphate buffer was prepared by adding 0.001g of BSA and 100 ul of gentamycin in 100 ml PBS.

2.2.4.3 Discontinuous Percoll gradient (Four gradients for 4 rats)

Stock: 10X M 199 + 1.0% BSA (30 ml M199 + 0.3 g BSA)

Solution A (Percoll 0%)

<table>
<thead>
<tr>
<th></th>
<th>Stock</th>
<th>Water</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock</td>
<td>12.5 ml</td>
<td>112.5 ml</td>
<td>125 ml</td>
</tr>
</tbody>
</table>

Solution B (Percoll 90%)

<table>
<thead>
<tr>
<th></th>
<th>Stock</th>
<th>Water</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock</td>
<td>15.5 ml</td>
<td>139.5 ml</td>
<td>155 ml</td>
</tr>
</tbody>
</table>

pH of solution A and B was maintained at 7.4

2.2.4.4 Gradient Steps

For four gradients (4 rats)

<table>
<thead>
<tr>
<th>% Percoll</th>
<th>Solution A (ml)</th>
<th>Solution B (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>8.8</td>
<td>27.2</td>
</tr>
</tbody>
</table>
43   18.8    17.2
35   22      14
20   28      8

**Preparation of Percoll Gradient**

Percoll gradient was layered starting from bottom of 50 ml centrifuge tube, using a long spinal needle in the following sequence;

8 ml each of solution A, Percoll 20 %, 35 %, 43 %, 68 % and 90 %.

**2.2.5 Reconstitution of Chemicals**

Following chemicals were reconstituted as their molar solution and concentration constituted are shown in pipetting protocol:-

- Corticosterone (soluble in DMSO)
- Cortisol (soluble in DMSO)
- Nor epinephrine (soluble in DMSO)
- Alpha tocopherol (soluble in DMSO)
- Ascorbic acid (soluble in ultra pure water)
- Cortisol (soluble in DMSO)
- Pregnenolone (soluble in DMSO)

**2.2.6 Procedure - Rat Leydig cells isolation and culture**

**Rats:**

The rats were of same specifications as mentioned in 2.1.5 above.
2.2.6.1 Orchidectomy

We took four male Sprague Dawley rats per experiment from NIH and kept them in our research lab for two days for acclimatization. The animals were fed on standard diet and water ad libitum. Dark and light cycles were maintained as per standards as mentioned before.

After anesthetizing the rats with ether, they were decapitated. The abdomen was opened by a midline incision. The testes were brought out and examined, if they were of same size and shape, only then the testes were dissected out and decapsulated. Then the testis were placed in eagles medium on ice, in a tray with ice crystals. (Testis were washed with Eagles Medium and exposed media was removed)

2.2.6.2 Collagenase dispersion of testicular interstitial cells

The testis after decapsulation were placed in eagles medium (EM) containing 0.25 mg/ml (10 ml) Collagenase in two 50 ml falcons tube. This falcon tube was then sealed with parafilm.

Then we incubated these falcon tubes at 37°C in shaking water bath at a speed of 75 cycles per min for 25 minutes. To stop the incubation process, 20 ml of EM was added and the contents were transferred to a beaker. Beaker was placed in kidney tray with ice, smallest magnet placed in beaker; beaker was covered with parafilm and then stirred with slow speed for 10 minutes.

Later, the contents were filtered through Nylon mesh. For that, we took funnel, mesh and falcon tube. Pasteur pipette was used and contents were shaken before filtering. Also the beaker was washed with medium at end to
remove the left over cells. After this, 15 ml falcon tube was centrifuged at a speed of 800 rpm for 10 minutes at 4°C to remove collagenase.

2.2.6.3 Purification of Leydig cells by density gradient Percoll centrifugation

The supernatant was discarded and pellet was resuspended in 24 ml Eagles medium. Preformed Percoll gradient (as described in 2.2.4.4) was there, with us at this stage. Then we layered the 6 ml cell suspension on the top of pre formed gradient (20, 35, 43, 68 and 90 % Percoll in M199 with BSA) with Pasteur pipette, taking care not to disturb gradient steps/layers for which we poured along walls with Pasteur pipette by rotating the falcon tube.

Gradient was then centrifuged at 800 g for 25 minutes at 27°C in eppendorf swinging rotor. Initially, needle and 10 ml syringe were cleaned with solution A. Then, the third fraction from the top of each gradient was collected through long needle (about 20 to 24 ml). Caution was exercised to prevent collection of erythrocytes from fourth layer. (We first removed last layer and then removed Leydig cell layer). That fraction was diluted in 2 volumes of M-199 with BSA and centrifuged at 800 rpm, at room temperature for 20 min. For one gradient, we resuspended cells in 32 ml M 199 with BSA

2.2.6.4 Leydig cell staining for 3-β-HSD

The presence of Leydig cells in the isolated cell fraction was confirmed by 3-β-HSD staining (Sharpe and Fraser, 1983). For that, 50 µL of cell suspension was mixed with 150 ul staining solution, incubated at 37°C for 60 minutes in incubator. To checked purity we put 50 µl of above mixture on haemocytometer and purity of the cells was checked by counting total number
of blue stained cells and unstained cells in WBCs counting chamber (16 small squares). The blue stained and the unstained cells represent presence and absence of 3-beta HSD, respectively. Percentage of the Leydig cells was calculated by following formula:

\[
\text{% age purity of LC} = \frac{\# \text{ of blue stained cells}}{\text{total # of cells}} \times 100
\]

Total number of blue stained cells = 131
Total number of unstained cells = 12
Total number of cells = 148

\[
\text{% age purity of LC} = \frac{131}{148} \times 100 = 88.51\%
\]

The purity of Leydig cells was 88.51%.

2.2.6.5 Preincubation:

Cell suspension was incubated at 34°C in shaking water bath in a conical flask at a speed of 75 cycles per min for 60 minutes to remove endogenous testosterone. After incubation, cell suspension was diluted in triple volume of PBS and then centrifugation was done at 900 rpm for 20 min, at room temperature. Then, we discarded the supernatant and resuspended the pellet in 10 ml EM. For this, the pellet was dispersed in 1 ml EM and then added to complete 10 ml EM.

2.2.6.6 Cell viability and cell count

Cell viability was checked by trypan blue exclusion method (Aldred and Cooke, 1983). To check viability of cells, 50 ul of CS and 50 ul of Trypan blue were mixed properly (Abdul-Saeed et al., 1995).

After charging the Neubauer Chamber, the cells were viewed under microscope. The number of unstained and stained cells represents viable and
damaged cells, respectively. The cells were counted, and the percentage of viable cells was calculated by following formula

\[
\% \text{ age of viable cells} = \frac{\# \text{ of unstained cells}}{\# \text{ of cells}} \times 100
\]

Cells were counted under microscope (40 x) as follows.

Total number of cells counted in four WBCs chamber = \(x\)

Cells/ml = \(x \times 2 \times 10,000\)

Total cells = cells/ml \times total volume

In our experiment \(x = 31\)

So cells/ml = \(31 \times 2 \times 10,000 = 580000\)

Total volume with us was 10 ml

So total cells = \(620000 \times 10 = 6200000\) or \(6.2 \times 10^6\)

According to the rule of 85,000 cells/200 ul ((Abdul-Saeed et al., 1995),

85,000 cells are present in 200 µl or 0.2 ml

Therefore 01 cell was present in \(0.2/85000\)

6.2 \(\times 10^6\) cells were present in \(0.2/85000 \times 6.2 \times 10^6 = 14.588\) ml

So we added more Eagle’s medium in cell suspension till total volume became 14.588 ml to get 85000 cells/200 µl.

**2.2.6.7 Incubation**

All culture tubes were numbered in triplicates. In all the tubes, 200 ul of cell suspension was pipetted to get 85,000 Leydig cells per tube

Cell sample were placed in culture tubes and different chemicals added as shown in pipetting protocol and then we incubated at 34°C in shaking water bath at 75 RPM. The reaction was then stopped after 03 hours by placing tubes
in 60°C water bath. After stopping reaction, the supernatant was pipetted in 1.5 ml eppendorf storing tubes and stored at -80°C till analysis.

Cell culture experiment was run in two steps. In first step we determined appropriate doses of each chemical/ antioxidant. Then cells were exposed to most appropriate dose of chemical. All the combinations were run with and without LH, but here only that components of pipetting protocol are shown whose results are narrated in this study i.e., with LH only (except with Isobutyl methyl xanthine and pregnenolone containing wells, where their mechanism of action is explored).
Table 2.1: Pipetting protocol for incubation of cultured Leydig cells from rats, step 1. (All were run in triplicate).

<table>
<thead>
<tr>
<th>Tube No</th>
<th>Cell suspension 200 µl - Sample (S)</th>
<th>Stimulaion</th>
<th>Chemical</th>
<th>Bio phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S</td>
<td>-</td>
<td></td>
<td>100 µl</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>LH 50 µl</td>
<td></td>
<td>50 µl</td>
</tr>
<tr>
<td>3</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 10 nM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>4</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 100 nM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>5</td>
<td>S</td>
<td>LH 50 µl</td>
<td>NE 10 µM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>6</td>
<td>S</td>
<td>LH 50 µl</td>
<td>NE 100 µM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>7</td>
<td>S</td>
<td>LH 50 µl</td>
<td>NE 1000 µM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>8</td>
<td>S</td>
<td>LH 50 µl</td>
<td>IBMX 0.1 mM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>9</td>
<td>S</td>
<td>LH 50 µl</td>
<td>IBMX 1 mM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>10</td>
<td>S</td>
<td>LH 50 µl</td>
<td>IBMX 10 mM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>11</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Pregnenolone 1 µM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>12</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Pregnenolone 10 µM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>13</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Pregnenolone 100 µM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>14</td>
<td>S</td>
<td>LH 50 µl</td>
<td>AA 10 µM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>15</td>
<td>S</td>
<td>LH 50 µl</td>
<td>AA 100 µM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>16</td>
<td>S</td>
<td>LH 50 µl</td>
<td>AA 200 µM: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>17</td>
<td>S</td>
<td>LH 50 µl</td>
<td>AT 10 µg/ml: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>18</td>
<td>S</td>
<td>LH 50 µl</td>
<td>AT µg/ml: 15 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>19</td>
<td>S</td>
<td>LH 50 µl</td>
<td>AT µg/ml: 15 µl</td>
<td>35 µl</td>
</tr>
</tbody>
</table>

LH: Luteinizing hormone; NE: Nor epinephrine; IBMX: Isobutyl Methyl Xanthine; AA: Ascorbic acid; AT: Alpha tocopherol.
Table 2.2: Pipetting protocol for incubation of cultured Leydig cells from rats, step 2. (All were run in triplicate).

<table>
<thead>
<tr>
<th>Tube No</th>
<th>Cell suspension 200 µl - Sample (S)</th>
<th>Chemical</th>
<th>Chemical</th>
<th>Bio phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S</td>
<td>LH 50 µl</td>
<td>VIT C 15 µl + VIT E 15 µl</td>
<td>-</td>
</tr>
<tr>
<td>2a</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl + NE 15 µl</td>
<td>-</td>
</tr>
<tr>
<td>2b</td>
<td>S</td>
<td>-</td>
<td>Corticosterone 15 µl</td>
<td>IBMX 15 µl</td>
</tr>
<tr>
<td>3a</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl</td>
<td>IBMX 15 µl</td>
</tr>
<tr>
<td>3b</td>
<td>S</td>
<td>-</td>
<td>NE 15 µl</td>
<td>IBMX 15 µl</td>
</tr>
<tr>
<td>4a</td>
<td>S</td>
<td>LH 50 µl</td>
<td>NE 15 µl</td>
<td>IBMX 15 µl</td>
</tr>
<tr>
<td>4b</td>
<td>S</td>
<td>-</td>
<td>Corticosterone 15 µl + NE 15 µl</td>
<td>IBMX 15 µl</td>
</tr>
<tr>
<td>5a</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl + NE 15 µl</td>
<td>IBMX 15 µl</td>
</tr>
<tr>
<td>5b</td>
<td>S</td>
<td>-</td>
<td>Corticosterone 15 µl</td>
<td>Pregnenolone 15 µl</td>
</tr>
<tr>
<td>6a</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl</td>
<td>Pregnenolone 15 µl</td>
</tr>
<tr>
<td>6b</td>
<td>S</td>
<td>-</td>
<td>NE 15 µl</td>
<td>Pregnenolone 15 µl</td>
</tr>
<tr>
<td>7a</td>
<td>S</td>
<td>LH 50 µl</td>
<td>NE 15 µl</td>
<td>Pregnenolone 15 µl</td>
</tr>
<tr>
<td>7b</td>
<td>S</td>
<td>-</td>
<td>Corticosterone 15 µl + NE 15 µl</td>
<td>Pregnenolone 15 µl</td>
</tr>
<tr>
<td>8</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl + NE 15 µl</td>
<td>Pregnenolone 15 µl</td>
</tr>
<tr>
<td>9</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl</td>
<td>AA 15 µl</td>
</tr>
<tr>
<td>10</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl</td>
<td>AT 15 µl</td>
</tr>
<tr>
<td>11</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl</td>
<td>AA 15 µl + AT 15 µl</td>
</tr>
<tr>
<td>12</td>
<td>S</td>
<td>LH 50 µl</td>
<td>NE 15 µl</td>
<td>AA 15 µl</td>
</tr>
<tr>
<td>13</td>
<td>S</td>
<td>LH 50 µl</td>
<td>NE 15 µl</td>
<td>AT 15 µl</td>
</tr>
<tr>
<td>14</td>
<td>S</td>
<td>LH 50 µl</td>
<td>NE 15 µl</td>
<td>AA 15 µl + AA 15 µl</td>
</tr>
<tr>
<td>15</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl + NE 15 µl</td>
<td>AA 15 µl</td>
</tr>
<tr>
<td>16</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl + NE 15 µl</td>
<td>AT 15 µl</td>
</tr>
<tr>
<td>17</td>
<td>S</td>
<td>LH 50 µl</td>
<td>Corticosterone 15 µl + NE 15 µl</td>
<td>AA 15 µl + AT 15 µl</td>
</tr>
</tbody>
</table>

LH: Luteinizing hormone; NE: Nor epinephrine; IBMX: Isobutyl Methyl Xanthine; AA: Ascorbic acid; AT: Alpha tocopherol.
2.3 ESTIMATION OF HORMONES

2.3.1 TESTOSTERONE (for In vivo samples)

KIT: ADALTIS- EIagen Testosterone- LI 4011 K.

It is an enzyme immune assay kit for measurement of Testosterone in the serum or plasma quantitatively.

2.3.1.1 Assay Principle

It is based on solid phase competitive enzyme immuno assay. Horseradish peroxidase labeled Testosterone (HRP-testosterone) competes with the testosterone present in sample for a fixed and limited number of antibody sites immobilized on the walls of microstrips. After completion of competitive immuno reaction, wells of microplate are washed and the amount of HRP testosterone that is in bound state with antibody in the solid phase is measured by adding a chromogen/ substrate solution that which converts it to blue compound by the bound conjugate. After 15 minutes of incubation, sulphuric acid is added to stop the enzymatic reaction and as a result the color of solution is changed to yellow color. At 450 nm, the absorbance of solution is measured photometrically. The value of absorbance which we obtain is related inversely to the concentration of testosterone which is present in the sample. A calibration curve is drawn to find out the final values of testosterone in our study sample.

2.3.1.2 REAGENTS

Testosterone Microplate

The microplate which is provided has 12 strips x 8 wells. Each well is coated with anti testosterone obtained from rabbit.
**Testosterone Calibrators**   The vials contain standard Testosterone concentration and 0.09 % Sodium Azide. Testosterone concentrations are as follows: 0 – 0.2 – 1 – 4 – 8 – 16 ng/ml. regarding calibrators volume, the volume of zero calibrator was to be kept 2 ml and volume of other calibrators was maintained at 0.5 ml.

**HRP Testosterone Conjugate**

The bottle has 22 ml horseradish peroxidase labeled testosterone in buffer which is supplemented by the bovine serum albumin (0.5 %) and the testosterone binding protein displacers.

**Washing solution. (10 x concentrated)**

A bottle of the washing solution 10 x has Tween 20 (0.1 %) and Amphotericin B (2.5 ug/ml) which is present in the citrate- borate buffer, 50 ml. Contents of this vial are to be diluted 500 ml distilled water and thorough mixing is also required.

**Substrate HS**

Another bottle of substrate HS containing 0.26 mg/ml of 3,3',5,5' Tetramethyl benzidin (TMB) along with 0.01 w/v Hydrogen peroxide, in citrate buffer. There is 13 ml volume of this container.

### 2.3.1.3 TESTOSTERONE ASSAY PROCEDURE

- All reagents and samples were brought to the room temperature.
- Enough strips in strip holder were placed so that all tests can be run adequately.
- For photometer blank, 100 μl of substrate along with 100 μl stop solution were pipetted into 1ˢᵗ well.
Then we prepared the washing solution by mixing the contents of bottle with 450 ml of distilled water.

Fifty μl of calibrators and samples were pipetted into appropriate wells of the strips.

Then, 200 μl of HRP testosterone conjugate was added to each well in sequence. Then incubated for 120 minutes at 37°C without covering the plate.

After incubation, the incubation solution was discarded, wells were rinsed with washing solution three times with automatic washer and residual fluid was removed.

Then immediately 100 μl of chromogen/substrate mixture was pipetted into the rinsed wells.

It was then incubated for 15 minutes at room temperature.

The reaction was stopped by pipetting 100 μl of stop solution into the wells in the same sequence adopted to dispense the chromogen/substrate mixture.

Microplate was then shaken gently to avoid splashing.

It was then read at 450 nm within one hour. (Ballester et al., 2005)

### 2.3.1.4 CALCULATION OF RESULTS

For all standards as well as samples, $B/ B_0$ % was calculated.

$$B/ B_0 \% = \frac{\text{Absorb of Sample} - \text{Absorb of Blank}}{\text{Absorb of C}_0 - \text{Absorb of Blank}}$$

Then the standard curve was plotted on graph paper with calibrators on y axis and standard values of Testosterone on x axis, to find out the Testosterone concentrations, as shown in figure 2.1. (Ballester et al., 2005)
Table 2.3: Absorbance and B/ B₀ % of standards of Testosterone.

<table>
<thead>
<tr>
<th>Name</th>
<th>Absorbance</th>
<th>B/ B₀ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.053</td>
<td>-</td>
</tr>
<tr>
<td>C₀</td>
<td>2.098</td>
<td>100%</td>
</tr>
<tr>
<td>C₁</td>
<td>2.045</td>
<td>97%</td>
</tr>
<tr>
<td>C₂</td>
<td>1.693</td>
<td>80%</td>
</tr>
<tr>
<td>C₃</td>
<td>1.423</td>
<td>67%</td>
</tr>
<tr>
<td>C₄</td>
<td>1.078</td>
<td>50%</td>
</tr>
<tr>
<td>C₅</td>
<td>0.861</td>
<td>39%</td>
</tr>
</tbody>
</table>
Figure 2.1: Testosterone standard calibration curves for *in vivo* analysis (ELISA)
2.3.2 TESTOSTERONE (For *In vitro* analysis)

Testosterone EIA kit - Cayman chemicals

Catalogue No: 582701.

Lot No: 181722

2.3.2.1 Principle of the assay

Competition between testosterone and a testosterone acetyl cholinesterase conjugate (testosterone tracer) for a restricted amount of testosterone anti serum is the basis of this test. As the concentration of testosterone is fixed, so the amount of testosterone tracer that has ability to bind with the testosterone serum will be inversely proportional to the concentration of that testosterone which is present in the well. This antiserum-testosterone complex is in bound state to the anti rabbit IgG obtained from mouse that has been previously attached to the well. After that, the plate is washed any unbound reagents are removed after which the Ellman’s Reagent (which contains substrate to acetyl cholinesterase) is added to the well. The product which is obtained from this enzymatic reaction has a distinctive yellow color and absorbs strongly at 412 nm. The amount of testosterone tracer bound to well is narrated by intensity of this color on spectrophotometer. This amount of testosterone tracer is inversely related to the amount of free testosterone that is present in the well during the incubation.

2.3.2.2 Buffer preparation

1 EIA Buffer preparation

We diluted the contents of one vial of EIA Buffer concentrate (10x) (Cat No: 400060) with 90 ml of ultra pure water. Caution was taken to rinse the bottle to remove any precipitated salts.
2 Wash Buffer preparation

We diluted 5 ml wash buffer concentrate (400 X) (cat No: 400062) to total volume of 2 liters ultra pure water and then added 1 ml Tween 20 (Cat No: 400035)

2.3.2.3 Preparation of Assay specific reagents

Testosterone standard

We equilibrated the pipette tip for which we used ethanol by repetitively filling and expelling the tip with ethanol. Then we transferred 100 ul of testosterone EIA standard (Cat No 482704) in a test tube that was clean and then we diluted with 900 ul ultra pure water to achieve a final conc of 5 ng/ml.

To prepare the standard, we took eight test tubes and labeled them from 1 to 8. Then we aliquoted 900 ul of EIA buffer to tube no 1, and 500 ul of EIA buffer into tubes no 2 to 8. Then 100 ul of the bulk standard was transferred to tube no 1 and mixed thoroughly. The serial dilution was done by pipetting 500 ul from tube 1 and transferring it to tube no 2. After thorough mixing, 500 ul removed from tube no 2 and placed in tube no 3. This process was repeated for tubes no 4 to 8.

Testosterone AChE Tracer

100 dtn Testosterone AChE Tracer (Cat No 482700) was reconstituted with 6 ml EIA Buffer.

Testosterone EIA Antiserum

100 dtn Testosterone EIA Antiserum (Cat No 482702) was reconstituted with 6 ml EIA Buffer.

2.3.2.4 ASSAY PROCEDURE

Reagents addition: Following reagents were added:
1. **EIA Buffer**

   We added 100 ul of EIA Buffer to those wells labeled as the Non Specific Binding (NSB) wells. Then, 50 ul of EIA Buffer was added to Maximum binding (B₀) well. In case of assay of cell culture wells, culture medium was substituted for EIA buffer both in the NSB and B₀ wells.

2. **Testosterone EIA Standard**

   From tube no 8 we added 50 micro liters to both of lowest standard wells. Then added fifty micro liters from tube no 7 to succeeding standard wells till all standards were aliquoted. We used the same pipette tip for all standards and pipette tip was equilibrated before pipetting.

3. **Samples:**

   Then 50 ul of sample added to each well.

4. **Testosterone Ache Tracer**

   Then we added 50 ul of tracer to each well. However total activity and blank wells were spared from this step.

5. **Testosterone EIA Antiserum**

   Then we added 50 ul of serum to each well. Here we spared the total activity well, NSB and Blank well.

**Incubation of plate**

Plate was then incubated for 2 hours at room temperature by covering it with plastic film.
Development of plate

1. Ellman’s reagent was reconstituted immediately before use. To develop 100 wells, 100 dtn vial of Ellman’s reagent was reconstituted with 20 ml ultra pure water.
2. Then wells were emptied with rinsed five times with wash buffer.
3. After this, 200 ul of Ellman’s reagent was added to each well.
4. Then 5 ul of tracer was added to total activity wells.
5. Then plate was covered with plastic film. Plates were placed in dark for 90 then minutes.

Reading the plate

1. Bottom of plate was cleaned to remove dirt and finger prints.
2. Plate cover was removed carefully to prevent splashing of Ellman’s reagent.
3. Plate was read at 405 to 420 nm.

Performance characteristics:

The assay range to determine the testosterone concentration with this kit was 3.9 to 500 pg/ml. The intra assay coefficient of variance was 9%. The inter assay coefficient of variance was 8%.

2.3.2.5 Calculations for testosterone estimation

1. Absorbance readings of blank wells were abstracted from all wells
2. Then the absorbance readings from NSB wells were averaged
3. After this, absorbance from B0 wells were averaged
4. NSB average was subtracted from B0 average. This is labeled as the corrected B0 or the corrected maximum binding.
5. Then % B/ B₀ was calculated for remaining wells. To do this, average NSB absorbance was subtracted from S1 absorbance and then divided by B₀.

6. Then it was multiplied by 100 which will give us the value of % B/ B₀.

Repeat for S2-S8 and all sample wells.

**Plotting the Standard curve.**

Standard curve was plotted for standards S1-S8 versus testosterone concentration using linear (y) along with the log (x) axis.
Testosterone **In vitro**

![Graph showing testosterone calibration curves](image)

**Figure 2.2** Testosterone standard calibration curves (for *in vitro* analysis)

**Equation:**

\[ y = -0.0006x + 0.2852 \]

**Correlation Coefficient:**

\[ R^2 = 0.7662 \]
2.3.3 Corticosterone EIA Kit

Catalogue No: 500651
Lot number: 0404965

2.3.3.1 Principle

This assay is based on competition between corticosterone and a corticosterone acetyl cholinesterase (AChE) conjugate (Corticosterone tracer) for a limited number of corticosterone specific rabbit anti serum binding sites. Because the concentration of the corticosterone tracer is held constant while the concentration of corticosterone varies, the amount of corticosterone tracer which is able to bind to rabbit anti serum is considered to be inversely proportional to the concentration of corticosterone in the well. This rabbit anti serum corticosterone (either free or tracer) complex binds to the mouse monoclonal anti rabbit IgG that was previously attached to the well. The plate is washed to remove any unbound agents and then Ellman’s Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymic reaction has discrete yellow color and absorbs strongly at 412 nm. The intensity of this color determined spectrophotometrically is in proportion to amount of corticosterone tracer bound to the well which is inversely proportional to the amount of free corticosterone present in well during incubation.

2.3.3.2 Buffer preparation

EIA Buffer preparation

We diluted the contents of one vial of EIA Buffer concentrate with 90 ml of ultra pure water. Caution was taken to rinse the bottle to remove any precipitated salts.
**Wash Buffer preparation**

We diluted 5 ml wash buffer concentrate to total volume of 2 liters ultra pure water and then added 1 ml Tween 20.

**2.3.3.3 Preparation of Assay specific reagents**

**Corticosterone standard**

We equilibrated the pipette tip using ethanol by repeatedly filling and expelling the tip with ethanol. Then we transferred 100 ul of corticosterone EIA standard into a clean test tube and diluted with 900 ul ultra pure water to gain conc of 100 ng/ml.

To prepare the standard, we took eight test tubes and labeled them from 1 to 8. then we aliquoted 900 ul of EIA buffer to tube no 1, and 750 ul of EIA buffer into tubes no 2 to 8. Then 100 ul of the bulk standard was transferred to tube no 1 and mixed thoroughly. The serial dilution was done by removing 500 ul from tube 1 and placing in tube no 2. After thorough mixing, 500 ul removed from tube no 2 and placed in tube no 3. This process was repeated for tubes no 4 to 8.

**Corticosterone AChE Tracer**

100 dtm corticosterone AChE Tracer (vial no 2) was reconstituted with 6 ml EIA Buffer.

**Corticosterone EIA Antiserum**

100 dtm corticosterone EIA Antiserum (vial no 1) was reconstituted with 6 ml EIA Buffer.

**2.3.3.4 ASSAY PROCEDURE:**

Addition of reagents:

The addition of reagents is explained below:
**EIA Buffer**

We added 100 ul of EIA Buffer to Non Specific Binding (NSB) wells. Then, 50 ul of EIA Buffer was added to Maximum binding ($B_0$) wells. In case of assay of cell culture wells, culture medium was substituted for EIA buffer in NSB and $B_0$ wells.

**Corticosterone EIA Standard**

We added 50 ul from tube no 8 to both of lowest standard wells. Then added 50 ul from tube no 7 to next standard wells until all standards aliquoted. Same pipette tip was used for all standards and pipette tip was equilibrated before pipetting.

**Samples:**

Then 50 ul of sample was added to each well.

**Corticosterone AchE Tracer**

Then we added 50 ul of tracer to each well except total activity and blank wells

**Corticosterone EIA Antiserum**

Then we added 50 ul of serum to each well, except the total activity well, NSB and Blank well.

**Incubation of plate**

Plate was then incubated for 2 hours at room temperature by covering it with plastic film

**Development of plate**

- Ellman’s reagent was reconstituted immediately before use. To develop 100 wells, 100 dtn vial Ellman’s reagent was reconstituted with 20 ml of ultra pure water.
- Then wells were emptied and then rinsed five times with wash buffer.
- After this, 200 ul of Ellman’s reagent was added to each well.
• Then 5 ul of tracer was added to total activity wells.

Then plate was covered with plastic film. Plates were placed in dark for 90 minutes.

**Reading the plate**

Bottom of plate was cleaned to remove dirt and finger prints.

Plate cover was removed carefully to prevent splashing of Ellman’s reagent.

Plate was read between 405 and 420 nm.

**Precision:**

The intra assay coefficient of variance and inter assay coefficient of variance for the concentration of 10,000 pg/ml was 18.6% and 17.1 % respectively. The intra assay coefficient of variance and inter assay coefficient of variance for the concentration of 1600 pg/ml was 18.1% and 7.3 % respectively. The intra assay coefficient of variance and inter assay coefficient of variance for the concentration of 41 pg/ml was 16% and 12 % respectively.

**Assay range:**

Assay range for this kit to determine the corticosterone is 16.4 pg/ml to 10000 pg/ml

**2.3.3.5 Calculations for corticosterone estimation**

Absorbance readings of blank wells were abstracted from all wells.

Then the absorbance readings from NSB wells were averaged.

After this, absorbance from B0 wells were averaged.

NSB average was subtracted from B0 average. This is the corrected B0 or corrected maximum binding.
Then % B/ B₀ was calculated for remaining wells. To do this, average NSB absorbance was subtracted from S1 absorbance and then divided by B₀.

Then it was multiplied by 100 to obtain % B/ B₀. Repeat for S2-S8 and all sample wells.

**Plotting the Standard curve.**

Standard curve was plotted % B/ B₀ for standards S1-S8 versus corticosterone concentration (usually in pg/ml) using linear (y) and log (x) axis.
Figure 2.3  Corticosterone standard calibration curves.
2.3.4  Nor adrenaline ELISA

Catalogue number: BA-E 5200 Labor Diagnostica Nord (LDN)

2.3.4.1  Principle

Enzyme immunoassay used for quantitative determination of nor adrenaline. Nor adrenaline is detected by using a cis-diol specific affinity gel, acylated and then derivatized enzymatically.

The competitive ELISA kit uses the microtiter plate format. The antigen is bound to solid phase of the micro titer plate. The derivatized standards, controls and samples and solid phase bound analyte compete for a fixed number of anti serum binding sites. After the system is in equilibrium, free antigen and free antigen – antiserum complexes are removed by washing. The antibody bound to solid phase is detected by an anti rabbit IgG peroxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm.

Quantification of unknown samples is achieved by comparing their absorbance with a reference curve prepared with known standard concentrations.

Kit contents

BA- D- 0032  Microtitre plate
BA- E – 0090  Adhesive foil
BA- E – 0030  Wash buffer concentrate
BA- E - 0040  Enzyme conjugate
BA- E - 0055  Substrate
BA- E - 0080  Stop solution
BA- E - 0231  Nor adrenaline micro titer strips
BA- E - 5210  Nor adrenaline antiserum
2.3.4.2 Preparation of reagents

Wash Buffer

We diluted 20 ml of wash buffer concentrate with distilled water to make a final volume of 1000 ml.

Acylation solution:

We diluted 20 ul of Acylation concentrate in 1.2 ml of acylation diluent.
2.3.4.3 PROCEDURE

Step 1: Extraction and acylation:

- Respective wells of extraction plate were filled with sample volume of 100 ul, 10 ul standard and 10 ul control. Distilled water was added to each well if final volume was less than 100 ul.
- Assay buffer 50 ul was pipetted into all wells
- Extraction buffer 50 ul was then pipetted into all wells
- Plate was covered with adhesive foil. Then it was shaked at 600 rpm (25 °C) for 60 minutes.
- Foil was removed, plate emptied and dried by tapping over adsorbent material.
- Wash buffer was then pipetted 1 ml in all wells. Plate was covered with adhesive foil.
- Then it was shaked at 600 rpm (25 °C) for 5 minutes.
- Foil was removed, plate emptied and dried by tapping over adsorbent material
- Washing repeated as in steps 6, 7, 8.
- Acylation buffer 150 ul was pipetted into all wells
- Acylation solution 25 ul was then pipetted into all wells
- Then it was shaked at 600 rpm (25 °C) for 20 minutes.
- Foil was removed, plate emptied and dried by tapping over adsorbent material
Wash buffer was then pipetted 1 ml in all wells. Plate was covered with adhesive foil

Then it was shaked at 600 rpm (25 °C) for 5 minutes.

Foil was removed, plate emptied and dried by tapping over adsorbent material

Washing repeated as in steps 14, 15, 16.

Then 100 ul Hydrochloric acid was pipetted into all wells.

Plate was the covered with adhesive foil. Then it was shaked at 600 rpm (25 °C) for 10 minutes.

**Step 2: Enzymatic Conversion**

Micro titer plate was used into which 90 ul of extracted standards, controls and samples were pipetted.

Then enzyme solution was prepared by reconstituting the contents of vial labeled enzyme with 1 ml distilled water which were mixed thoroughly. Then we added 0.3 ml of coenzyme after which we added 0.7 ml of adjustment buffer. Thus a total volume of 2.0 ml was obtained.

Then, 25 ul of enzymatic solution was added to all wells.

Plate was the covered with adhesive foil. Then it was shaked at 600 rpm (25 °C) for 1 minute.

Then it was incubated for 2 hours at 37°C.

**Step 3: Noradrenaline ELISA**

Enzyme plate was then used to pipette 100 ul of standards, controls and samples, into precoated Microtitre strips.
Then, 50 ul of nor adrenaline antiserum was pipetted into all wells.

Plate was then covered with adhesive foil. Then it was incubated for 1 min on Room temperature on a shaker.

After this, 15 hours (over night) incubation was done at 2-8 °C.

Foil was then removed, contents aspirated and each well was washed four times with 300 ul of wash buffer. The plate was dried by tapping it over dry tissue paper.

Then 100 ul of enzyme conjugate was pipetted into all wells.

Plate was then covered with adhesive foil and incubated for 30 min at room temperature on a shaker with rpm of 600.

Foil was then removed, contents aspirated and each well was washed four times with 300 ul of wash buffer. The plate was dried by tapping it over dry tissue paper

Then 100 ul of substrate was pipetted into all wells.

Then plate was incubated for 30 min at room temperature on a shaker with rpm of 600.

In the end, 100 ul of stop solution was pipetted into all wells

Then the absorbance was measured by ELISA reader at wavelength of 450 nm.

**Assay characteristics:**

Analytic specificity for nor epinephrine was 100%, and for epinephrine was 0.14%. The analytical sensitivity was 0.2 ng/ml x correction factor (0.013). Intra assay coefficient of variance was 11.7% at 1.3 ng/ml and 8.4% for a concentration of 0.5 ng/ml.
2.3.4.3 Results calculation

Calibration curve was obtained by plotting the absorbance readings, measured for the standards (linear y axis) against standard concentrations (x axis). The standards which were referred were as follows:

- Standard A  0 ng/ml
- Standard B  0.45 ng/ml
- Standard C  1.5 ng/ml
- Standard D  4.5 ng/ml
- Standard E  15 ng/ml
- Standard F  45 ng/ml

Concentration of samples obtained form standard curve was multiplied with correction factor.

Correction factor = 10 ul (volume of standards extracted) / sample volume extracted.
Figure 2.4 Nor epinephrine standard curve (ELISA)

\[ y = -0.0153x + 0.9 \]
\[ R^2 = 0.5332 \]
2.3.5  LH ESTIMATION

Kit: LH EIA.

Catalogue number: 11-LUTHU-E01

Manufacturer: ALPCO Diagnostics

2.3.5.1 Principle of the method

It is a two step capture or sandwich type assay. This assay makes use of two highly specific monoclonal antibodies. A monoclonal antibody specific for LH is immobilized on micro well plate and another monoclonal antibody specific for a different region of LH is conjugated to the horse radish peroxidase (HRP). LH from sample and standards are permitted to bind to plate, washed afterwards and incubated with HRP conjugate. After a second washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of stopping solution. The absorbance is measured on Microtitre plate reader. The intensity of color formed is in direct proportion to the concentration of LH in the sample. A set of standards is used to plot a standard curve from which the amount of LH in sample and control is measured.

2.3.5.2 REAGENTS

Mouse anti LH antibody coated micro well plate – Break Apart Wells

96 wells

Mouse anti LH antibody - Horse radish peroxidase (HRP) conjugate concentrate

240 ul of HRP in 12 ml assay buffer

LH CALIBRATORS

Calibrator A 0 IU/L 2.0 ml

Calibrator B 1 IU/L 0.5 ml
Calibrator C  4 IU/L  0.5 ml
Calibrator D  10 IU/L  0.5 ml
Calibrator E  40 IU/L  0.5 ml
Calibrator F  100 IU/L  0.5 ml

Control

Ready to use

Wash buffer concentrate

50 ml of wash buffer was diluted with 450 ml of distilled water

Assay buffer

Ready to use

TMB substrate

Ready to use, bottle contains tetra methyl Benzidine and hydrogen peroxide.

Stopping solution

One vial containing 1 M sulfuric acid, ready to use

Performance characteristics:

Sensitivity of the kit for LH was 0.2 IU/L. The intra assay coefficient of variance for the concentration of 4.8 IU/L was 4.5 % and for a concentration of 53.28 IU/L was 2.9%. The inter assay coefficient of variance for the concentration of 5.15 IU/L was 5.1 % and for a concentration of 51.50 IU/L was 9.2%.

2.3.5.3 ASSAY PROCEDURE

- After bringing all samples and reagents to room temperature, working solutions for cortisol-HRP conjugate and wash buffer were prepared.
- Micro well strips were taken out of seal
25 ul of calibrator, control and samples were pipetted into respective wells

Then 100 ul of assay buffer was pipetted into all wells

Incubation was done for 30 minutes on plate shaker at 200 rpm.

Wells were washed three times with 300 ul diluted wash buffer per well and plate was tapped firmly against absorbent paper to make it dry

Then, 100 ul of conjugate working solution was put into all wells.

Incubation was done for 30 minutes on plate shaker at 200 rpm at room temperature

Wells were washed three times with 300 ul diluted wash buffer per well and plate was tapped firmly against absorbent paper to make it dry

The 100 ul of TMB substrate was pipetted into all wells

Then it was incubated on plate shaker for 15 to 20 minutes at room temperature.

Stop solution 50 ul was then pipetted in to all wells

Then absorbance was read on micro plate reader at 450 nm

2.3.5.4  CALCULATIONS

Mean optical density for each calibrator was calculated in duplicate

Then mean optical density of each unknown was calculated

Then we subtracted the mean absorbance value of ‘0’calibrator from the mean absorbance values of calibrators, control and serum samples.

Calibration curve was drawn on semi Log paper with mean optical densities on y axis and calibrator concentrations on x axis.

Values were read directly from calibration curve
Figure 2.5  Standard calibration curve for LH.

\[ y = 0.0186x + 0.1898 \]

\[ R^2 = 0.9703 \]
2.3.6 MALONDIALDEHYDE ESTIMATION

TBARS Essay kit- Cayman chemical company

Catalogue No: 10009055

Lot No: 181722

2.3.6.1 Principle of the assay

Thio Barbituric Acid Reactive Substances-(TBARS) assay provides a simple and standard method for assaying lipid peroxidation in various body fluids including plasma, serum, urine, tissue homogenates, and cell lysates. The chemical reaction is

\[ \text{MDA} + \text{TBA} \rightarrow \text{MDA-TBA Adduct} \]

The MDA-TBA adduct formed by above reaction under high temperature (90-100°C) and acidic conditions is measured colorimetrically at 530-540 nm. (Huang et al., 2002)

2.3.6.2 Preparation of Reagents

**Thio Barbituric Acid (TBA.)**

Each vial contains 2 g of TBA. It is ready to use to prepare the color reagent. One vial is sufficient to evaluate 24 wells.

**TBA – Acetic acid**-

The vial contains concentrated acetic acid. To prepare the solution, 40 ml of TBA Acetic acid was added slowly to 160 ml of HPLC grade water. This solution was used to prepare the color reagent.

**TBA-Sodium Hydroxide (10 X)**

20 ml of TBA Sodium Hydroxide was diluted with 180 ml of HPLC grade water. This solution will be used to prepare the color reagent.
TBA- Malondialdehyde standard.

This vial contains 500 μM MDA in water. It was ready to use

TBA SDS Solution.

This vial contains sodium dodecyl sulfate (SDS). It is ready to use preparation.

Preparation of color reagent

We weighed 530 mg of TBA and it was added to 50 ml of diluted TBA Acetic acid solution in 500 ml beaker. Then 50 ml of diluted TBA Sodium Hydroxide solution was added and mixed until TBA completely dissolved. Then other three vial were also processed in a similar way.

2.3.6.3 PERFORMING THE ASSAY

- In order to prepare MDA Standards, 250 μl of MDA standard was diluted with 750 μl of water to obtain a stock solution of 125 μM. Glass tubes, 8 in number were taken and labeled from A to H. Water and 125 μM MDA was added to tubes as described in table 3.3.
- Vial caps were labeled with identification numbers.
- Then, 100 μl of sample or standard was added to labeled vials.
- After this, 100 μl of SDS solution was added to vials and mixed on maxi mixer.
- In each vial, 4 ml of color reagent was added
- Vials were caped and placed in holder to keep them upright.
- Vials were then placed in vigorously boiling water and allowed to boil for 1 hour.
After one hour, vials were removed and placed in ice bath for 10 minutes to stop the reaction.

After 10 minutes vials were centrifuged for 10 minutes at 1600 x g at 4°C.

Vials were brought to room temperature and 150 μl from each vial was taken to plate

Absorbance was read at 545 nm using a plate reader (Huang et al., 2002).

**Precision:**

The intra assay coefficient of variation was 5.5% and interassay coefficient of variation was 5.9%

**Assay range:**

Assay range of this kit for determining MDA is 0 - 50 uM. (μM = umole/L = nmol/ml)

**2.3.6.3 CALCULATION OF RESULTS**

Average absorbance of each standard and sample was calculated.

Standard A absorbance was subtracted from all to get the value of corrected absorbance as shown in table 2.5

3. Standard curve was plotted by plotting corrected absorbance value and MDA concentration as shown in figure 2.6.

4. From standard curve, results were drawn.
Table 2.4: Concentrations for the preparation of Malondialdehyde standards.

<table>
<thead>
<tr>
<th>Test tube</th>
<th>MDA (μl)</th>
<th>Water (μl)</th>
<th>MDA concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>995</td>
<td>0.625</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>990</td>
<td>1.25</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>980</td>
<td>2.5</td>
</tr>
<tr>
<td>E</td>
<td>40</td>
<td>960</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>80</td>
<td>920</td>
<td>10</td>
</tr>
<tr>
<td>G</td>
<td>200</td>
<td>800</td>
<td>25</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>600</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 2.5: Absorbance and concentrations of Malondialdehyde standards.

<table>
<thead>
<tr>
<th>Name of standard</th>
<th>Absorbance</th>
<th>MDA concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0.009</td>
<td>0.625</td>
</tr>
<tr>
<td>C</td>
<td>0.011</td>
<td>1.25</td>
</tr>
<tr>
<td>D</td>
<td>0.014</td>
<td>2.5</td>
</tr>
<tr>
<td>E</td>
<td>0.020</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>0.028</td>
<td>10</td>
</tr>
<tr>
<td>G</td>
<td>0.057</td>
<td>25</td>
</tr>
<tr>
<td>H</td>
<td>0.103</td>
<td>50</td>
</tr>
</tbody>
</table>
Figure 2.6 Malondialdehyde EIA standard calibration curve.

The equation for the calibration curve is:

\[ y = 0.0021x + 0.0028 \]

with a correlation coefficient of \( R^2 = 0.9791 \).
2.3.7 Super oxide dismutase:

Superoxide dismutase assay kit- Cayman chemical company
Catalogue No: 706002

2.3.7.1 Principle:

This kit utilizes a tetrazolium salt for detection of superoxide radicals generated by Xanthine oxidase and hypo xanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD assay measures all three types of SOD, (Cu/Zn, Mn, and Fe-SOD). This assay provides a simple, reproducible and fast tool for assaying SOD activity in serum, plasma etc.

2.3.7.2 PRE ASSAY PREPARATION

Preparation of reagents

1 Assay buffer

We diluted 2 vials of assay buffer concentrate in 54 ml of HPLC grade water. This preparation was used to dilute radical detector.

2 Sample buffer

Two vials of sample buffer were diluted in 36 ml of HPLC grade water. It was used to prepare

3 Radical detector

The vial contains tetrazolium salt. Transfer 100 ul of solution in a vial and add 39.90 ml of diluted assay buffer. Then it was covered with tin foil.
SOD Standard:

The vial contains a solution of bovine erythrocyte SOD. It is ready to use preparation.

6 Xanthine Oxidase

These vials contain a solution of Xanthine Oxidase. We diluted 100 ul of enzyme with 3.90 ml of sample buffer. It was stored on ice.

2.3.7.3 Assay procedure

We diluted 20 ul of SOD standard with 1.8 ml of sample buffer to obtain SOD stock solution. Then seven glass tubes were taken and marked from A to G. Then we added SOD and sample buffer to each tube as described in table SOD

<table>
<thead>
<tr>
<th>Tube</th>
<th>SOD Stock ul</th>
<th>Sample buffer ul</th>
<th>Final SOD activity U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>980</td>
<td>0.025</td>
</tr>
<tr>
<td>C</td>
<td>40</td>
<td>960</td>
<td>0.05</td>
</tr>
<tr>
<td>D</td>
<td>80</td>
<td>920</td>
<td>0.1</td>
</tr>
<tr>
<td>E</td>
<td>120</td>
<td>880</td>
<td>0.15</td>
</tr>
<tr>
<td>F</td>
<td>160</td>
<td>840</td>
<td>0.2</td>
</tr>
<tr>
<td>G</td>
<td>200</td>
<td>800</td>
<td>0.25</td>
</tr>
</tbody>
</table>

SOD Standard wells. We added 200 ul of diluted radical detector and 10 ul of standard tubes per well in designated wells on plate.
Sample wells: we added 200 ul of diluted radical detector and 10 ul of samples to the wells
The reaction was initiated by adding 20 ul of diluted xanthine oxidase to all wells, and this step was performed quickly.
Plate was shaken carefully for few seconds to mix it well
Plate was then incubated at room temperature for 20 minutes and absorbance was read at 450 nm using a plate reader.

Assay range:
Dynamic range of this kit is .025 -0.25 units/ml super oxide dismutase.

Precision:
The intra assay coefficient of variance was 3.2 % and the inter assay coefficient of variance was 3.7%.

2.3.7.4 CALCULATION OF RESULTS
We calculated the average absorbance of each standard and sample.
Then we divided the standard A’s absorbance by itself and standard A’s absorbance by all the other standards and samples absorbances to yield the linearized rate.
Then we plotted the linearized SOD standard rate as a function of final SOD activity (U/ml).
Then we calculated the SOD activity of samples using the equation obtained from linear regression of standard curve substituting the linearized rate for each sample.

STATISTICAL ANALYSIS
Data were analyzed on SPSS version 13. The arithmetic mean and standard error of mean of all samples was calculated. One way ANOVA was applied to observe the level of significance among groups followed by Post Hoc Tukey’s test for multiple
comparisons. Difference in mean among the control and treated groups was calculated by 'Independent sample t test' for 2 group comparisons. The difference was considered significant if p value was found less than 0.05. (Sengupta et al., 2004)
Figure 2.7  Standard Calibration curve for Super oxide dismutase

\[ y = 11.689x + 1.0116 \]

\[ R^2 = 0.9915 \]
RESULTS

In vivo results

The in vivo component of this project was done on eighty male Sprague Dawley rats by dividing them into five groups with 16 rats in each group. All rats remained alive and healthy for whole period of study and took their feed properly.

Acute stress for six hours in mesh wire restrainer resulted in a significant decrease in levels of serum testosterone \((p \text{ value} < 0.01)\), while serum corticosterone \((p \text{ value} < 0.01)\) levels significantly increased. The levels of plasma nor-epinephrine were also increased in significant amount \((p \text{ value} < 0.001)\) after exposure to restraint stress.

There was statistically insignificant \((p \text{ value} > 0.05)\) change in the levels of serum LH. The lipid peroxidation increased after stress exposure quite significantly \((p \text{ value} < 0.01)\) which is expressed in our study in terms of serum malondialdehyde levels. Stress exposure also resulted in a significant fall in superoxide dismutase activity \((p \text{ value} < 0.01)\) as shown in table 3.1.

Effects of one month supplementation with ascorbic acid on stress induced changes:

The results shown in table 3.2 reveal that one month supplementation with ascorbic acid was unable to prevent the acute restraint stress induced decline in serum testosterone levels \((p \text{ value} > 0.05)\). Serum corticosterone \((p \text{ value} > 0.05)\) and plasma nor epinephrine \((p \text{ value} > 0.05)\) showed insignificant change as compared to non-supplemented group. The levels of serum LH were also unchanged \((p \text{ value} > 0.05)\) after ascorbic acid supplementation.
The lipid peroxidation which is expressed in our study in terms of serum malondialdehyde levels remained high after stress exposure quite significantly ($p \text{ value} > 0.05$) despite ascorbic acid supplementation. However ascorbic acid supplementation resulted in a significant rise in superoxide dismutase activity despite the exposure to stress ($p \text{ value} < 0.05$).

Effects of one month supplementation of alpha tocopherol on stress induced changes

The results shown in table 3.3 reveal that one month supplementation with alpha tocopherol significantly prevented the acute restraint stress induced decline in serum testosterone levels ($p \text{ value} < 0.01$). The serum corticosterone ($p \text{ value} < 0.01$) decreased in the group of rats supplemented with alpha tocopherol. The plasma nor epinephrine ($p \text{ value} > 0.05$) showed insignificant change as compared to non-supplemented group. The levels of serum LH were also unchanged ($p \text{ value} > 0.05$) after alpha tocopherol supplementation.

The lipid peroxidation in terms of serum malondialdehyde levels, decreased significantly ($p \text{ value} < 0.05$) in alpha tocopherol supplemented group after stress exposure. Also in alpha tocopherol supplemented group, there was a significant rise in superoxide dismutase activity despite the exposure to stress ($p \text{ value} < 0.05$).
Table 3.1: Effects of 06 hours restraint stress on serum Testosterone, serum Corticosterone, plasma Nor epinephrine serum LH, serum Malondialdehyde and serum Super oxide dismutase levels in male Sprague Dawley rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I Control (n=16) Mean ± SEM</th>
<th>Group II Acute restraint stress (n=16) Mean ± SEM</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Testosterone (ng/ml)</td>
<td>6.09 ± 0.15</td>
<td>5.18 ± 0.19</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Serum Corticosterone (pg/ml)</td>
<td>8870.00 ± 175.35</td>
<td>9688.75 ± 219.18</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Plasma Nor epinephrine (ng/ml)</td>
<td>2.59 ± 0.18</td>
<td>5.06 ± 0.31</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum LH (IU/L)</td>
<td>9.10 ± 0.08</td>
<td>9.17 ± 0.09</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum Malondialdehyde (nmol/ml)</td>
<td>9.71 ± 0.59</td>
<td>14.77 ± 1.18</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Serum Super oxide dismutase (U/ml)</td>
<td>0.14 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

*p value less than 0.05 is taken as significant.
Table 3.2: Effects of one month, ascorbic acid supplementation (AAS) on changes in serum Testosterone, serum Corticosterone, plasma Nor epinephrine, serum Luteinizing hormone, serum Malondialdehyde and Superoxide Dismutase levels in male Sprague Dawley rats exposed to six hours restraint stress

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group II Acute restraint stress (n=16) Mean ± SEM</th>
<th>Group III AAS and restraint stress (n=16) Mean ± SEM</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Testosterone (ng/ml)</td>
<td>5.18 ± 0.19</td>
<td>5.29 ± 0.20</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum Corticosterone (pg/ml)</td>
<td>9688.75 ± 219.18</td>
<td>9229.38 ± 240.95</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Plasma Nor epinephrine (ng/ml)</td>
<td>5.06 ± 0.31</td>
<td>5.45 ± 0.21</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum LH (IU/L)</td>
<td>9.17 ± 0.09</td>
<td>9.26 ± 0.11</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum Malondialdehyde (nmol/ml)</td>
<td>14.77 ± 1.18</td>
<td>13.88 ± 0.96</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum Sup oxide dismutase (U/ml)</td>
<td>0.08 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

*p value less than 0.05 is taken as significant.
Effects of one month supplementation of ascorbic acid and alpha tocopherol on stress induced changes:

The results shown in table 3.4 reveal that one month supplementation with combination of ascorbic acid and alpha tocopherol significantly prevented the acute restraint stress induced decline in serum testosterone levels ($p$ value $< 0.01$). The serum corticosterone ($p$ value $< 0.01$) were decreased in the group of rats supplemented with combination of antioxidants. The plasma nor epinephrine ($p$ value $> 0.05$) showed the insignificant change even in this group also. The levels of serum LH were also unchanged ($p$ value $> 0.05$) after combined antioxidants supplementation.

The lipid peroxidation in terms of serum malondialdehyde levels decreased significantly ($p$ value $< 0.01$) in ascorbic acid and alpha tocopherol supplemented group after stress exposure. There was also a significant rise in superoxide dismutase activity of this combination supplementation group rats despite the exposure to restraint stress for six hours ($p$ value $< 0.01$).
Table 3.3: Effects of one month alpha tocopherol supplementation (ATS) on changes in serum Testosterone, serum Corticosterone, plasma Nor epinephrine, serum Luteinizing hormone, serum Malondialdehyde and Superoxide Dismutase levels in male Sprague Dawley rats exposed to six hours restraint stress

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group II Acute restraint stress (n=16) Mean ± SEM</th>
<th>Group IV ATS and restraint stress (n=16) Mean ± SEM</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Testosterone (ng/ml)</td>
<td>5.18 ± 0.19</td>
<td>6.80 ± 0.23</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Serum Corticosterone (pg/ml)</td>
<td>9688.75 ± 219.18</td>
<td>8710.63 ± 149.15</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Plasma Nor epinephrine (ng/ml)</td>
<td>5.06 ± 0.31</td>
<td>5.28 ± .17</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum LH (IU/L)</td>
<td>9.17 ± 0.09</td>
<td>9.18 ± 0.08</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum Malondialdehyde (nmol/ml)</td>
<td>14.77 ± 1.18</td>
<td>10.84 ± 1.12</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Serum Superoxide dismutase (U/ml)</td>
<td>0.08 ± 0.01</td>
<td>0.13 ± 0.07</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

*p value less than 0.05 is taken as significant.
Table 3.4: Effect of combined ascorbic acid and alpha tocopherol supplementation on changes in serum Testosterone, serum Corticosterone, plasma Nor epinephrine, serum Luteinizing hormone, serum Malondialdehyde and Superoxide Dismutase levels in male Sprague Dawley rats exposed to six hours restraint stress

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group II Acute restraint stress (n=16) Mean ± SEM</th>
<th>Group V AAS + ATS and restraint stress (n=16) Mean ± SEM</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Testosterone (ng/ml)</td>
<td>5.18 ± 0.19</td>
<td>7.06 ± 0.15</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Serum Corticosterone (pg/ml)</td>
<td>9688.75 ± 219.18</td>
<td>8776.25 ± 157.71</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Plasma Nor epinephrine (ng/ml)</td>
<td>5.06 ± 0.31</td>
<td>5.29 ± 0.32</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum LH (IU/L)</td>
<td>9.17 ± 0.09</td>
<td>9.15 ± 0.08</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serum Malondialdehyde (nmol/ml)</td>
<td>14.77 ± 1.18</td>
<td>10.27 ± 0.99</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Serum Superoxide dismutase (U/ml)</td>
<td>0.08 ± 0.01</td>
<td>0.13 ± 0.007</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

*p value less than 0.05 is taken as significant.
**IN VITRO FINDINGS:**

All samples were run in triplicate. Therefore, all findings are based on mean of three results.

**Effect of LH on testosterone release by Leydig cells**

Basal testosterone production was 48.67 ± 3.85 pg/well and with LH stimulation raised to 189.78 ± 1.47 pg/ well (*p value* < 0.001) as shown in figure 3.1. Therefore, LH significantly increased testosterone production by isolated Leydig cells placed in well.

**Effects of different concentrations of corticosterone in the presence of LH on testosterone production by Leydig cells:**

The mean value of the testosterone produced under the effect of LH alone was 189.78 ± 1.47 pg/ well. The mean value of testosterone production was 190.89 ± 4.34 pg/ well when exposed to 10 nM concentration of corticosterone, while addition of corticosterone in concentration of 100 nM resulted in mean testosterone production of 112.56 ± 2.94 pg/ well as shown in figure 3.2. The production of testosterone in the presence of LH was significantly reduced by 100 nM concentration of corticosterone with a *p value* < 0.05.

**Effects of different concentrations of nor epinephrine in the presence of LH, on testosterone production by Leydig cells:**

The mean value of the testosterone produced under the effect of LH alone was 189.78 ± 1.47 pg/well. The mean value of testosterone production was 220.89 ± 6.76 pg/ well when exposed to 10 µM concentration of nor epinephrine, while addition of nor epinephrine in concentration of 100 µM resulted in mean
Figure 3.1: Effect of LH stimulation on testosterone production by cultured Leydig cells (*p value < 0.001 as compared to basal, without LH)
testosterone production of 205.33 ± 6.94 pg/ well. When we added 1000 µM concentration of nor epinephrine, the mean value of testosterone was 196.44 ± 4.84 pg/ well, as shown in figure 3.3. The production of testosterone in the presence of LH was significantly increased by 10 µM concentration of nor epinephrine with a p value < 0.05.

Effects of different concentrations of Iso butryl methyl xanthine (IBMX) in the presence of LH, on testosterone production by Leydig cells:

The mean value of the testosterone produced under the effect of LH alone was 189.78 ± 1.47 pg / well. The mean value of testosterone production was 197.55 ± 4.34 pg/ well when exposed to 0.1 mM concentration of IBMX, while addition of IBMX in concentration of 1 mM resulted in mean testosterone production of 229.78 ± 8.73 pg/ well. When we added 10 mM concentration of IBMX, the mean value of testosterone was 191.44 ± 3.09 pg/ well. The production of testosterone in the presence of LH was significantly increased by 1 mM of IBMX with a p value < 0.05 as shown in figure 3.4.
Figure 3.2: Effect of different concentrations of corticosterone on testosterone production by cultured Leydig cells incubated with LH (* p value < 0.05 as compared with the well incubated with LH alone)

Figure 3.3: Effect of different concentrations of nor epinephrine on testosterone production by cultured Leydig cells incubated with LH (*p value< 0.05 as compared with the well incubated with LH alone)
Figure 1.4: Effects of different concentrations of Isobutyl methyl xanthine (IBMX) on testosterone production by cultured Leydig cells incubated with LH (*p value < 0.05 as compared with the well incubated with LH alone)
Effects of different concentrations of pregnenolone in the presence of LH, on testosterone production by Leydig cells:

The mean value of the testosterone produced under the effect of LH alone was 189.78 ± 1.47 pg/well. The mean value of testosterone production was 192 ± 7.70 pg/well when exposed to 0.1 µM concentration of pregnenolone, while addition of pregnenolone in concentration of 1 µM resulted in mean testosterone production of 241.44 ± 8.18 pg/well. When we added 10 µM concentration of pregnenolone, the mean value of testosterone was 199.22 ± 6.41 pg/well, as shown in figure 3.5. The production of testosterone in the presence of LH was significantly increased by 1 µM of pregnenolone with a p value< 0.05.

Effects of different concentrations of ascorbic acid in the presence of LH, on testosterone production by Leydig cells:

The mean value of the testosterone produced under the effect of LH alone was 189.78 ± 1.47 pg/tube. The mean value of testosterone production was 194.22 ± 4.84 pg/tube when exposed to 10 µM concentration of ascorbic acid, while addition of ascorbic acid in concentration of 100 µM resulted in mean testosterone production of 207.00 ± 6.31 pg/tube. When we added 200 µM concentration of ascorbic acid, the mean value of testosterone was 153.67 ± 5.36 pg/tube, as shown in figure 3.6. The production of testosterone in the presence of LH was insignificantly changed by low doses of ascorbic acid, while high dose has inhibitory effect (p value< 0.01).
Figure 3.5  Effect of different concentrations of pregnenolone on testosterone production by cultured Leydig cells incubated with LH  (* p value< 0.05 as compared with the well incubated with LH alone)

Figure 3.6  Effect of different concentrations of ascorbic acid (AA) in the presence of LH on testosterone production by cultured Leydig cells incubated with LH. (p value< 0.01 as compared with the well incubated with LH alone)
Effects of different concentrations of alpha tocopherol in the presence of LH, on testosterone production by Leydig cells:

The mean value of the testosterone produced under the effect of LH alone was 189.78 ± 1.47 pg/well. The mean value of testosterone production was 256.45 ± 7.22 pg/well when exposed to 10 µg/ml concentration of alpha tocopherol, while addition of alpha tocopherol in concentration of 100 µM resulted in mean testosterone production of 201.45 ± 7.22 pg/well. When we added 1000 µg/ml concentration of alpha tocopherol, the mean value of testosterone was 158.11 ± 6.26 pg/well, as shown in figure 3.7. The production of testosterone in the presence of LH was significantly increased by 10 µg/ml of alpha tocopherol with a p value < 0.01.

Effects of combination of corticosterone and nor epinephrine in the presence of LH, on testosterone production by Leydig cells:

The mean value of the testosterone produced under the effect of LH alone was 189.78 ± 1.47 pg/well. However, exposure of Leydig cells in the experimental well containing LH along with corticosterone in concentration of 100 nM and nor epinephrine 10 µM resulted in significant reduction (p value < 0.05) in mean testosterone production to 136.45 ± 6.83 pg/well. These results are shown in figure 3.8.
Figure 3.7: Effect of different concentrations of alpha tocopherol (AT) in the presence of LH on testosterone production by cultured Leydig cells incubated with LH. (*p value< 0.01 as compared with the well incubated with LH alone)
Figure 3.8: Effect of combination of corticosterone and nor epinephrine on testosterone production by LH stimulated cultured Leydig cells incubated with LH. (*p value < 0.05 as compared with the well incubated with LH alone)
Effect of IBMX on basal testosterone production by cultured Leydig cells:

The mean value of the testosterone produced without LH stimulation was 48.67 ± 3.85 pg/well. However, addition of IBMX to the well containing Leydig cells without LH resulted in significant increase (p value< 0.001) in mean testosterone production to 140.89 ± 6.76 pg/well as shown in figure 3.9.

Effect of corticosterone on IBMX induced increase in basal testosterone production by cultured Leydig cells:

The mean value of the testosterone produced under the effect of IBMX was 140.89 ± 6.76 pg/well. The corticosterone in conc of 100 nM resulted in a significant decrease (p value< 0.05) in mean testosterone production to 113.67 ± 6.01 pg/well as shown in figure 3.10.

Effect of corticosterone on IBMX induced increase in testosterone production by cultured Leydig cells in the presence of LH:

The mean value of the testosterone produced under the effect of IBMX was 229.78 ± 8.73 pg/well. The corticosterone in conc of 100 nM resulted in a significant decrease (p value< 0.01) in mean testosterone production to 161.47 ± 6.19 pg/well as shown in figure 3.11.

Effect of nor epinephrine on IBMX induced increase in basal testosterone production by cultured Leydig cells:

The mean value of the testosterone produced under the effect of IBMX was 140.89 ± 6.76 pg/well. The nor epinephrine in conc of 10 µM, as shown in figure 3.12 resulted in a significant increase (p value<0.05) in mean testosterone production to 169.78 ± 8.01 pg/well.
Effect of nor epinephrine on IBMX induced increase in testosterone production by cultured Leydig cells in the presence of LH:

The mean value of the testosterone produced under the effect of IBMX in the presence of LH was $229.78 \pm 8.73$ pg/well. The nor epinephrine in conc of 10 µM resulted in an insignificant change ($p \text{ value} > 0.05$) in mean testosterone production with value of $221.45 \pm 12.56$ pg/well as shown in figure 3.13.
Fig 3.9: Effect of IBMX on testosterone production by Leydig cells incubated without LH. (*p value < 0.001 as compared with the well incubated without LH with basal testosterone production)

Fig 3.10: Effect of corticosterone on IBMX induced increment in testosterone production by Leydig cells incubated without LH. (*p value < 0.05 as compared with the well incubated with IBMX alone)
Fig 3.11: Effect of corticosterone on IBMX induced increment in testosterone production by Leydig cells incubated with LH. (*p value < 0.01 as compared with the well incubated with IBMX and LH)

Fig 3.12: Effect of nor epinephrine on IBMX induced increment in testosterone production by Leydig cells incubated without LH. (*p value < 0.05 as compared with the well incubated with IBMX)

Fig 3.13: Effect of nor epinephrine on IBMX induced increment in testosterone production by Leydig cells incubated with LH (*p value > 0.05 as compared with well containing IBMX and LH)
Effect of pregnenolone on basal testosterone production by cultured Leydig cells:

The mean value of the testosterone produced without LH stimulation was 48.67 ± 3.85 pg/well. However, addition of pregnenolone to the well containing Leydig cells without LH resulted in significant increase (p value< 0.01) in mean testosterone production to 95.89 ± 5.80 pg/well as shown in figure 3.14.

Effect of corticosterone on pregnenolone induced increase in basal testosterone production by cultured Leydig cells:

The mean value of the testosterone produced under the effect of pregnenolone was 95.89 ± 5.80 pg/well. The mean testosterone produced in the experimental well where we added corticosterone in conc of 100 nM to pregnenolone was 100.89 ± 6.01 pg/well, which showed an insignificant change (p value> 0.05). These results are shown in figure 3.15.

Effect of corticosterone on pregnenolone induced increase in testosterone production by cultured Leydig cells in the presence of LH:

The mean value of the testosterone produced under the effect of pregnenolone was 241.44 ± 8.18 pg/well. The effect of corticosterone as shown in figure 3.16, in conc of 100 nM which resulted in a significant decrease (p value< 0.01) in mean testosterone production to 160.89 ± 6.26 pg/well.
Effect of nor epinephrine on pregnenolone induced increase in basal testosterone production by cultured Leydig cells:

The mean value of the testosterone produced under the effect of pregnenolone was 95.89 ± 5.80 pg/well. The nor epinephrine in conc of 10 µM resulted in an insignificant change (p value > 0.05) and value of mean testosterone production is 98.11 ± 3.05 pg/well as shown in figure 3.17.

Effect of nor epinephrine on pregnenolone induced increase in basal testosterone production by cultured Leydig cells in the presence of LH:

The mean value of the testosterone produced under the effect of pregnenolone in the presence of LH was 241.44 ± 8.18 pg/well. The nor epinephrine in conc of 10 µM resulted in an insignificant change (p value > 0.05) in mean testosterone production with value of 271.45 ± 7.22 pg/well as shown in figure 3.18
Fig 3.14: Effect of pregnenolone on testosterone production by Leydig cells incubated without LH. (*p value < 0.01 as compared with the well incubated without LH with basal testosterone production)

Fig 3.15: Effect of corticosterone on pregnenolone induced increment in testosterone production by Leydig cells incubated without LH. (*p value > 0.05 as compared with pregnenolone alone).

Fig 3.16: Effect of corticosterone on pregnenolone induced increment in testosterone production by Leydig cells incubated with LH. (*p value < 0.01 as compared to pregnenolone with LH)
Fig 3.17: Effect of nor epinephrine on pregnenolone induced increment in testosterone production by Leydig cells incubated without LH. (*p value > 0.05 as compared to pregnenolone alone).

Fig 3.18: Effect of nor epinephrine on pregnenolone induced increment in testosterone production by Leydig cells incubated with LH. (*p value > 0.05 as compared to pregnenolone and LH).
Effect of ascorbic acid on corticosterone induced changes in testosterone production by cultured Leydig cells in the presence of LH.

Corticosterone, as mentioned before in a conc of 100 nM resulted in a fall of testosterone synthesis by cultured Leydig cells. The addition of ascorbic acid in 100 µM conc to our experimental well containing cultured Leydig cells exposed to corticosterone resulted in a significant rise (p value< 0.01) of testosterone production to a value of 198.11 ± 9.35 pg/well as shown in figure 3.19.

Effect of alpha tocopherol on corticosterone induced decline in testosterone production by cultured Leydig cells in the presence of LH.

The addition of alpha tocopherol in 10 µg/ml conc to our experimental well containing cultured Leydig cells exposed to corticosterone resulted in a significant rise (p value< 0.001) of testosterone production to a value of 197.00 ± 4.19 pg/well as shown in figure 3.20.

Effect of addition of both ascorbic acid and alpha tocopherol on corticosterone induced changes in testosterone production by cultured Leydig cells in the presence of LH.

The addition of ascorbic acid in 100 µM conc and alpha tocopherol in 10 µg/ml conc in the presence of LH to our experimental well containing cultured Leydig cells exposed to corticosterone resulted in a significant rise (p value< 0.001) of testosterone production to a value of 209.22 ± 7.78 pg/well as shown in figure 3.21.
Fig 3.19: Effect of ascorbic acid on corticosterone induced decline in testosterone production by isolated and cultured Leydig cells incubated with LH. (*p value < 0.01 as compared with corticosterone in presence of LH)

Fig 3.20: Effect of alpha tocopherol on corticosterone induced decline in testosterone production by isolated and cultured Leydig cells incubated with LH. (*p value < 0.001 as compared with corticosterone in the presence of LH)

Fig 3.21: Effect of both ascorbic acid and alpha tocopherol on corticosterone induced decline in testosterone production by isolated and cultured Leydig cells incubated with LH. (*p value < 0.001 as compared with corticosterone in the presence of LH)
Effect of ascorbic acid on nor epinephrine induced changes in testosterone production by cultured Leydig cells in the presence of LH.

Nor epinephrine, as mentioned before in a conc of 10 µM resulted in increase in testosterone synthesis by cultured Leydig cells. The addition of ascorbic acid in 100 µM conc to our experimental well containing cultured Leydig cells exposed to nor epinephrine resulted in insignificant change (p value > 0.05) of testosterone production to a value of 220.33 ± 3.46 pg/well as shown in figure 3.22.

Effect of alpha tocopherol on nor epinephrine induced changes in testosterone production by cultured Leydig cells in the presence of LH.

The addition of alpha tocopherol in 10 µg/ml conc to our experimental well containing cultured Leydig cells exposed to nor epinephrine resulted in a significant rise (p value < 0.01) of testosterone production to a value of 258.67 ± 4.41 pg/well as shown in figure 3.23.

Effect of addition of both ascorbic acid and alpha tocopherol on nor epinephrine induced changes in testosterone production by cultured Leydig cells in the presence of LH.

The addition of ascorbic acid in 100 µM conc and alpha tocopherol in 10 µg/ml conc in the presence of LH to our experimental well containing cultured Leydig cells exposed to nor epinephrine resulted in a significant rise (p value < 0.05) of testosterone production to a value of 243.11 ± 3.64 pg/well. These results are presented in figure 3.24.
Fig 3.22: Effect of ascorbic acid on nor epinephrine induced changes in testosterone production by isolated and cultured Leydig cells incubated with LH (*p value > 0.05 as compared with nor epinephrine in the presence of LH).

Fig 3.23: Effect of alpha tocopherol on nor epinephrine induced changes in testosterone production by isolated and cultured Leydig cells incubated with LH. (*p value < 0.01 compared with nor epinephrine in the presence of LH)

Fig 3.24: Effect of both ascorbic acid and alpha tocopherol on nor epinephrine induced changes in testosterone production by isolated and cultured Leydig cells incubated with LH. (*p value < 0.05 compared with nor epinephrine in the presence of LH)
Effect of ascorbic acid on corticosterone and nor epinephrine induced changes in testosterone production by cultured Leydig cells in the presence of LH.

Addition of both corticosterone, in a conc of 100 nM and nor epinephrine in a conc of 10 μM resulted in decrease in testosterone synthesis by cultured Leydig cells to a value of 136.45 ± 6.83 pg/well. The addition of ascorbic acid in 100 μM conc to our experimental well containing cultured Leydig cells exposed to both corticosterone and nor epinephrine resulted in testosterone production to a value of 197.55 ± 4.94 pg/well (p value< 0.01) as shown in figure 3.25.

Effect of alpha tocopherol on corticosterone and nor epinephrine induced changes in testosterone production by cultured Leydig cells in the presence of LH.

Addition of both corticosterone, in a conc of 100 nM and nor epinephrine in a conc of 10 μM resulted in decrease in testosterone synthesis by cultured Leydig cells to a value of 136.45 ± 6.83 pg/well. The addition of alpha tocopherol in 10 μg/ml conc to our experimental well containing cultured Leydig cells exposed to both corticosterone and nor epinephrine resulted in testosterone production to a value of 198.11 ± 3.89 pg/well (p value< 0.01) as shown in figure 3.26.

Effect of addition of both ascorbic acid and alpha tocopherol on nor epinephrine induced changes in testosterone production by cultured Leydig cells in the presence of LH.

Addition of both corticosterone, in a conc of 100 nM and nor epinephrine in a conc of 10 μM resulted in decrease in testosterone synthesis by cultured Leydig cells to a value of 136.45 ± 6.83 pg/well. The addition of both ascorbic acid in 100
µM conc and alpha tocopherol in 10 µg/ml conc to our experimental well containing cultured Leydig cells exposed to both corticosterone and nor epinephrine resulted in testosterone production to a value of 200.33 ± 3.47 pg/well (p value< 0.01) as shown in figure 3.27.
Fig 3.25: Effect of ascorbic acid on corticosterone and nor epinephrine induced decline in testosterone production by isolated and cultured Leydig cells incubated with LH. (*p value< 0.01 compared with nor epinephrine and corticosterone in the presence of LH)

Fig 3.26: Effect of alpha tocopherol on corticosterone and nor epinephrine induced decline in testosterone production by isolated and cultured Leydig cells incubated with LH. (*p value< 0.01 compared with nor epinephrine and corticosterone in the presence of LH)

Fig 3.27: Effect of ascorbic acid and alpha tocopherol on corticosterone and nor epinephrine induced decline in testosterone production by isolated and cultured Leydig cells incubated with LH. (*p value< 0.01 compared with nor epinephrine and corticosterone in the presence of LH)
Effect of stress hormones in the presence of LH on lipid peroxidation in terms of Malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity at Leydig cell level:

There was insignificant effect of LH alone on MDA as well as SOD activity (p value > 0.05) in cultured Leydig cells. The predominant steroid in rat, i.e., corticosterone significantly increased the lipid peroxidation (p value < 0.05) in terms of MDA and caused the SOD activity to decline significantly (p value < 0.05) as shown in table. However, the other important hormone nor epinephrine has shown no effect on MDA as well as SOD activity of cultured Leydig cells. But the combination of corticosterone and nor epinephrine has resulted in a significant rise of MDA (p value < 0.05) and also a statistically significant fall in SOD (p value < 0.01) activity in cultured Leydig cells of our study wells as shown in table 3.5.

Effect of antioxidants ascorbic acid (AA) and alpha tocopherol (AT) in the presence of LH on levels of Malondialdehyde (MDA) and superoxide dismutase (SOD) at Leydig cell level:

There was no significant effect (p value > 0.05) of ascorbic acid and alpha tocopherol separately on lipid peroxidation in terms of MDA activity of Leydig cells. However combination of AA and AT resulted in a significant (p value < 0.05) reduction in levels of MDA.

Ascorbic acid (p value < 0.05) and alpha tocopherol (p value < 0.05) has caused a significant rise in superoxide dismutase activity. This was also significantly raised (p value < 0.05) by combination of AA and AT, as shown in table 3.6.
Table 3.5: Effect of stress hormones in the presence of LH on lipid peroxidation in terms of Malondialdehyde (MDA) levels and Superoxide dismutase (SOD) activity at Leydig cell level:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Well Contents</th>
<th>Mean ± SEM</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehyde (nmol/well)</td>
<td>LH only</td>
<td>6.52 ± 0.63</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + Corticosterone 100 nM</td>
<td>9.14 ± 0.28</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + Nor epinephrine NE 10 µM</td>
<td>5.33 ± 0.28</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + Corticosterone 100 nM + Nor epinephrine NE 10 µM</td>
<td>9.46 ± 0.42</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Serum Superoxide dismutase (U/well)</td>
<td>LH only</td>
<td>0.10 ± 0.004</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + Corticosterone 100 nM</td>
<td>0.07 ± 0.006</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + Nor epinephrine NE 10 µM</td>
<td>0.09 ± 0.008</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + Corticosterone 100 nM + Nor epinephrine NE 10 µM</td>
<td>0.06 ± 0.005</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

*p value less than 0.05 is taken as significant.
Table 3.6: Effect of antioxidants ascorbic acid (AA) and alpha tocopherol (AT) in the presence of LH on levels of Malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity at Leydig cell level:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Well Contents</th>
<th>Mean ± SEM</th>
<th>*p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehyde (nmol/Well)</td>
<td>LH only</td>
<td>6.52 ± 0.63</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + AA 100 µM</td>
<td>5.01 ± 0.69</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + AT 10 µg/ml</td>
<td>4.70 ± 0.57</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + AA 100 µM + AT 10 µg/ml</td>
<td>3.74 ± 0.42</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Serum Superoxide dismutase (U/well)</td>
<td>LH only</td>
<td>0.10 ± 0.004</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + AA 100 µM</td>
<td>0.13 ± 0.008</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + AT 10 µg/ml</td>
<td>0.15 ± 0.006</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>LH + AA 100 µM + AT 10 µg/ml</td>
<td>0.15 ± 0.009</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

*p value* less than 0.05 is taken as significant.
Effect of antioxidants on stress hormone induced changes in Malondialdehyde (MDA) at Leydig cell level:

Effect of ascorbic acid on stress hormones induced changes in MDA levels

The levels of MDA in corticosterone added well was 9.14 ± 0.28 nmol /well and addition of ascorbic acid to this well containing LH and corticosterone resulted in significant reduction of MDA to 6.76 ± 0.27 nmol /well (p value < 0.01). Addition of ascorbic acid to NE exposed cells resulted in MDA levels of 5.08 ± 0.31 nmol /well, which is an insignificant change as compared to NE addition alone (p value > 0.05). These results are shown in figure 3.28. However ascorbic acid significantly reduced lipid peroxidation to 5.33 ± 0.73 nmol /well (p value < 0.01) in the corticosterone and NE added well. These results are shown in figure 3.28.

Effect of alpha tocopherol on stress hormones induced changes in MDA levels

The levels of MDA in corticosterone added well was 9.14 ± 0.28 nmol /well and addition of alpha tocopherol to this well containing LH and corticosterone resulted in significant reduction of MDA to 6.29 ± 0.56 nmol /well (p value < 0.05). Addition of alpha tocopherol to NE exposed cells resulted in decline in MDA levels to 3.75 ± 0.42 nmol /well, (p value < 0.05). Also, alpha tocopherol significantly reduced MDA to 6.29 ± 0.56 nmol /well (p value < 0.05) in the corticosterone and NE added well. These results are shown in figure 3.29.
Effect of ascorbic acid and alpha tocopherol combination on stress hormones induced changes in MDA levels

The levels of MDA in corticosterone added well was 9.14 ± 0.28 nmol /well and ascorbic acid and alpha tocopherol addition to this well containing LH and corticosterone resulted in significant reduction of MDA to 6.12 ± 0.42 nmol /well (p value < 0.01). Addition of ascorbic acid and alpha tocopherol to NE exposed cells resulted in fall in MDA levels to 3.75 ± 0.32 nmol /well, (p value < 0.05). Similarly, ascorbic acid and alpha tocopherol significantly reduced MDA levels to 7.24 ± 0.55 nmol /well (p value < 0.01) in the corticosterone and NE added well as shown in figure 3.30.
Figure 3.28: Effect of ascorbic acid (AA) on stress hormones corticosterone and norepinephrine (NE) induced changes in MDA levels

*p value < 0.01 as compared with corticosterone with LH
**p value > 0.05 as compared with nor epinephrine with LH
***p value < 0.01 as compared with corticosterone and nor epinephrine with LH

Figure 3.29: Effect of alpha tocopherol (AT) on stress hormones corticosterone and norepinephrine (NE) induced changes in MDA levels

*p value < 0.05 as compared with corticosterone with LH,
**p value < 0.05 as compared with nor epinephrine with LH
***p value < 0.05 as compared with corticosterone and nor epinephrine with LH
Figure 3.30: Effect of ascorbic acid (AA) and alpha tocopherol (AT) combination on stress hormones corticosterone and norepinephrine (NE) induced changes in MDA levels

* $p$ value < 0.01 as compared with corticosterone with LH

** $p$ value < 0.05 as compared with nor epinephrine with LH

*** $p$ value < 0.01 as compared with corticosterone and nor epinephrine with LH
Effect of antioxidants on stress hormone induced changes in superoxide dismutase (SOD) at Leydig cell level:

Effect of ascorbic acid on stress hormones induced changes in SOD levels

The levels of SOD in corticosterone added well was $0.07 \pm 0.005$ U/well and addition of ascorbic acid to this well containing LH and corticosterone resulted in significant rise of SOD to $0.11 \pm 0.01$ U/well (p value < 0.05). Addition of ascorbic acid to NE exposed cells resulted in SOD levels of $0.14 \pm 0.006$ U/well, which is a significant rise as compared to NE addition alone (p value < 0.05). Also, ascorbic acid significantly increased SOD to $0.11 \pm 0.008$ U/well (p value < 0.05) in the corticosterone and NE added well as shown in figure 3.31.

Effect of alpha tocopherol on stress hormones induced changes in SOD levels

The levels of SOD in corticosterone added well was $0.07 \pm 0.006$ U/well and addition of alpha tocopherol to this well containing LH and corticosterone resulted in significant rise of SOD to $0.15 \pm 0.008$ U/well (p value < 0.05). Addition of alpha tocopherol to NE exposed cells resulted a significant rise in SOD levels to $0.13 \pm 0.005$ U/well, (p value < 0.05). Also, alpha tocopherol cause a significant rise in SOD to $0.12 \pm 0.01$ U/well (p value < 0.05) in the corticosterone and NE added well. These results are shown in figure 3.32.

Effect of ascorbic acid and alpha tocopherol combination on stress hormones induced changes in SOD levels

The ascorbic acid and alpha tocopherol addition to the well containing LH and corticosterone resulted in significant rise of SOD to $0.07 \pm 0.006$ U/well (p value < 0.01). Addition of ascorbic acid and alpha tocopherol to NE exposed cells
resulted in a significant rise in SOD levels to \(0.15 \pm 0.01\) U/well (p value < 0.05). Similarly, ascorbic acid and alpha tocopherol significantly increased SOD levels to \(0.14 \pm 0.01\) U/well (p value < 0.05) in the corticosterone and NE added well as shown in figure 3.33.
Figure 3.31: Effect of ascorbic acid (AA) on stress hormones corticosterone and norepinephrine (NE) induced changes in SOD levels

*p value < 0.05 as compared with corticosterone with LH

** p value < 0.05 as compared with nor epinephrine with LH

*** p value < 0.05 as compared with corticosterone and nor epinephrine with LH

Figure 3.32: Effect of alpha tocopherol (AT) on stress hormones corticosterone and norepinephrine (NE) induced changes in SOD levels.

*p value < 0.05 as compared with corticosterone with LH

** p value < 0.05 as compared with nor epinephrine with LH

*** p value < 0.05 as compared with corticosterone and nor epinephrine with LH
Figure 3.33: Effect of ascorbic acid (AA) and alpha tocopherol (AT) combination on stress hormones corticosterone and norepinephrine (NE) induced changes in SOD levels

*p value < 0.01 as compared with corticosterone with LH

** p value < 0.05 as compared with nor epinephrine with LH

*** p value < 0.05 as compared with corticosterone and nor epinephrine with LH
DISCUSSION

Stress is an extensively prevalent problem, the contributing factors of which include changed life styles along with industrialization and pollution (Eskiocak et al., 2005). Stress participates in the pathophysiology of various diseases (McEwen, 2000). Stress of various origins has inhibitory effect on male reproductive functions (Hardy et al., 2005). We examined the effects of stress by the in vivo study using mesh wire restraint immobilization stress protocol on adult male Sprague Dawley rats. This immobilization stress model, serves as a mixture of psychological and physical stressors by isolating the experimental animal from its group and by restricting its movements (Pacak and Palkovits, 2001). Restraint stress as comprehended by shansky et al., not only stimulates a number of neurological changes but may also induce various molecular, biochemical and hormonal changes (Shansky et al., 2006).

We also designed an in vitro model of isolated and cultured Leydig cells to explore the effects of two important stress hormones in rat, i.e., corticosterone and nor epinephrine on testosterone synthesis by Leydig cells. The stress induced changes in lipid peroxidation and superoxide dismutase activity were probed. The individual as well as combined effects of antioxidants, ascorbic acid and alpha tocopherol to prevent the stress induced alterations in above parameters were investigated in both in vivo and in vitro models.

Our results of acute stress have shown that LH levels remained unchanged. It is supported by various studies which claim that acute stress mediated effects are not via changes in hypothalamo pituitary gonadal axis, therefore there is no change in
LH as a result of acute stress. Acute stress affects the steroidogenesis at the level of Leydig cells only. However, stress exposure of more than ten days may result in decline of LH levels (Maric et al., 1996). Hence the suppression of whole hypothalamo pituitary gonadal axis by acute stress is ruled out by the data of present study.

Stress of various origins suppresses male reproductive functions (Ozawa et al., 2002). Exposure of our experimental animals to restraint stress for six hours resulted in rise in corticosterone and a fall in testosterone. These findings are in accordance with study by Dong et al., done on mice in which they exposed the mice to immobilization stress for three hours with resultant fall in testosterone and rise of corticosterone, the predominant steroid of rodents (Dong et al., 2004). We made it sure that timings for sampling remain the same between 11 am and 12 noon, for sampling serum corticosterone in control and experimental groups (Debigare et al., 2003).

The group of rats exposed to restraint stress, showed a significant rise in the corticosterone. The literature supports the concept that even two hours of immobilization stress to rats may decrease the testicular steroidogenesis and also there is an increase in the level of serum corticosterone. It is thought that this rise of corticosterone is responsible for decline in testosterone (Hales and Payne, 1989). A study done by Kosti et al., revealed that immobilization stress decreases the testosterone levels but hypothalamo pituitary gonadal axis is not affected (Kosti et al., 1997). Another study by Taylor et al., points towards the fact that immobilization stress causes a decline in testicular androgens (Taylor et al., 1994).
An important point of consideration here is that, not only immobilization stress, but stress of multiple origin like various diseases, exercise and even psychosocial interactions may also result in suppression of male reproductive functions (Chrousos and Gold, 1992). For example, testosterone levels are lower in patients of Cushing’s syndrome which were further depressed by dexamethasone given for diagnostic reasons (Contreras et al., 1996; Vierhapper et al., 2000). Similarly, in humans the severe psychological stress due to death of a relative or spouse lowers sperm count which is most likely caused by stress induced decline in testosterone (Fenster et al., 1997).

As testosterone is produced by Leydig cells, we designed an in vitro model of isolated and cultured Leydig cells with three hours incubation time, to investigate the effects of stress hormone corticosterone on Leydig cells. When we incubated the Leydig cells with corticosterone 100 nM, there was a significant decline in synthesis of testosterone. Therefore, it is evident that corticosterone decreases the steroidogenesis at Leydig cell level.

Synthesis of testosterone is dependent on optimal functioning of Leydig cells, located in testicular interstitium and stress induced escalation in corticosterone can induce apoptosis in Leydig cells. However, the apoptotic effect is evident only after 12 hours of stress exposure; therefore the fall in testosterone as a consequence of apoptosis induced damage to Leydig cells shows no correlation with our study (Gao et al., 2002). Acute immobilization stress induced fall in testosterone is also suggestive that these effects may be produced by corticosterone are non-genomic, because it involves relatively rapid response which may not involve the protein synthesis. These responses may include change in permeability
of ion channels, involvement of cAMP and calcium channels (Wehling, 1997; Dong et al., 2004). Steroidogenic acute regulatory (StAR) protein is also thought to be suppressed by glucocorticoids (Wang et al., 2000).

In order to investigate the mechanism of action of corticosterone, we used phosphodiesterase inhibitor, 3 iso butryl -1-methyl xanthine (IBMX). It blocks the activity of phosphodiesterases which may cause hydrolysis of cAMP. Therefore, we can say that IBMX increases the availability of cAMP at cell level by decreasing its breakdown. The cAMP is involved in biosynthetic pathways of testicular steroidogenesis (Soderling et al., 1998). Initially we observed that, IBMX produced significant rise in basal testosterone synthesis at concentration of 1 mM, which is in accordance with literature (Shiow et al., 1997). Also IBMX led to increased testosterone production in the presence of LH.

When corticosterone was added to the well containing Leydig cells with IBMX, there was a fall in concentration of testosterone as compared to IBMX alone stimulated cells. Similarly, the effects of stimulation of LH and IBMX both were also diminished by corticosterone. Based on these findings, it is suggested that corticosterone would have decreased the cAMP production in Leydig cells, (both basal and LH stimulated) due to which the effects of IBMX were not evident. These findings support the study done by Dong et al, in which they measured cAMP and found that direct action of corticosterone is to decrease cAMP. We used IBMX to check the cAMP instead of measuring cAMP levels (Dong et al, 2004).
Addition of pregnenolone increased the production of testosterone. It is due to the fact, that pregnenolone is an important precursor in the pathway of testicular steroidogenesis, because after the conversion of cholesterol to pregnenolone, it is converted to progesterone with the help of 3 beta hydroxyl steroid dehydrogenase (3βHSD), which by action of 17 α hydroxylase is converted to 17 α hydroxyl progesterone. Action of C-17-20 lyase will result in formation of androstenedione, which is then converted to testosterone (Zirkin, and Chen, 2000).

In our study, the pregnenolone was unable to replenish the fall of testosterone synthesis induced by corticosterone, because addition of corticosterone resulted in a decline in testosterone synthesis both in basal and LH stimulated cells, in which pregnenolone was added.

It is well know that steroidogenesis involve conversion of cholesterol to pregnenolone as a rate limiting step catalyzed by the mitochondrial cholesterol side chain cleavage enzyme (Payne and Hales, 2004) For rapid regulation of steroid synthesis, transfer of cholesterol from outer to inner mitochondrial membrane involves steroid acute regulatory (StAR) protein (Stocco, 2001). Inability of pregnenolone to replenish decrement in testosterone induced by corticosterone is suggestive that synthesis of pregnenolone is not affected or we can say that activity of StAR protein may not be affected by corticosterone at Leydig cell level during three hours of incubation. Therefore we suggest that corticosterone could have inhibited the enzymes like 3βHSD or 17 α hydroxylase or C-17-20 lyase down in steroidogenesis pathway. But 3βHSD inhibition is excluded by study done by Orr et al, in which they estimated the levels of progesterone and 17 α hydroxyl progesterone. Their study revealed that after 03 hours of immobilization stress,
nine fold rise of corticosterone was accompanied by the increase in the levels of progesterone by 33 percent while there was 47 percent decline in levels of 17 α hydroxyl progesterone. This suggests that conversion of progesterone to 17 α hydroxyl progesterone is seriously impaired by corticosterone. It means that the enzyme involved in above step is blocked by corticosterone, which they confirmed by estimation to be 17 α hydroxylase. Also it was suggested that the activity of C-17-20 lyase is also suppressed. (Orr et al., 1994) Our study validates the findings of Orr et al., that enzymatic pathways are also blocked by stress hormones. However, we obtained this inference on cultured Leydig cells by adding pregnenolone and corticosterone together, in the well containing cultured Leydig cells contrast to Orr et al., because they measured levels of these enzymes.

Along with increase in the glucocorticoids, another neuroendocrine component of stress response include increased secretion of epinephrine and nor epinephrine from sympathetic nervous system and adrenal medulla (Carrasco and VanderKar, 2003). Restraint stress exposure to our experimental animals significantly increased the nor epinephrine levels. Increase in catecholamines like nor epinephrine is well known entity of stress, which results in stimulation of adrenal cortex and locus coeruleus, with resultant increase in nor epinephrine (Montoro et al., 2009). Increase in nor epinephrine is also reported after cold induced stress (Dronjak et al., 2004). Similarly, immobilization stress may result in rise in catecholamines like nor epinephrine (Collu et al., 1984). Immobilization stress is thought to decrease the catecholamines stores in central and peripheral tissues (Dronjak and Gavrilovic, 2006). Therefore, the rise of nor epinephrine
confirms that our stress protocol was efficient enough to produce the effects of stress.

Involvement of catecholamines in testicular steroidogenesis is an important fact to be considered because a direct autonomic pathway of innervation from spinal cord to testicular interstitium may become active during stressful conditions (Lee et al., 2002). Fewer studies point towards role of catecholamines during stress at Leydig cell level (Hardy et al., 2005). Catecholamines are generally assumed to increase steroidogenesis in vitro (Mayerhofer et al., 1993). Beta blockers which decrease sympathetic activity decrease testicular steroidogenesis (Khan et al., 2004). Now, stress involves rise of nor epinephrine and fall in testosterone values. Therefore, we investigated the role of nor epinephrine at Leydig cell level.

Nor epinephrine significantly increased the testosterone production in LH stimulated Leydig cells in our study. These findings are in accordance with studies done by Anakwe and Moger who have shown that catecholamines stimulate testicular steroidogenesis in vitro on isolated cultured Leydig cells. They have shown that the beta agonist isoproterenol, increased steroid production to 174 percent after three hours of incubation of Leydig cells. Similar proportionate increase was observed with LH (Anakwe and Moger, 1986). Likewise, the in vitro study done by Meyerhofer et al., on isolated Leydig cells of Siberian Hamster have shown that nor epinephrine is one of the most potent stimulus to stimulate testosterone production in vitro (Mayerhofer et al., 1993).

As mentioned before, that phosphodiesterase inhibitor IBMX increases the availability of cAMP, therefore we exposed the purified Leydig cells with nor
epinephrine in the presence of IBMX. Addition of nor epinephrine with IBMX, significantly stimulated the testosterone production in Leydig cells. However nor epinephrine failed to produce an upsurge in IBMX stimulated testosterone production in the presence of LH in our study. It shows that presence of LH decreased responsiveness of Leydig cells to nor epinephrine. One reason may be this, that the Leydig cells may respond after delay to nor epinephrine in the presence of IBMX and LH both and three hours culture was insufficient to produce such effect. There could also be a possibility that enough stimulation and release of cAMP was achieved by IBMX alone and adding of LH has given no additional surge (Cooke et al., 1982). From this, it is suggested that nor epinephrine have stimulated the cAMP pathway and presence of IBMX resulted in more potent steroidogenesis at Leydig cell level.

On the other hand, there were insignificant effects produced by addition of nor epinephrine to pregnenolone added wells, both in LH stimulated and non-stimulated Leydig cells. Thus, we can suggest that nor epinephrine may be affecting the enzymes like 17 α hydroxylase or C-17-20 lyase down in steroidogenesis pathway. We are also uncertain about the effect of nor epinephrine on 3 β HSD activity. The measurement of levels of these enzymes after exposure of Leydig cells to nor epinephrine can give further clue to the mechanism of action of nor epinephrine.

However, investigators have worked on other possible mechanisms of nor epinephrine which may involve the presence of alpha and beta receptors on Leydig cells (Mayerhofer et al., 1992). That’s why; studies have shown that beta blockers
like atenolol and propranolol may decrease the testicular steroidogenesis (Khan et al., 2004; Mayerhofer et al., 1992).

Another important point of consideration regarding actions of corticosterone and nor epinephrine is that, in vivo both are increased after stress and as a result the testosterone decreased. However, in vitro study revealed that corticosterone decrease testicular steroidogenesis while nor epinephrine increase it. The query is how do they interact? To solve this, we added both corticosterone and nor epinephrine to our wells containing LH stimulated Leydig cells. The combination of both, resulted in a significant fall of testosterone, similar to that occurs in in vivo after acute stress.

The question arises that if nor epinephrine is increasing testicular steroidogenesis in vitro, then why stress decreases the levels of testosterone in males. One of our in vitro experiments data helps to resolve this problem, that when we added both corticosterone and nor epinephrine, the effects of corticosterone were predominant with a resultant fall in testosterone levels.

Another possible explanation is that in vivo, the rise of nor epinephrine can induce vasoconstriction which may restrict the access of LH to testicular interstitium to act on Leydig cell, with consequent fall in levels of testosterone in blood (Ogilive and Rivier, 1998; Turnbull and Rivier, 1997).

As LH is also not changed in acute stress, which means hypothalamo-pituitary gonadal axis is not involved in acute stress, therefore there is another possibility that stress may cause release of chemical substances in circulation or cause their local production in testis that directly decreases the testicular
steroidogenesis (Selvage et al., 2004). Also the investigators have worked out an efferent neural pathway from hypothalamus to testis (Lee et al., 2002; Selvage and Rivier, 2003). It is proposed that this pathway work independently from pituitary axis (Turnbull and Rivier 1997). This pathway was mapped in a retrograde fashion from testis via spinal segments T_{10} - L_{1}, L_{5} - S_{1} to brain stem. Hypothalamic areas may include para ventricular nucleus and lateral hypothalamus (Gerendai et al., 2000).

Some researchers are also of the opinion that activation of this pathway leads to inhibition of Leydig cell’s responsiveness to gonadotropins. Interestingly, this pathway is activated by intra cerebroventricular injection of beta agonists like nor epinephrine (Turnbull and Rivier, 1997; Ogilvie and Rivier, 1998). Therefore, it is possible that stress and disturbed emotions via this efferent pathway could also adversely affect the testicular steroidogenesis and the isolated increase of testicular steroidogenesis by nor epinephrine (shown by in vitro studies) is largely nullified by these mechanisms and net result of stress is decrease in testicular steroidogenesis.

Another important point of consideration is that immobilization stress protocol as followed by us, can lead to oxidative stress (Nadeem et al., 2006). Oxidative stress may occur due to imbalance between prooxidants and antioxidants. This imbalance may result from either a decrease in level of antioxidants or an increased production of prooxidants (Halliwell and Whiteman, 2004). Increase in the production of reactive oxygen species and generation of free radicals constitute the oxidative stress. When reactive oxygen species exceed body natural antioxidant defense, and protective mechanisms become unable to prevent
stress induced damages, then damage to macro molecules such as DNA, proteins and lipids occur (Bartsch and Nair, 2000).

The reactive oxygen species most commonly target polyunsaturated fatty acids of cell membrane with resultant oxidation of lipids called lipid peroxidation (Makker et al., 2009). The degradation of lipid peroxides of the biological membranes may result in formation of a number of aldehydes such as malondialdehyde (MDA), which may cause damage to cells by reacting with proteins, lipids and nucleic acids (Sim et al., 2003).

Endogenous antioxidant systems that may act as free radical scavengers may have important role to combat against stress induced lipid peroxidation. One of important endogenous antioxidant defense system is an enzyme, superoxide dismutase (SOD). The SOD helps body to combat with the deleterious effects of reactive oxygen species (Jones et al., 1981). The levels of SOD may thus indicate the activity of natural intrinsic anti-oxidant defense system of body, and considering its importance, we estimated the levels of SOD in this study.

Exposure of rats to the restraint sessions is a time tested classic model to induce stress (Shansky et al., 2006). In present study, the restraint stress to Sprague Dawley rats for six hours in mesh wire restrainer led to the increase in levels of MDA, which suggests that the stress protocol has resulted in per oxidative damage with increased lipid peroxidation. In addition there was fall in antioxidant enzyme activity as manifested by fall in SOD levels in group II thus, suggesting the balance in favor of oxidative stress. As the testicular tissue is highly susceptible to stress because testicular membranes are rich in polyunsaturated fatty acids (Chainy et al.,
1997). Similar findings were obtained by *in vitro* results of our study, where addition of corticosterone and corticosterone plus nor epinephrine together has resulted in rise of lipid peroxidation in terms of elevated MDA levels and fall in antioxidant enzyme activity as is evidenced by reduced SOD levels.

Therefore, it can be said that the oxidative stress induced damage to testis at cellular level could also be responsible for the decline in levels of testosterone in our study.

As antioxidants obtained from dietary sources constitute an essential component of human antioxidant defense system (Agarwal and Prabakaran, 2005). Many epidemiologic and clinical trials have reported that supplementation with antioxidant vitamins is associated with a reduction in the incidence of chronic disease morbidity and mortality (Weber *et al.*, 1996; Enstrom, 1997; Gey, 1998). Therefore we investigated the effects of supplementation of two important, FDA approved dietary antioxidants i.e., ascorbic acid and alpha tocopherol, both *in vivo* and *in vitro*.

In our *in vivo* component of study, the supplementation of rats with ascorbic acid 500 mg/L drinking water for one month was unable to prevent restraint stress induced rise in corticosterone and decline in testosterone. Also there was insignificant change in nor epinephrine levels. Release of LH was also unchanged in ascorbic acid supplemented group. However, despite insignificant change in MDA, SOD activity increased in ascorbic acid supplemented group. This shows that antioxidant capability via increase in SOD is enhanced but stress induced lipid peroxidation was not prevented by ascorbic acid administration.
High levels of ascorbic acid are thought as a defense line against oxidative stress. Also increased testosterone levels are reported after ascorbic acid supplementation at a dose of 500 mg/kg/day (Sonmez et al., 2005). Ascorbic acid supplementation in high doses is shown to reduce oxidative stress and consequent damage in arsenic induced stress in mice (Chang et al., 2007). Smokers are at increased risk of oxidative stress, and various studies have shown that supplementation with ascorbic acid, decreases stress with consequent reduced lipid peroxidation as is evidenced by decline in MDA levels (Naidoo and Lux 1998; Wen et al, 1997).

Our previous study has shown that ascorbic acid do not affect the basal testosterone levels in rats (Lodhi et al., 2009). Although numerous studies point towards antioxidant role of ascorbic acid, but some studies also point that ascorbic acid may not ameliorate or prevent all stressors, like alloxan induced stress was increased by ascorbic acid supplementation (Dillard et al., 1982).

In our study, there was insignificant difference in levels of MDA between the stressed group and ascorbic acid alone supplementation group, exposed to same stressor for same duration. This is in accordance with a study on male humans in which they have suggested that ascorbic acid has no effect on MDA concentration (Bayer-Eder et al., 2004). But the levels of SOD were increased in ascorbic acid supplemented group, which suggested the increased antioxidant capability of ascorbic acid. Therefore we cannot deny the antioxidant role of ascorbic acid, contrary to the study done by Anderson et al. in which they had shown that ascorbic acid increases the per oxidative damage (Anderson et al, 1994).
In our *in vitro* experimental model, addition of different doses of ascorbic acid to the wells containing the cultured Leydig cells have shown no significant effect, rather high doses adversely affected testosterone synthesis. However, the corticosterone induced decline in testosterone synthesis was restored to normal by addition of ascorbic acid. The addition of ascorbic acid also restored decline in testosterone induced by combination of corticosterone and nor epinephrine.

Ascorbic acid decreased MDA insignificantly, while SOD activity was increased significantly in the presence of LH. Therefore, based on these findings it is suggested that in basal conditions, without exposure to stress hormones, ascorbic acid increases antioxidant enzyme activity.

Ascorbic acid alone in our study had no favorable effect on MDA level in our study wells containing LH and Leydig cells, but when combined with alpha tocopherol, the antioxidant effect of alpha tocopherol got enhanced, which suggests some synergism in their actions. This can be explained on the basis of available literature which supports that ascorbic acid increases the availability of alpha tocopherol to the body. For example, when ascorbic acid is added to cultured hepatocytes, it prevented the loss of alpha tocopherol and thus increased its availability (Halpner *et al.*, 1998).

Among stress hormones, corticosterone alone and in combination with nor epinephrine caused a significant rise in MDA and fall in SOD. But, nor epinephrine has caused no significant changes in MDA and SOD, based on which we can infer that norepinephrine is not causing lipid peroxidation. The derangements in levels of
MDA and SOD were not seen in those corticosterone containing wells in which we added ascorbic acid.

As reviewed by Padayatty et al., many studies suggest that ascorbic acid has failed to prove prevention against oxidative stress in humans, but it has shown antioxidant capability in the *in vitro* experiments (Padayatty *et al.*, 2003). Increase in plasma concentration of ascorbic acid increases its concentration in adrenal gland and this local increase in ascorbic acid has been assigned an interesting role of quenching the oxidants which are produced during steroidogenesis (Rapoport *et al.*, 1995). Therefore, based on our *in vitro* experiments, it is suggested that ascorbic acid can act as an efficient antioxidant at Leydig cell level also.

However, the results of *in vivo* component of our study are not favoring the international studies in which ascorbic acid is proved as an antioxidant. Ascorbic acid is thought to prevent the oxidative stress induced derangements, like study by Ergul *et al.*, which narrates that ascorbic acid may reduce the oxidative damage to liver caused by anti tuberculous drug isoniazid (Ergul *et al* 2010). This difference could be due to dose difference, because Ergul and colleagues used a dose of 100 mg/kg/day via oral route but we used ascorbic acid in a dose of 500mg/l drinking water.

Another study done by Sengupta *et al.*, reveals that ascorbic acid restored the cadmium induced decline in testosterone levels. However, they used ascorbic acid via subcutaneous route in a dose of 400 mg/100 g BW for 48 h period (200 mg at 24 h before and 200 mg at 24 h after cadmium injection). This difference of
route and dose of administration of ascorbic acid in our case could be responsible for inefficiency of ascorbic acid to reduce stress effects (Sengupta et al., 2004).

This is supported further by another study of Peters et al., which have given two different doses of ascorbic acid, 500 mg/day and 1500 mg/day to marathon runners before race and estimated the levels of stress hormones, cortisol and adrenaline immediately after the race. It was observed that ascorbic acid at 1500 mg/day dose significantly reduced the both cortisol and adrenaline, while 500 mg/kg was ineffective. Based on this we can say that although ascorbic acid can act as free radical scavenger, but very high doses are required to have antioxidant effect. (Peters et al., 2001)

High doses are recommended because high doses are not toxic and it is well known fact, discovered long ago that the excessive ascorbic acid is rapidly eliminated in urine within 24 hours (Sneer et al., 1968). Also a comprehensive review has strongly suggested that ascorbic acid RDA should be increased for more beneficial effects (Carr and Frei, 1999). Therefore, based on our findings, it is suggested that ascorbic acid at high doses can act as antioxidant to prevent oxidative stress induced infertility.

Supplementation of the rats of group IV with alpha tocopherol for one month before stress exposure, significantly prevented restraint stress induced rise of corticosterone and fall in testosterone. However LH and nor epinephrine remained unchanged. There was also positive effect on oxidative stress with reduced lipid peroxidation and consequent fall in MDA and rise of antioxidant capability in terms of rise of SOD activity.
Alpha tocopherol is an important component of anti-oxidant defense system of our body and protects the tissues against oxidative damage induced by reactive oxygen species (Naziroglu et al., 2003). Our findings are indicative that oxidative stress induced by restraint has resulted in decline in testosterone, which was significantly protected by alpha tocopherol and oxidative stress effects were also reduced as is evidenced by values of MDA and SOD. However, alpha tocopherol decreased only corticosterone, while it has no effect on nor epinephrine levels.

Our results to assess antioxidant preventive role of alpha tocopherol are supported by various international studies which have confirmed the anti-oxidant role of alpha tocopherol. For example, administration of alpha tocopherol protects the rats against various stressors like homocystine induced oxidative stress (Sonmez et al., 2005), Cadmium induced toxicity (Ognjanovic, et al., 2003), nicotine induced damaging effects (Hijazi et al., 2002) and ethane dimethane sulfonate induced testicular toxicity (Sahinturk, et al., 2007).

Similarly some recent studies also point towards this antioxidant role of alpha tocopherol. A study by Hong et al., has suggested that supplementation of the diets of goat with alpha tocopherol significantly increased SOD activity while MDA levels were decreased in testicular tissue. Therefore it is suggested that alpha tocopherol protect their testis from peroxidative damage (Hong et al., 2010). In another study, intra peritoneal administration of alpha tocopherol in a dose of 50 mg/kg/day to streptozotacin induced diabetic rats significantly lowered the MDA and increased SOD activity in the nervous tissue of rats (Kabay et al., 2009).
Alpha tocopherol on cultured Leydig cells in the presence of LH stimulated the testosterone synthesis in a dose of 10 µg/ml. However, high doses lead to suppression at Leydig cell level. This is supported by research work of Mather et al., which claims that alpha tocopherol has stimulant effect on testosterone synthesis by Leydig cells (Mather et al., 1983) Some authors believe that alpha tocopherol may decrease the plasma testosterone levels (Hartman et al., 2001) but in our view, in this study, Hartman et al have given alpha tocopherol to older men in which testosterone is already lower than normal.

In the cultured Leydig cells, alpha tocopherol decreased MDA insignificantly, while SOD activity was increased significantly in the presence of LH. Therefore, based on these findings, it is suggested that in basal conditions, without exposure to stress hormones, alpha tocopherol increases antioxidant enzyme activity. However, alpha tocopherol prevented decline in testosterone synthesis in the presence of corticosterone and there was fall in MDA and rise in SOD activity, when we added alpha tocopherol to corticosterone and NE added wells.

Alpha tocopherol is mandatory to combat against stress. It was verified by experiments of Burczynski et al, in which they given the rats a diet deficient in alpha tocopherol and rat adrenal mitochondria showed increased lipid peroxidation after exposure to stress (Burczynski et al., 1999). Our in vitro results are also pointing the preventive roles of alpha tocopherol against corticosterone induced derangements. It is also well supported by literature that alpha tocopherol can reduce lipid peroxidation in cultured Leydig cells and may increase testosterone synthesis (Chen et al., 2005).
The effectiveness of alpha tocopherol as an anti-oxidant to prevent the testicular tissues against various diseases is well supported by literature (Sahinturk et al., 2007). If we see effects of ascorbic acid and alpha tocopherol, we can see that alpha tocopherol exerted more potent effect in reducing lipid peroxidation and our results are in agreement with research outcomes of Massaeli et al., who also narrated that alpha tocopherol is more potent in this regard (Massaeli et al., 1999). Therefore, based on our in vivo and in vitro findings, we hereby suggest that alpha tocopherol is very effective anti-oxidant against restraint stress induced derangements in testicular steroidogenesis.

Supplementation of our experimental animals with a combination of ascorbic acid and alpha tocopherol for one month before stress exposure, significantly prevented restraint stress induced rise of corticosterone and fall in testosterone as shown in table 3.4. However LH and nor epinephrine remained unchanged in this group too. Oxidative stress was also reduced as narrated by reduction in lipid peroxidation and consequent drop in MDA and escalation of antioxidant capability as expressed by rise of SOD activity.

These findings are supported by various international studies. Krishnamoorthy et al., have reported that Polychlorinated biphenyls - PCB (Aroclor 1254) which is an endocrine disruptor present in paints, oils etc., has damaging effects on reproductive parameters of rats. The sperm count and motility decreased with evident emblems of oxidative stress in terms of raised MDA and fall in SOD activity. However, supplementation with ascorbic acid and alpha tocopherol combination effectively restored the fertility parameters as well as stress markers to their normal physiological range. Therefore, they narrated these
antioxidants as excellent tools to normalize both the enzymic and non-enzymic antioxidant activity (Krishnamoorthy et al., 2007). The preventive role of ascorbic acid and alpha tocopherol supplementation against various types of stressors is well documented in literature (Akturk, et al., 2006; Judge et al., 2007).

As the steroid levels were decreased in our study and testosterone was significantly increased in this combination supplemented group. Similar research outcomes are narrated in the study of Schroder et al., in which they have seen that in athletes, combination of antioxidants results in statistically significant increase in testosterone and a fall in glucocorticoid activity seen (Schroder et al., 2001). In ascorbic acid and alpha tocopherol supplemented group, serum MDA levels were decreased. These results are similar to the study of Guney et al., in which rats were exposed to fluoride toxicity and supplementation with ascorbic acid and alpha tocopherol reduced the levels of MDA (Guney et al., 2007b). Therefore ascorbic acid and alpha tocopherol combination is effective in ameliorating the oxidative stress, manifested by the decline in MDA levels and increase in SOD activity.

An important point of consideration evident from our study data is that, rise of testosterone and fall of corticosterone is in almost same significance range in alpha tocopherol supplemented group, but considering the values of MDA and SOD, we can see that there is more significant fall in MDA and rise in SOD in the group of rats supplemented with a combination of ascorbic acid and alpha tocopherol as compared to alpha tocopherol alone. Therefore, it is suggested that, combination of antioxidants ascorbic acid and alpha tocopherol has synergistic effect in reducing oxidative stress.
Although some authors are of the opinion that antioxidants combination have no added advantage like that of study by Huang et al., in which they have given antioxidants in combination to humans and their results revealed no added advantage of combination than either alone (Huang et al., 2002). Contrary to this, many studies are favoring our findings. When ascorbic acid was added to cultured hepatocytes, it prevented the loss of alpha tocopherol and thus increased its availability (Halpner, et al., 1998). Another study shows that ascorbic acid supplementation lead to enhanced levels of alpha tocopherol (Eichenberger et al., 2004). Also, the documented evidence is there, that alpha tocopherol increases tissue ascorbic acid concentration (Sztanke et al., 2004). Therefore this could be the reason of our results that both antioxidants in combination affected more potently and led to an increase in testosterone with more marked reduction in lipid peroxidation as suggested by decreased MDA levels.

Our in vitro findings are confirming our in vivo results, that combination of ascorbic acid and alpha tocopherol in the presence of stress hormones not only prevented fall in testosterone, but also has positive effect on oxidative balance. Therefore, the combination of antioxidants is effective in the presence of stress hormones in decreasing lipid peroxidation in terms of fall in MDA and an increased anti-oxidant i.e., SOD activity.

Our in vitro results are endorsed by various research works. A study on cultured erythrocytes by Durak et al., has revealed that an organophosphate pesticide Malathion induce oxidative stress in erythrocytes and combination of ascorbic acid and alpha tocopherol prevented the malathion induced oxidative stress (Durak et al., 2009).
Similarly a study by Murugesan et al., in which they have noted the harmful effects of an important environmental contaminant i.e., polychlorinated biphenyl called Aroclor 1254, on Leydig cell steroidogenesis. They performed experiments in vivo on rats and in vitro on cultured Leydig cells of rat. Aroclor caused oxidative stress and contrary to our studies, the levels of LH were also reduced by Aroclor. The reason is that, oxidative stress exposure was for thirty days, leading to chronic stress and chronic stress may affect the hypothalamo pituitary gonadal axis as discussed before. The results of this study have substantiated that co-administration of ascorbic acid and alpha tocopherol prevented the decrement in testosterone levels induced by Aroclor 1254 (Murugesan et al., 2007). Therefore, based on the results of this group and in vitro component where we added both antioxidants, it is proposed that the combination of antioxidants (ascorbic acid & alpha tocopherol) is useful in preventing the stress induced decline in testicular steroidogenesis as well as the derangements in MDA/SOD balance and thus confer protection against the oxidative stress.

As mentioned before, exposure of our experimental animals to restraint stress for six hours caused increased lipid peroxidation as is evidenced by rise in MDA and there was also fall in SOD activity. If there is increase in MDA, it is an evidence of enhanced lipid peroxidation (Lata et al., 2004). Increase in lipid peroxidation may result in rise of glucocorticoid activity as a result of stimulation of stress axis (Gupta et al., 2004). Similarly a fall in antioxidant enzyme activity showing decline in SOD also points towards oxidative stress (Kleczkowski et al., 2003). Therefore, we can propose that our experimental protocol induced the oxidative stress, as a result of which there was decline in testicular steroidogenesis.
The oxidative stress has multi-dimensional damaging effects on body. Oxidative stress involves generation of free radicals, which are atoms and molecules with an unpaired electron. These free radicals have the capability to damage molecules that are important for functions of the cell, resulting in impaired functions. Superoxide radicals are formed during reduction of oxygen and site of formation is inner mitochondrial membrane. They can initiate the chain reaction in fatty acids of phospholipids which may lead to lipid peroxidation in membrane (Evans, 2000).

The peroxidation of membrane lipids can be very detrimental because it is a self-propagating chain reaction and oxidation of few molecules of lipids may result in significant damages. This lipid peroxidation may modify the biological properties of membranes, like the degree of fluidity of membrane. It can also lead to inactivation of various receptors and enzymes which may lead to increased tissue permeability and normal cellular functions are blighted (Dalle-Donne et al., 2006; Zaidi et al., 2003). Such damage by oxidative stress is due to production of reactive oxygen species which may damage the macromolecules including DNA (Guney et al., 2007a).

Free radicals which are generated in response to stress may also lead to an increase in protein oxidation (Dohm and Louis, 1978). Various reactive oxygen species may include superoxide anions, hydroxyl radicals, and hydrogen peroxide etc. These reactive oxygen species released during oxidative stress are recognized as damaging agents for various components of steroidogenic pathways including steroidogenic enzymes (Murugesan et al., 2005).
At cell level, various mechanisms are operating to decrease the damage or neutralize the effects of oxidative stress. Although these intrinsic mechanisms are normally operating in body to respond to stressor in order to neutralize the adverse effects of stress, but restraint stress is capable of generating severe oxidative stress which may disrupt the defense mechanism and reactive oxygen species are liberated (Zaidi et al., 2003). Also, there is documented evidence that stress of various origins may cause depletion of antioxidant sources of body (Sconberg et al., 1993). Our results have also shown similar trend with a significant fall in SOD activity.

One of the antioxidant which we investigated was ascorbic acid which has the ability to react with various reactive oxygen species like superoxide, hydroperoxyl radicals, aqueous peroxyl radicals, nitrogen peroxide, nitroxide radicals and hypochlorous acid (Buettner, 1993). Ascorbic acid is hydrophilic and is a very important free-radical scavenger in extracellular fluids. It traps the radicals in the aqueous phase and thereby confers protection of bio membranes from per oxidative damage (Harapanhalli et al., 1996). Ascorbic acid is thought to interact with plasma membrane too by donating electrons to alpha tocopheroxyl radical and a trans plasma membrane oxidoreductase activity (May, 1999) as reviewed by Padayatty et al., ascorbic acid by donating electron is a reducing agent and its antioxidant actions are also dependent on this property, because by donating electrons it prevents oxidation of other compounds. Reactive and harmful free radicals react with ascorbic acid and form less reactive ascorbyl radical which is then reduced to dehydroascorbic acid which may be hydrolyzed or reduced back to
ascorbic acid. Reduction of reactive free radicals with less reactive radicals is called free radical scavenging property of ascorbic acid (Padayatty et al., 2003).

The other antioxidant of our study alpha tocopherol is considered as a major chain breaking anti-oxidant and as first line of defense against oxidative stress. It is also recognized as a potent scavenger of free radicals like superoxide and peroxynitrite. One of its core functions is to scavenge lipid peroxyl radicals by virtue of which, it protects the polyunsaturated fatty acids (PUFAs) from oxidation. If sufficient quantity of alpha tocopherol is accessible at site of oxidative stress where there is free radical generation, it would neutralize the deleterious effects of ROS (John et al., 2001). Alpha tocopherol supplementation provides more than normal levels of alpha tocopherol in blood, and thus reacts with membrane phospholipids bilayers, thereby breaking the chain reaction initiated by ROS. By these actions, alpha tocopherol could have protected Leydig cell membrane structures against oxidative injury (Therond et al., 1996). The improvement of antioxidant enzyme activities could be the result of the free radical scavenging effects of alpha tocopherol (Gurel et al., 2005).

Both antioxidants are important to combat against stressors of various origins. For example, when alpha tocopherol deficient diet was given to guinea pigs that were ascorbic acid deficient, death occurred in few days (Hill et al., 2003). Documentary evidence is there that simultaneous administration of ascorbic acid and alpha tocopherol maintains the expression of steriodogenic enzymes in Leydig cells (Murugesan et al., 2007). Along with its antioxidant effects, ascorbic acid is also involved in the regeneration of tocopherol from tocopheroxyl radicals in the membrane. Therefore, ascorbic acid and alpha tocopherol can have
interactive but protective effects against generation of reactive oxygen species and lipid peroxidation (Stoyanovsky et al., 1995). The antioxidants supplementation may also protect the protein structures (Schroder et al., 2001) receptors and enzymes which are then not allowed to be reactivated by ROS, and there is stabilization the cell membranes.

Due to these abilities of antioxidants, Leydig cells were protected from detrimental effects of stress with consequent protection against derangements in testicular steroidogenesis induced by oxidative stress. Therefore, it is concluded that the antioxidants ascorbic acid and alpha tocopherol confer protection at Leydig cell level against restraint induced oxidative stress.
Limitations of our study

1. Due to non-availability of male Sprague Dawley rats at National Institute of health, we had to perform in vivo component of project with 16 rats in each group instead of 30 rats per group.

2. We have investigated effects of stress hormones in short term culture only. It is due to the reason that the cell culture technique was first time established by us in our institution and we had to rely on 3 hours incubation, because facilities for long term incubation of cultured Leydig cells are not yet established.

3. We had also a plan to see the effects of androstenedione while investigating the mechanism of action of corticosterone and nor epinephrine on cultured Leydig cells, but unfortunately we could not import it and it was not available locally.
**Conclusion**

The data of our study led us to conclude that:

1. Acute stress suppresses steroidogenesis at Leydig cell level.

2. Corticosterone in acute stress decreases the Leydig cell steroidogenesis by inhibiting cAMP as well as enzymatic pathways whereas nor epinephrine increases the testosterone synthesis at Leydig cell level by stimulating cAMP or the enzymes involved in testosterone synthesis.

3. Ascorbic acid at low dose is not effective as antioxidant, but alpha tocopherol is an effective antioxidant against restraint stress induced derangements in testicular steroidogenesis.

4. Combinations of antioxidants, ascorbic acid and alpha tocopherol prevents the stress induced fall in testicular steroidogenesis and have synergistic effect in reducing oxidative stress by decreasing lipid peroxidation and increasing the antioxidant enzyme activity.
Future directions

1. Mechanism of action of nor epinephrine needs further clarification. The measurement of levels of cAMP in the presence of nor epinephrine in cultured Leydig cells or working on other intra cellular pathways with the help of blockers may further reveal the insights into the mechanism of action of nor epinephrine.

2. Stress response in rats fed on antioxidants deficient diet needs to be elucidated which will further explain the definite role of these antioxidants to combat the stress induced derangements.

3. The possible role of genetic basis (especially DNA) in the stress response needs further elucidation.
REFERENCES


Cacioppo, J.T., Malarkey, W.B., Kiecolt-Glaser, J.K et al., (1995) Heterogeneity in neuroendocrine and immune responses to brief psychological stressors as a function of autonomic cardiac activation. Psychosom. Med. 57: 154 - 64,


Miller, D.B and O’Callaghan, J.P. (2002). Neuroendocrine Aspects of the Response to Stress Metabolism. 51(6), Suppl 1. pp 5-10


Naziroglu, M., Kokcam, I and Yilmaz, S. (2003). Beneficial effects of intraperitoneally administered alpha-tocopheryl acetate on the levels of lipid peroxide and activity of


Peters EM, Anderson R, Nieman DC, Fickl H and Jogessar V. (2001) Vitamin C supplementation attenuates the increases in circulating cortisol, adrenaline and anti-


Sullivan, M.H. and Cooke, B.A. (1986). The role of calcium in steroidogenesis in Leydig cells. Stimulation of intracellular free \( \text{Ca}^{2+} \) by lutropin (LH), luliberin (LHRH) agonist and cyclic AMP. Biochem. J. 236: 45–51


144: 183-97.
## Composition of Pelleted Diet for Rats

<table>
<thead>
<tr>
<th>INGREDIENTS</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Wheat Flour</td>
<td>2.85 Kg</td>
</tr>
<tr>
<td>2. Wheat Brawn</td>
<td>2.85 Kg</td>
</tr>
<tr>
<td>3. Dried skimmed milk Powder</td>
<td>2.00 Kg</td>
</tr>
<tr>
<td>4. Soyebean Oil</td>
<td>0.50 Liter</td>
</tr>
<tr>
<td>5. Mollasen</td>
<td>0.15 Kg</td>
</tr>
<tr>
<td>6. Fish meat</td>
<td>1.50 Kg</td>
</tr>
<tr>
<td>7. Salt (common).</td>
<td>0.05 Kg</td>
</tr>
<tr>
<td>8. Vitamins/ Minerals/amino acids (Premix)*</td>
<td>0.10 Kg</td>
</tr>
</tbody>
</table>

| total weight.                           | 10.00 Kg |

This diet is prepared at National Institute of Health (N.I.H), Islamabad according to the standard approved by the Universities Federation for Animals Welfare.

* = Composition of premix is given in the next appendix-2
## Composition of Vitamins/ Minerals/amino acids (Premix)

Mixed in the Diet, for Rats.

<table>
<thead>
<tr>
<th>INGREDIENTS</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vitamin A</td>
<td>10,000 IU/Kg*</td>
</tr>
<tr>
<td>2. Vitamin D</td>
<td>5,000 IU/Kg</td>
</tr>
<tr>
<td>3. Vitamin E</td>
<td>50 mg/Kg</td>
</tr>
<tr>
<td>4. Choline</td>
<td>800 mg/Kg</td>
</tr>
<tr>
<td>5. Methionine</td>
<td>500 mg/Kg</td>
</tr>
<tr>
<td>6. Sodium chloride</td>
<td>5 g/Kg</td>
</tr>
<tr>
<td>7. Dibasic Calcium Phosphate</td>
<td>9.5 g/Kg</td>
</tr>
<tr>
<td>8. Zinc Sulphate</td>
<td>24 mg/Kg</td>
</tr>
<tr>
<td>9. Potassium Iodide</td>
<td>3 mg/Kg</td>
</tr>
</tbody>
</table>

Total weight = amount of the premix added in 10 kg of the Pelleted diet prepared.

* = amount added/ kg of the diet prepared.