Chapter 1: General Introduction and Review of Literature

1. Introduction and Review of Literature

1.1 Hypothalamic-pituitary-gonadal-hepatic axis

The reproductive hormonal axis consists of four main components: (A) the hypothalamus, (B) the pituitary gland, (C) the gonads (ovary or testis) and (D) Liver (steroid metabolism). Regulation of this axis impacts on the steroid-sensitive end organs apart from primary sex organs. This axis normally functions in a tightly regulated manner to produce concentrations of circulating steroids required for normal sexual development, sexual function and fertility.

1.1.1 Hypothalamus-Pituitary axis

The integrating center of the reproductive hormonal axis is the hypothalamus (Figure 1). It consists of a collection of nuclei and areas located at the base of the brain, ventral to the sub-thalamus. The median eminence of the hypothalamus is the site at which anterior pituitary regulating hypothalamic neurons release their secretions into the capillaries of the primary plexus of the hypophyseal portal system. The median eminence has three components; **neural**, consisting of nerve terminals and neurons in passage; **vascular**, consisting of the primary capillary plexus and the portal veins; and **epithelial,** consisting of the pars tuberalis of the anterior pituitary gland. Two types of tuberhypophyseal neurons project into the median eminence, peptide secreting (peptidergic) (e.g.: GnRH, GHRH, TRH, LHRH and Somatostatin) and bioaminergic the most important of which are dopaminergic. Some peptides in nerve endings are not released into the hypophyseal-portal circulation but instead function to regulate the secretion of other nerve terminals.

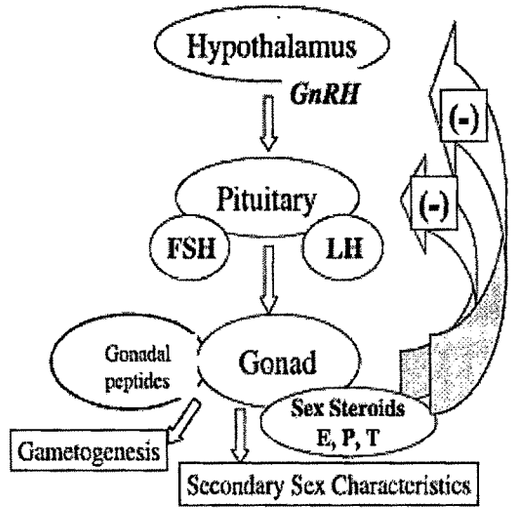

Figure 1: The hypothalamic-pituitary-gonadal axis.

GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E, estrogen; P, progesterone; T, testosterone.

Molecular structure of gonadotrophin-releasing hormones (GnRHs)

GnRH was first isolated and sequenced from mammals (Schally, 1999), and now 13 distinct forms of GnRH have been found in various species (Carolsfeld *et al.* 2000). In fish, amphibians, reptiles and birds, there are two or more forms of GnRH within the brain of single species (Sherwood *et al.* 1993, White *et al.* 1995). The GnRH genes consist of four exons and three introns and exist as part of a larger precursor gene (Sherwood *et al.* 1993, White *et al.* 1998). The precursor cDNA consists of GnRH that is extended at the N-terminus by a signal peptide and at the C- terminus by a Gly- Lys-Arg sequence, characteristic of an enzymatic amidation and precursor processing site, followed by a GnRH associated peptide (GAP).

Functions of GnRH

Gonadotropin-releasing hormone (GnRH) plays a major role in the endocrine control of reproduction. GnRH, released from the hypothalamus, acts upon the pituitary to stimulate LH and FSH secretion. In addition, GnRH also functions as a local regulator in a number of tissues and cell lines. GnRH

interacts with a membrane receptor which belongs to the G-protein-coupled receptor family. The response of the pituitary to GnRH is influenced by gonadal steroids. GnRH has a very short half-life in the blood (approximately 2 to 5 minutes). The pituitary gland is therefore exposed to high levels of GnRH in hypophyseal-portal blood for brief periods of time.

Regulation of GnRH secretion

The area of the brain responsible for the GnRH pulse generator is in the arcuate nucleus of the medial basal hypothalamus. The rhythmic nature of GnRH pulse generator is regulated by the hormonal milieu. Progesterone also slows the pacemaker whereas estrogen has no effect on the pacemaker. The fact that LH and FSH secreted in short pulsatile bursts led to the assumption that GnRH release is also pulsatile. GnRH is secreted in a pulsatile fashion at intervals of 70 to 90 min. In all species, gonadotropin responsiveness to GnRH increases at midcycle either because of sensitization of pituitary gonadotrophs by estrogens or because of stimulation of GnRH secretion, or both, which in turn upregulates GnRH receptors. GnRH secretion is enhanced at midcycle in rodents and rhesus monkey (Pau *et al.*, 1993). Since GnRH neurons do not have estrogen receptors, enhanced GnRH secretion is not caused by direct action of estrogens. Neural control of GnRH secretion is mediated by signals from four classes of neurotransmitters-bioamines, neuropeptides, excitatory amino acids and gaseous neurotransmitters. Excitatory factors include norepinephrine acting through β1 receptors, neuropeptide Y, galanin, NT, substance P and other tachykinins, glutamic acid, NO, transforming growth factor α and prostaglandin E2. Epinephrine and norepinephrine increase GnRH release, whereas dopamine and serotonin and endogenous opiod peptides are inhibitory (Shivers *et al.*, 1983). Other hormones in particular gut related peptide hormones also modulate GnRH release.

Gonadotropins

Gonadotropins belong to glycoprotein hormone family made up of 2 subunits α andβ. α-subunit is common to both LH and FSH. α-subunit gene is

located on chromosome 6 having 4 exons and 3 introns. Prohormone is synthesized as 116 amino acids and mature molecule having 92 amino acids with two N-terminal oligosachharide units.

The human LHβ subunit gene is located on chromosome 19q 13.32 and is close to the hCG β genes. Each of the glycoprotein β subunit is coded by a single gene except hCGβ. There are atleast seven hCG β genes and pseudogenes, and only primates and horses are known to have these genes. The LH and hCG β genes are approximately 1.5 kb size and consists of three exons and two introns. The LH β gene encodes a prohormone with a 24-aminoacid leader sequence a mature peptide of 121 amino acids. The hCG β protein differs from other β subunits in having a 24 aminoacid -COOH terminal extensions, and a longer 5' untranslated portion. The 5'-flanking region of the LH β gene contains a putative estrogen response element.
The human FSH β gene is located on chromosome 11p13 and contains three exons and two introns. The nucleotide and aminoacid sequences of the coding regions of the rat and human FSH β subunits show 79 and 80% homology, respectively. In contrast to a single mRNA in rat and cow, the human FSH β subunit gene is transcribes into four mRNA, as the result of alternate splicing of exon 1 and two polyadenylation sites.

The half time of immunoreactive FSH is greater than that of LH. Similarly metabolic clearance of FSH is less than that of LH, being 6 to 14 ml/min in women and 4 to 12 ml/min in men. Gonadal function does not affect the metabolic clearance rate. The liver and kidney degrade circulating LH and FSH; small amounts of intact LH and FSH are excreted in the urine (3 to 5% for FSH). The content of gonadotropins is low in the pituitary of prepubertal children. In men and in menstruating women the pituitary contains approximately 700 IU of FSH. After menopause the content of pituitary LH rises to approximately 1700 IU but there is no change in FSH content. The range of both serum LH and FSH in normally cycling adult woman is 5 to 20 IU/L. However, on or about the day of ovulation, levels of FSH and LH may be two to three times normal values. A serum level of FSH

higher than 40 IU/L is diagnostic of ovarian failure. Gonadal maturation is regulated by gonadotropins. These hormones are also responsible for the timing and control of pubertal development and sexual maturation. Before puberty the release of FSH is greater than that of LH, the relationship is reversed at puberty. The differential release of LH or FSH is not well understood, but may reflect the effects of inhibin. The major restraint on prepubertal GnRH secretion originates in central nervous system.

Effect of neurotransmitters on H-P-G axis

Serotonin: Serotonin mediates the influence of short photoperiod on reproduction in temperate mammalian species: darkness stimulates the production of hypothalamic and/or pineal indoleamines, which via the hypothalamic suprachiasmatic nucleus and pars tuberalis inhibit hypothalamic GnRH production and release. They thereby regulate gonadotropin output. It has been observed that serotonin injection increases the LH and FSH uptake by the gonadotropin receptor on rat ovaries (Trentini *et al.*, 1976). *In vitro* experiments strongly demonstrated direct effects: it stimulates progesterone and estradiol secretion by cultured rat preovulationary follicles (Tanaka *et al.*, 1993) and human granulosa cells (Bodis *et al.*, 1992) and progesterone release by isolated porcine granulosa (Sirotkin, 1995). Further more, serotonin treatment stimulates oxytocin and cGMP and inhibits vasopressin and cAMP output by porcine granulosa cells (Sirotkin, 1995).

Catecholamines: Dopamine can act both as a neurotransmitter and as a hormone. Dopamine is released from synaptic vesicles to effect nerve cells and is released by the hypothalamus into the portal blood system of the pituitary to primarily inhibit the activity of the acidophilic cells of the anterior pituitary in their production of prolactin. Dopamine also inhibits LH, FSH, and TSH production in the basophilic cells. Dopamine has a less clear role but is thought to slightly reduce GnRH secretion. Norepinephrine exhibits stimulatory effects on LH secretion and appears to be dependant on the presence of ovarian steroids, particularly estradiol.

1.3 Ovarian Structure and functions

The primary function of an adult ovary is to produce the steroid and protein hormones needed to support (1) the development and maturation of ovarian follicles (i.e., folliculogenesis) including oocytes, (2) ovulation, and (3) the initiation and maintenance of pregnancy in mammals. In order for this to collectively occur, the secretion of ovarian hormones is tightly regulated by the neuroendocrine system and is locally controlled by many intraovarian feedback mechanisms. Two major developmental events within the ovary are follicular assembly (i.e., the formation of primordial follicles) and the primordial-to-primary follicle transition (the "initial" recruitment). The initial phase of folliculogenesis is regulated by paracrine growth factors and influenced by local concentrations of steroid hormones, with a more limited role of follicle stimulating hormone (FSH) and luteinizing hormone (LH). It is widely accepted that a pool of primordial follicles (i.e., the ovarian follicular reserve) is formed within the developing fetus or neonate, and once established, primordial follicles do not proliferate. Thus, it is the ovarian reserve of primordial follicles that comprises the source of female gametes for life. This transition enables the growth of some primordial follicles, while others remain quiescent for months, years, or decades depending upon the species. Improper activation of follicular transition is proposed to be the cause of certain reproductive disorders.

Folliculogenesis

Folliculogenesis begins with the recruitment of a primordial follicle into the pool of growing follicles and ends with either ovulation or death by atresia (Fig. 2).

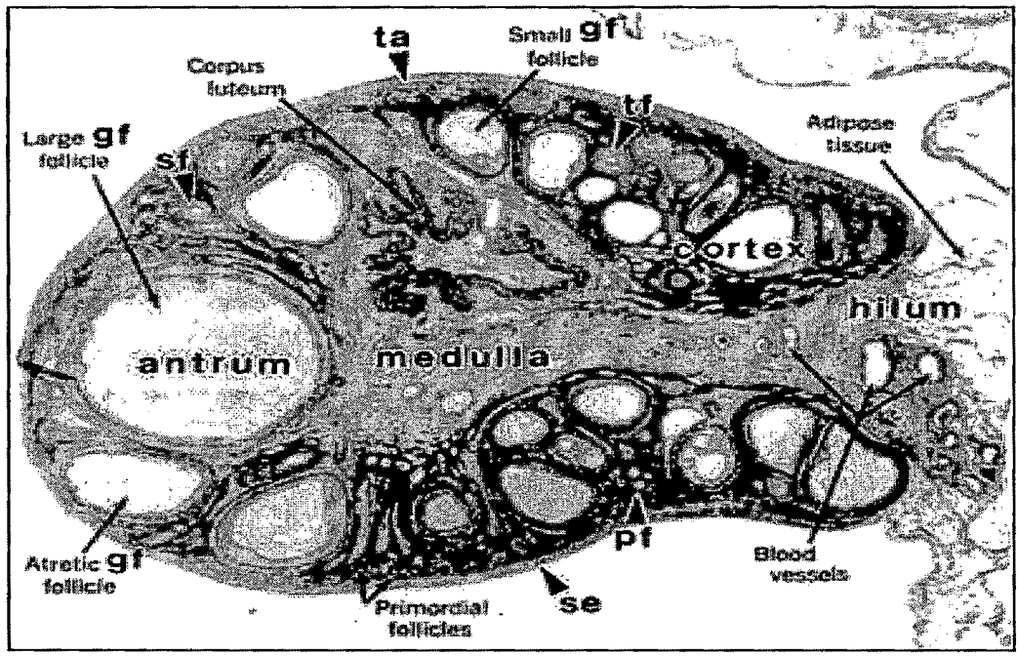

Figure 2: The adult ovary can be subdivided into three regions: the cortex, medulla, and hilus regions. The cortex consists of the surface epithelium (se), tunica albuginea (ta), ovarian follicles (primordial, primary (pf), secondary (sf), small, medium, large Graafian follicle (gf)) and corpora lutea (cl).

Folliculogenesis can be divided into two phases: The first phase, termed the preantral or gonadotropin-independent phase, is characterized by the growth and differentiation of the oocyte. The second, termed the antral (Graafian) or gonadotropin-dependent phase, is characterized by the tremendous increase of the size of the follicle itself (up to approximately 25 mm). The preantral phase is controlled by locally produced growth factors through autocrine/paracrine mechanisms. The second phase is regulated by FSH and LH as well as by growth factors. A primary follicle is defined by the presence of one or more cuboidal granulosa cells that are arranged in a single layer surrounding the oocyte (Fig. 3).

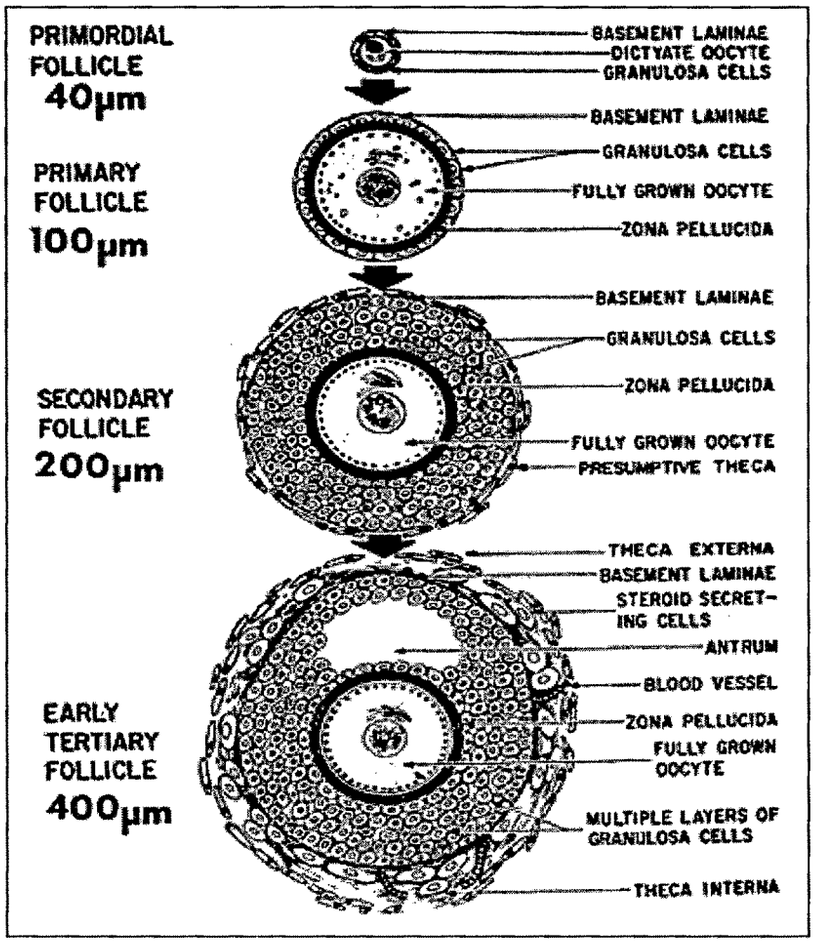

Figure 3: The major histological changes that accompany the gonadotropin-independent period of preantral folliculogenesis

The major developmental events that occur in the primary follicle include FSH receptor expression and oocyte growth and differentiation. During primary follicle development, the granulosa cells express FSH receptors. In rodents, activin produced by the granulosa cells appears to play a role in stimulating the expression of FSH receptors through autocrine/paracrine mechanisms (Fig. 4).

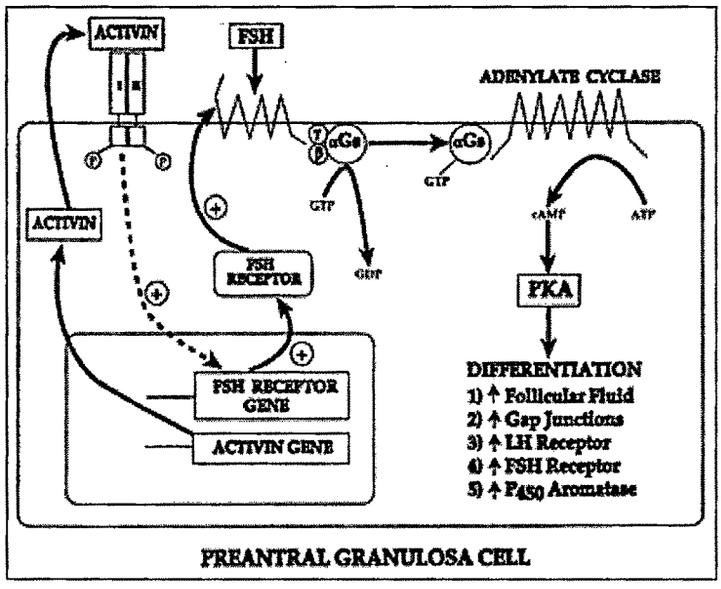

Figure 4: The early differentiation of the granulosa cells during preantral folliculogenesis involves the expression of FSH receptors.

The major changes occur during secondary follicle development include the accumulation of increased numbers of granulosa cells, and the acquisition of a theca. The development of a primary to a full-grown secondary follicle results from an active autocrine/paracrine regulatory process that involves growth factors produced by the oocyte. Secondary follicle development is also characterized by thecal development. Theca development is accompanied by the neoformation of numerous small vessels, presumably through angiogenesis. A Graafian follicle is characterized by a cavity or antrum containing a fluid termed follicular fluid or liquor folliculi. Accordingly, the term antral follicle is used correctly as a synonym for Graafian follicle. Follicular fluid is an exudate of plasma and is conditioned by secretory products from the oocyte and granulosa cells. It is the medium in which the granulosa cells and oocyte reside and through which regulatory molecules must pass on their way to and from this microenvironment.

The proliferation of the follicle cells also contributes to follicle size. In a dominant follicle, the granulosa and theca cells proliferate extensively (as much as 100-fold) concomitant with the antrum becoming filled with follicular fluid (Fig. 5). Thus, increased follicular fluid accumulation and cell proliferation are responsible for the tremendous growth of the dominant follicle during the follicular phase of the cycle. It is the cessation of follicular fluid formation and mitosis that limits the size of the atretic follicle.

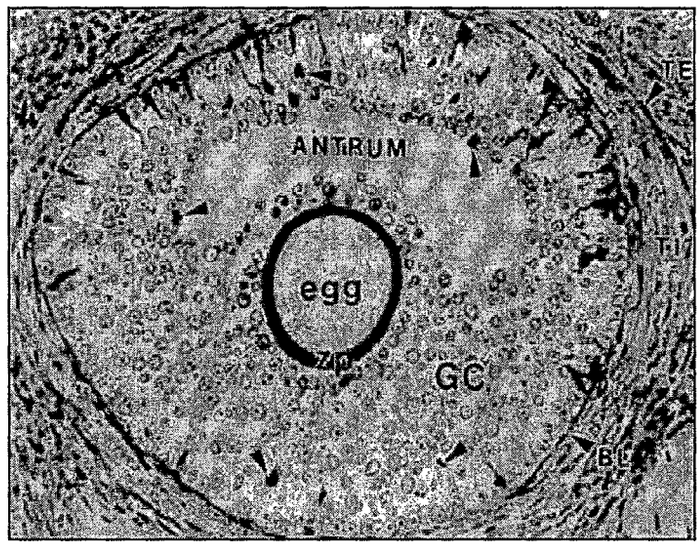

Figure 5: Photomicrograph of an early tertiary follicle 0.4 mm in diameter at the cavitation or early antrum stage. zona pellucida (ZP); granulosa cells (GC); basal lamina (BL); theca interna (TI); theca externa (TE); granulosa mitosis (arrow heads).

The theca externa (Fig.5) consists of concentrically arranged smooth muscle cells, which are innervated by autonomic nerves. The theca interna have the ultrastructural characteristics typical of active steroid producing cells, i.e., the cytoplasm is filled with lipid droplets, smooth endoplasmic reticulum and mitochondria with tubular cristae. The theca interstitial cells possess cell receptors for LH and insulin. In response to LH and insulin stimulation, they produce high levels of androgens, most notably androstenedione. The theca interna is richly vascularized by a loose capillary

network that surrounds the Graafian follicle during its growth. In the Graafian follicle, the granulosa cells and oocyte are distributed as a mass of precisely shaped and precisely positioned cells. All the granulosa cells express FSH receptors during Graafian follicle development; however, each group of granulosa cells is influenced by its position to express a specific differentiated state in response to FSH stimulation.

Atresia

In mammals, 99.9% of all the follicles (oocytes) die by atresia. A fundamental property of atresia is the activation of apoptosis in the oocyte and granulosa cells. Apoptosis is a complex process involving ligand signaling pathways that are coupled to cell death. The importance of FSH in preventing apoptosis has led to the concept that FSH is a follicle survival factor. Understanding the mechanisms underlying atresia continues to be a priority in female reproductive research.

Apoptosis

Apoptosis is a mechanism that allows cells to self-destruct and operates at all developmental stages and in all cell-types (Raff, 1992). Apoptosis can be initiated for several reasons, such as when a cell is no longer needed or when it becomes a threat to the health of the organism. Controlled removal of cells is necessary in embryonic development as well as in the daily maintenance of a mature organism. The term apoptosis is derived from a Greek word meaning "falling off" in the sense of leaves falling off the trees in autumn. Apoptotic death requires co-ordinated activation and propagation of several sub-programs (Hengartner, 2000). Two groups of proteins constitute the framework of the apoptotic program: the caspase family of proteases and the Bcl-2 family of regulatory proteins (Fig. 6). Caspases are the executioners of the apoptotic pathway (Hengartner, 2000) and function as proteases cleaving target proteins after an aspartic acid residue. There are at least three mechanisms for caspase activation, including 1) processing by an upstream caspase, 2) association with cofactors and 3) cleavage induced by a high local

concentration of caspases, associated with activated death receptors (Fig. 6). One function of caspases is to activate the endonuclease CAD (Caspase-Activated DNase). CAD and its inhibitory subunit ICAD are constantly expressed in the cells. Caspase-mediated cleavage of the inhibitory subunit results in release and activation of the endonuclease. The resulting internucleosomal DNA fragmentation is one of the classical hallmarks used for apoptosis detection. The Bcl-2 family of apoptotic regulators comprises both anti- and pro-apoptotic proteins. Members of the Bcl-2 family can homodimerise or heterodimerise with other family members, thereby regulating each other's activity. The main function of the Bcl-2 family seems to be to regulate the release of pro-apoptotic factors, in particular cytochrome c, from mitochondria into the cytosol (Antonsson *et al.*, 2000). Execution of the apoptotic program converges on the mitochondria, where pro- and anti-apoptotic members of the Bcl-2 family interact at the surface. Excess of pro-apoptotic activity will cause the release of an array of molecules from the mitochondrial compartment. The most important of these is cytochrome c, which associates with APAF-1 and procaspase-9 to form the apoptosome complex. This complex subsequently activates downstream caspases, such as caspase-3. Downstream of caspase-3, the apoptotic program branches off into a multitude of subprograms, one of which is exposure of phosphatidyl serine on the cell surface. Another effect of caspase activation is cleavage of ICAD (Inhibitor of Caspase-Activate DNase) resulting in the release of CAD (Caspase-Activated DNase), the endonuclease responsible for internucleosomal DNA fragmentation.

Regulation of ovarian apoptosis

The susceptibility to apoptosis, as well as the regulators of follicle survival, changes in the course of follicle development. This results in a gradual reduction of the number of follicles as they differentiate and grow towards ovulation (Markström *et al.*, 2002). The main physiological regulators of ovarian follicle survival are the gonadotropins, although a number of locally produced growth factors have also been demonstrated to affect follicle cell survival (Fig.6).

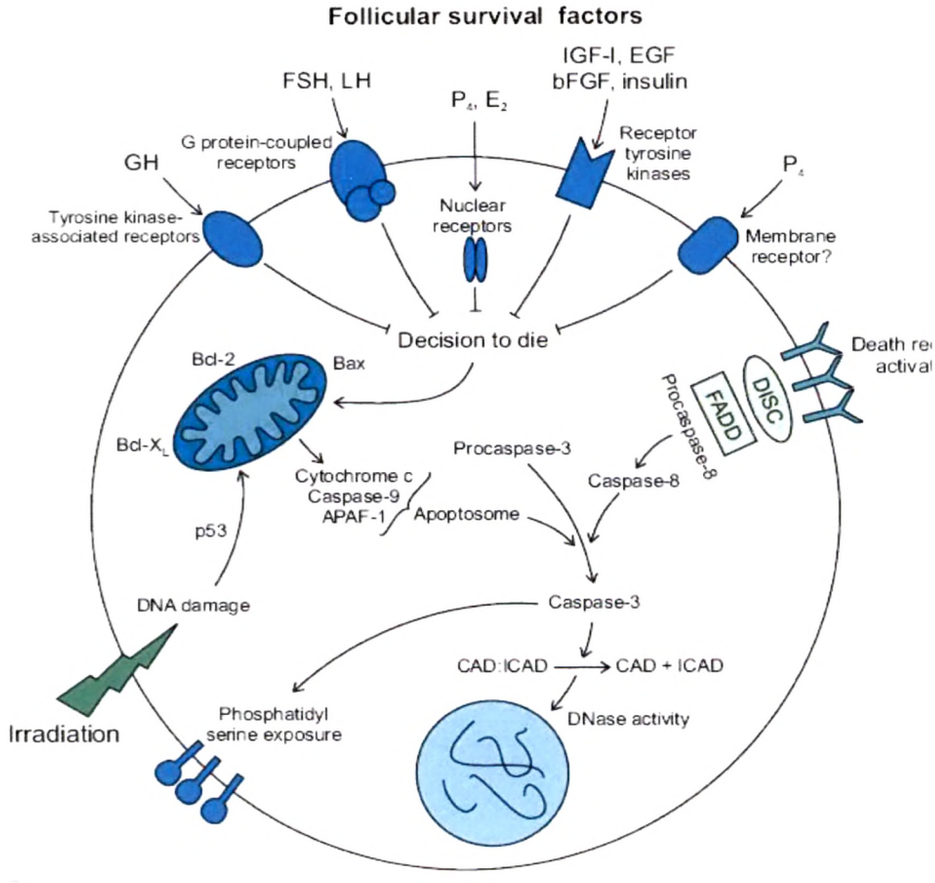

Figure 6: Schematic overview of the apoptotic process in follicular granulosa cells

Examples of survival factors include oestrogens, insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), fibroblast growth factor (FGF), interleukin-1β (IL-1β) and nitric oxide. Pro-apoptotic factors include androgens, gonadotropin-releasing hormone-like peptide (GnRH-like peptide) and interleukin-6 (IL-6) (Billig *et al.*, 1996).

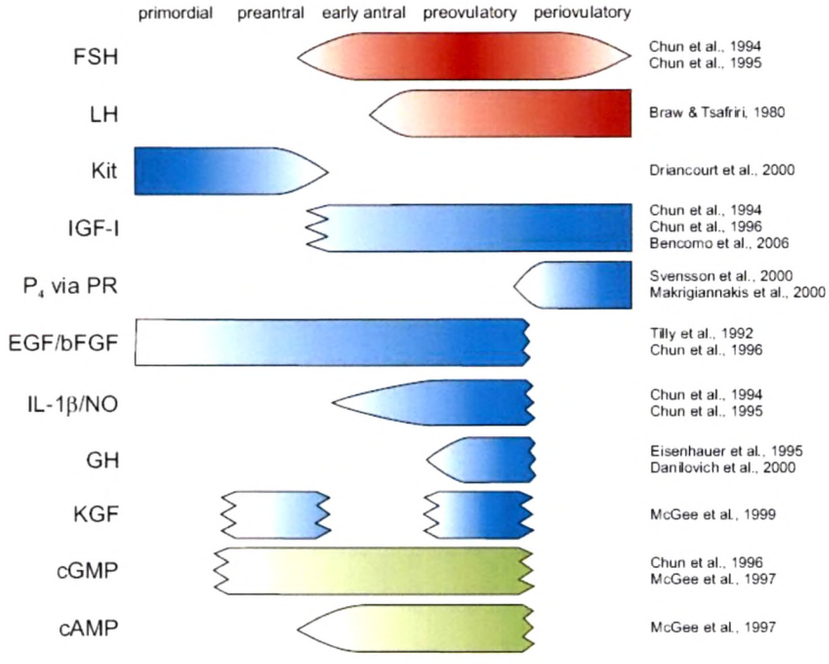

Figure 7: Factors regulating stage-dependent survival of ovarian follicles.

Some of the factors promoting survival during the growth and differentiation of follicles are summarised in (Fig. 7). Although FSH has the ability to enhance steroidogenic enzyme expression in preantral follicles (Dunkel *et al.*, 1994, Rannikki *et al.*, 1995) it has no effect on apoptosis in cultured rat follicles (McGee *et al.*, 1997). Follicles that have differentiated to the antral stage or further express FSH receptors and are dependent on sufficient FSH concentrations for survival. Due to lack of FSH support, many follicles never pass this point in their development. In the adult human ovary,

it has been suggested that the degree of atresia is highest in antral follicles of a diameter greater than 5 mm, 77% of which have been estimated to undergo atresia (Gougeon, 1986). Locally produced factors of importance for the survival of isolated early antral rat follicles include IGF-I, EGF, FGF, activin, and the cytokine IL-1β (Chun *et al.*, 1996). At the preovulatory stage of development, the ovarian follicles express LH receptors and are able to respond to the coming LH surge. Both FSH and LH suppress the degree of apoptosis in isolated preovulatory rat granulosa cells in a way that may be partially mediated by endogenously produced IGF-I (Chun *et al.*, 1994). Recently, IGF-I has also been shown to be a survival factor after the LH surge in periovulatory follicles (Bencomo *et al.*, 2006).

Progesterone

The steroid hormone progesterone is a key component in the regulation of female reproduction and targets several organs. The main functions of progesterone are 1.) In the ovaries and uterus: ovulation, facilitation of implantation and maintenance of pregnancy by promoting uterine growth and suppressing myometrial contractility; 2.) in the mammary glands: development of the ducts during pregnancy and suppression of milk protein synthesis before parturition; and 3.) in the brain: mediation of signals for sexually responsive behaviour (Graham & Clarke, 1997). The main production site for progesterone is the corpus luteum and, during pregnancy, the placenta. Like all steroid hormones, progesterone is synthesized from cholesterol.

The progesterone receptor

Progesterone exerts its functions by interacting with a specific nuclear progesterone receptor (PR) protein. The PR belongs to the superfamily of nuclear receptors. All members share structural domain organisation with a highly conserved DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD) and a more variable N-terminal domain. The steroid receptors contain at least two transcription activation function domains that are important for interaction with coactivators. Upon ligand binding the receptor-

ligand complex translocates to the nucleus and functions as a transcription factor, thereby regulating the expression of target genes. The receptor-mediated action of progesterone has been extensively characterised by O'Malley and colleagues (Li & O'Malley, 2003). Upon progesterone binding the PR undergoes conformational changes, causing dissociation from the multi-protein chaperone, dimerisation, increased phosphorylation, and binding to steroid response elements (SREs) within target gene promoters (Tsai & O'Malley, 1994). The consensus sequence of the response element is 5'-TGTTCT-3', a semi-palindromic half-site usually separated by three base pairs. As each individual response element is weak, there are usually several SREs near the regulatory region of steroid controlled genes. Active PR interacts with co-activators that facilitate transcription in two ways; by interacting with the general transcription machinery and by promoting local chromatin remodeling (Edwards *et al.*, 2002).

FSH Signaling cascade in granulosa cells

The FSH receptor is part of a large family of transmembrane receptors that regulate the heterotrimetric G proteins.

The human FSH receptor contains 678 amino acids. It is organized into three domains: 1) the extracellular NH2 terminal ligand binding domain with six potential N-linked glycosylation sites and a cluster of cysteines at the junction between the extracellular and transmembrane domains; 2) the transmembrane spanning domain composed of seven hydrophobic a helicies that anchor the receptor to the plasma membrane; and 3) the intracellular COOH-terminal domain with a relatively high proportion of serine and threonine residues. The regulated phosphorylation of these amino acids in the intracellular domain plays a role in desensitization and down regulation of the FSH receptor. The FSH signaling cascade in the granulosa cell is illustrated in (Fig. 8). The FSH control of differential gene activity in the granulosa cells is the basis of the process of dominant follicle growth and development to the pre-ovulatory stage.

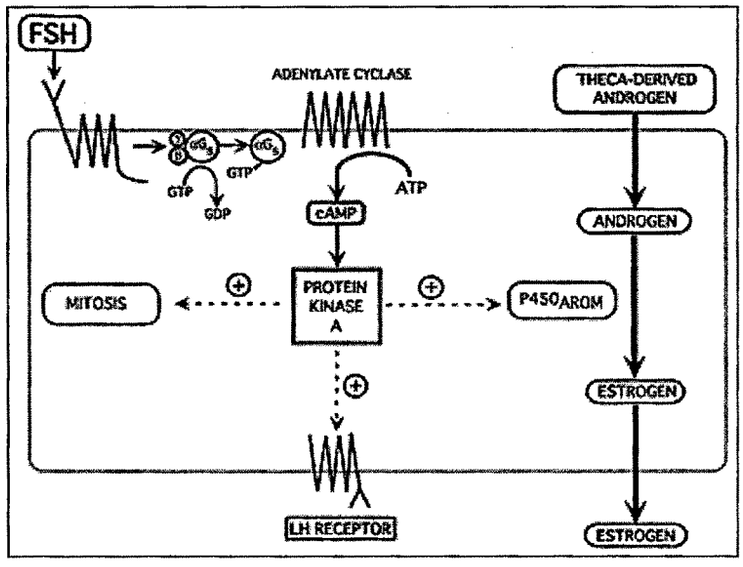

Figure 8: FSH signal transduction pathway in granulosa cells of a dominant follicle.

In women, FSH is a stimulator of granulosa cell proliferation. FSH probably acts with growth factors to regulate proliferation of human granulosa cells. As the dominant follicle grows, the granulosa cells acquire the potential to produce large amounts of estradiol. The FSH induction of P450arom (the CYP19 gene) expression in the granulosa cells is causal to the acquisition of the estrogen potential of the follicle. The P450arom is detected when a follicle reaches ~1mm in diameter or the class 2 stage, and it is seen in only the dominant follicle. The P450arom activity increases progressively, reaching very high levels in the granulosa cells of the preovulatory follicle in the late follicular phase. The type I 17ß-hydroxysteroid dehydrogenase (17ß-HSD) appears to be constitutively expressed in the granulosa cells in follicles from the primary to the preovulatory stage. By virtue of the expression of P450arom and 17ß-HSD, the granulosa cells become highly active in converting theca-derived androstenedine to estradiol. It is the progressive increase in the level of P450arom gene expression that makes it possible for the dominant follicle to secrete the increasing amounts of in estradiol during days 7 to 12 of the menstrual cycle.

During the follicular phase, the granulosa cells also acquire the potential to produce increasing amounts of progesterone. Several operative processes have been found to be involved in the acquisition of luteinization potential. The continued stimulation of the granulosa cells by FSH is believed to be involved in this progressive process. When luteinization actually occurs, granulosa cells express large amounts of Steroid Acute Regulatory Protein (StAR), the P450 side chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD). Despite the fact that a progressive increase in the potential for luteinization occurs, it remains suppressed until just prior to ovulation. It is clear from studies in animals that the inhibition is caused by oocyte-derived luteinization inhibitors present in follicular fluid. These luteinization inhibitors include GDF-9, BMP-6, and BMP-15. They operate to specifically inhibit the expression of StAR, P450scc and 3β-HSD in the granulosa cells during folliculogenesis.

Competence of the preovulatory follicle to respond to the inductive stimulus of the LH surge by undergoing ovulation involves the expression of LH receptors in the granulosa cells. FSH plays a vital role in LH receptor induction in the granulosa cells. Similar to StAR, P450scc and 3ß-HSD, the expression of LH receptors remain suppressed until late in the follicular phase of the cycle. There is compelling evidence in laboratory animals that oocyte-derived inhibitors inhibit FSH- induced LH receptors in granulosa cells. The interpretation is that the oocyte is responsible for inhibiting the expression of granulosa LH receptors in the developing Graafian follicle until the onset of the preovulatory stage.

LH Signaling cascade in granulose cells

The human LH receptor cDNA has been cloned and sequenced. Like the FSH receptor, it is a part of a large family of transmembrane receptors that regulate the heterotrimeric G proteins. The mature LH receptor has a long extracellular NH2 terminus ligand binding domain, a transmembrane domain

containing seven hydrophobic a helices that integrate the LH receptor to the membrane, and an intracellular COOH-terminus domain that interacts with residues of the third intracellular loop I3 to activate the G proteins. The signal transduction mechanisms of LH receptors are coupled to G proteins (Fig. 9).

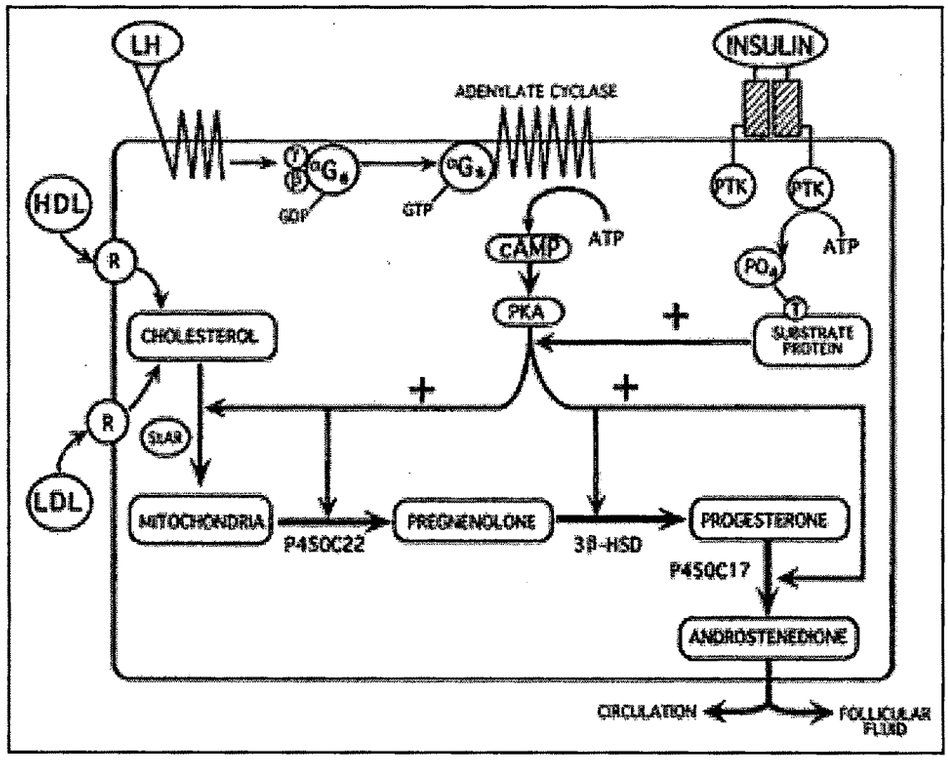

Figure 9: The regulatory mechanisms of androgen production by theca interstitial cells

Stimulation of Androgen Production

At about the time of antrum formation, the theca interstitial cells begin to express their differentiated state. This event involves the expression of a battery of genes, including LH receptors, insulin receptors, lipoprotein receptors (HDL, LDL), StAR, P450scc, 3ß-HSD, and P450c17. By virtue of the expression of these genes, the theca interstitial cells have the capacity to produce androstenedione (Fig.9). LH is the most important effector of theca interstitial cytodifferentiation, but insulin and lipoproteins can act in synergy with LH to amplify this process.

Two-cell two-gonadotropin concept

The physiological mechanism by which the dominant follicle produces estradiol is called "the two-cell two-gonadotropin concept" (Fig.10). The delivery of LH to the theca interstitial cell leads to the synthesis and secretion of androstenedione. The amount of androgen secretion will reflect the presence within the theca of other regulatory molecules including insulin, IGF-I, lipoproteins, activin, and inhibin. Some androstenedione diffuses into the follicular fluid where is accumulates at very high concentrations. In response to the P450arom induced in the granulosa cells by FSH stimulation, the androstenedione is aromatized to estrone, which then is converted to estradiol by the 17ß-HSD.

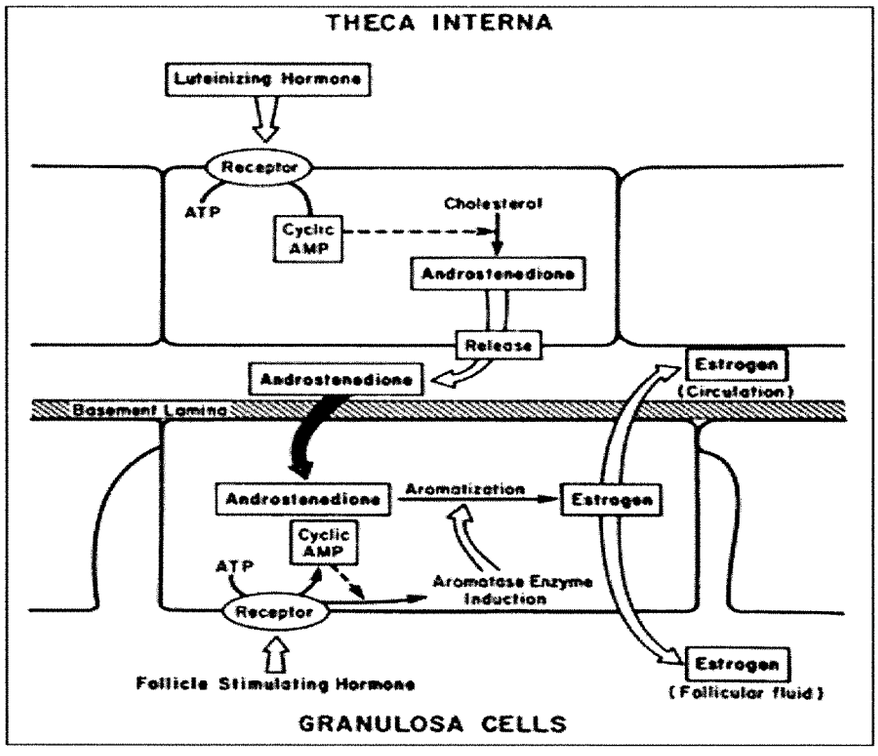

Figure 10: "Two Gonadotropin-Two Cell Concept"

Biogenesis of steroid hormones

Steroid hormone biosynthesis is mainly regulated by events that ultimately affect steroid production through four parameters or processes: 1) Steroidogenic enzyme level as determined by transcription, stability and translation of the mRNAs encoding the enzymes, 2) Steroidogenic enzyme activity, determined by the conditions of the intracellular milieu, cofactor availability, or the post-translational modification of the enzymes, 3) Substrate availability, generally determined by cholesterol mobilization and transport to the mitochondrial P450scc that catalyzes the first step in the pathways of steroid biosynthesis and 4) Tissue growth, determined by cell division and multiplication, as in the corpus luteum formation. All the steroids of the ovary are derived from the precursor-cholesterol. Cholesterol can be derived from three main sources: 1) Preformed cholesterol circulating in blood in form of lipoproteins 2) cholesterol synthesized de novo within the ovary from 2C units 3) cholesterol liberated from cholesterol esters stored within the lipid droplets.

In ovary, LH stimulates activity of adenylate cyclase causing the release of cyclic AMP, which serves as secondary messenger to stimulate an increase in mRNA for LDL receptor, thereby increases binding and uptake of LDL-Cholesterol and cholesterol esters (Strauss *et al.*, 1999). Cholesterol is then transported from outer to the inner mitochondrial membrane by cAMP activated StAR protein (Steroidogenesis acute regulatory Protein). StAR is a 30 Kd mitochondrial protein, which is believed to be key mediator of acute induction of steroidogenesis (Clark *et al.*, 1995). StAR has two functional domains namely C-terminal domain which increases cholesterol movement to cytochrome P450scc by promoting sterol desorption from the sterol rich outer mitochondrial membrane, driving it to the relatively poor inner membrane (Fig. 11). Other domain is N-terminal domain which has mitochondrial targeting sequence that directs the StAR protein to the mitochondria (Arakane *et al.* 1996). StAR is expressed in the granulosa cells, cells of corpus lutea (Thompson *et al.*, 1999). There is a nuclear receptor protein steroidogenesis

factor -1 (SF-1), which is a cis acting cognate response element in StAR's promoter regions and causes a several fold increase in the expression of the gene and parallel conversion of cholesterol to pregnenolone.

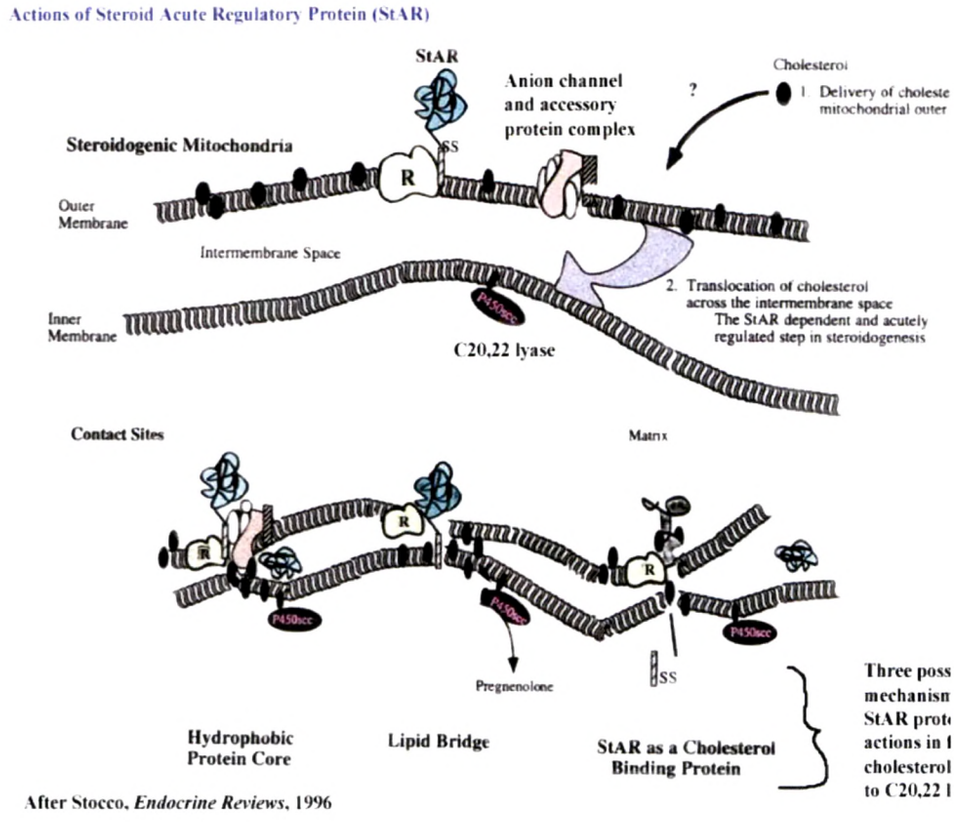

Figure 11: Functions of Steroidogenic Acute Regulatory Protein (StAR)

Principal steroid producing cells of the ovary - Granulosa cell, Theca and Corpus Luteum, which possess complete enzymatic complement required for steroid hormone synthesis (Figure 12). The main pathway of steroid synthesis involves conversion of pregnenolone to progesterone. In ovarian follicle, Δ^5 are preferred pathway for the formation of androgen to estrogen because thecal cell can easily metabolize 17- hydroxy pregnenolone more than 17-hydroxyprogesterone. Predominant steroid produced, differs

among each cell type- thecal, stromal cell secretes androgen, corpus luteum cells mainly progesterone, and granulosa cells mainly estrogen. The factors that determine which steroid is secreted by each cell type include levels of gonadotropin, gonadotropin receptors, expression of steroidogenic enzymes and availability of LDL- Cholesterol.

Rate of steroid production during the estrus cycle is function of content of 4 key enzymes- Cholesterol side chain cleavage enzyme (CYP11A1), 3 β Hydroxy Steroid Dehydrogenase, Steroid 17 α Hydroxylase (CYP 17) and Aromatase (CYP19). These enzymes catalyze conversion of cholesterol to pregnenolone, pregnenolone to progesterone, pregnenolone to androgens, androgens to estrogen.

P450 Side Chain Cleavage (P 450scc) - This enzyme catalyzes the first and rate-limiting step in the biosynthesis of steroid hormones (Jefcoate *et al.*, 1992). It converts cholesterol to pregnenolone in three successive monooxygenations (hydroxylation at C-22, followed by C-20, and finally cleavage of the C-20, 22). Hydroxylated intermediates of cholesterol bind very tightly to P450scc and do not show significant dissociation from the enzyme (Orme-Johnson, 1990). In contrast, the final product pregnenolone has a dissociation constant 40-600 fold higher than those of intermediates, facilitating its release from the enzyme (Orme-Johnson, 1990). In the ovary it is expressed in the theca interna; its expression in the granulosa cells depends on the stage of growth of the follicle. Expression of P450scc in rat granulosa cells is initiated with a delay after expression in theca cells, and takes place close to the time of LH surge (Zlotkin *et al.*, 1986). P450 scc expression differs according to stage of ovarian development. The sequential expression of P450scc in the different follicular cells could result from a gradient of gonadotropin responsiveness in the follicle.

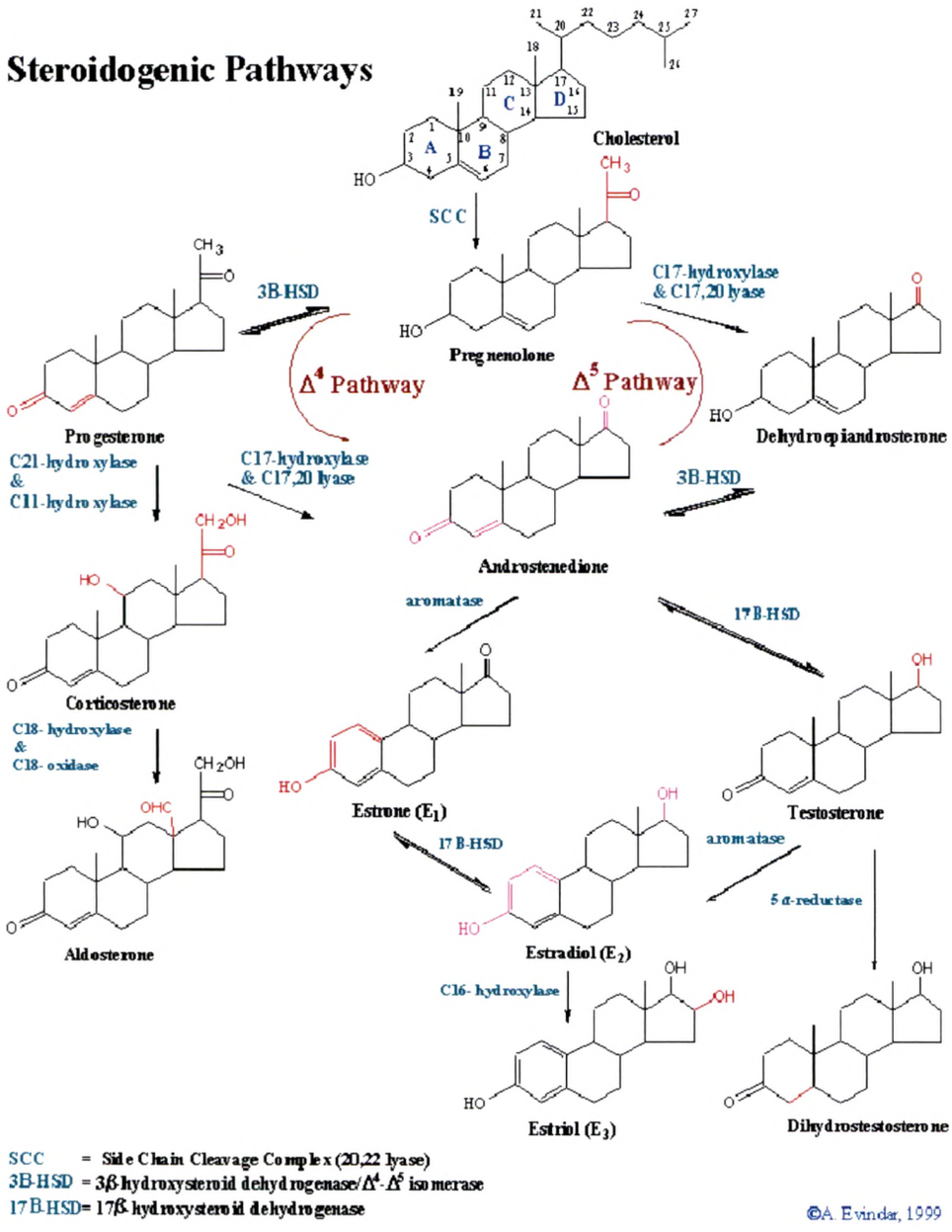

Figure 12: Steroidogenic Pathways

3β-Hydroxy Steroid Dehydrogenase (3β-HSD) enzyme has two major catalytic activities, which in concert convert 3β-hydroxy-5-ene steroids into 3-keto-4-ene steroids. In contrast to steroidogenic P450s each of which is encoded by a single gene, in the human, rat and mouse genomes there are at least 2-3 homologous genes encoding 3β-HSD's that share 80-94% sequence identity within each species (Labrie *et al.*, 1992). Two types of 3β-HSD's from human and rat can use pregnenolone, 17-OH-pregnenolone or DHEA as substrates (Labrie *et al.*, 1992). The different types have different K_m values for the same substrates. However, each type of enzyme can use pregnenolone or DHEA as substrates with similar K_m values (Labrie *et al.*, 1992). Rat type I 3β-HSD also shows 17β-HSD activity with 5α-androstane steroids but not with estradiol, estrone, androstenedione, or testosterone. Type II 3β-HSD is expressed in the human adrenal cortex and gonads, but not placenta, whereas in the rat, both type I and II are found in these tissues (Labrie *et al.*, 1992).

Aromatase (P450arom): P450arom catalyzes conversion of testosterone into 17β-estradiol. In the ovary P450arom is expressed in granulosa cells which are the major site of estrogen production in females (Sasano *et al.*, 1989). However, this enzyme is widely expressed in many tissues besides gonads, e.g. adipocytes, breast, central nervous system, skin and placenta. The gene encoding P450arom is the longest one among steroidogenic P450 genes. This gene is also unique among P450 genes in having alternative promoters that are utilized in a tissue specific manner (Simpson *et al.*, 1992).

Studies of the steroidogenic capacities of isolated granulosa cells and thecal cells led to propose 2-cell gonadotropin theory, which proposed that thecal cell produce C_{19} steroids in response to LH and FSH, which stimulates granulosa cells to aromatize those C19 steroids produced by thecal cells to estrogen. CYP19 mRNA (Aromatase) is localized in the granulosa cells, which is increased markedly in estrogen biosynthesis before ovulation. CYP19 mRNA is highly concentrated in corpus luteum (Fig. 12).

17β-Hydroxy Steroid Dehydrogenase (17β-HSD): This enzyme is also referred as 17-keto-steroid reductase. It catalyzes the reversible conversion of

the 17-keto and 17β-hydroxy groups in androgens and estrogens, including androstenedione, Dihydroepiandrosterone (DHEA), and 17β-estradiol. Direction of the reaction depends on the substrate and cofactor. There are multiple 17β-HSD isozymes with androgen or estrogen specificity (Inano *et al.*, 1990; Luu-The *et al.*, 1990; Martel *et al.*, 1992).

17β-HSD, which converts androstenedione and testosterone as well as estrone and estradiol, consists of 5 isoenzymes. Each isoenzyme has a preferred substrate, cofactor and equilibrium. 17 βHSDH type I is responsible for converting estrone to estradiol in ovary and placenta, it is localized in follicles and Corpus luteum. Type II converts estradiol to estrone, localized in corpus luteum. Cholesterol side chain cleavage enzyme (CYP 11A1) and 3β-HSD expressed in granulosa cells and thecal cells of antral and preovulatory follicles and in luetinized granulosa and thecal cells of corpus luteum. In contrast, C $_{17\text{-}20}$ Lyase (CYP 17) is expressed only in thecal cells of antral and preovulatory follicle and in luetinized theca cells of Corpus luteum. Consequence is that androstenedione is synthesized in theca cells and diffuses into granulosa cell, which is converted to estrone and estradiol (Zhang *et al.*, 1996). 17β HSD Type 1 is expressed in the granulosa cells of the ovary, which convert the theca cells derived androgens to estradiol with the help of P450 Aromatase. Correlation of 17β HSD type 1 mRNA expression with 17β HSDH activity as well as estrogen production in granulosa cells demonstrated that the type 1 enzyme is the 17β HSDH predominantly involved in ovarian Estradiol biosynthesis. The expression of 17β-HSD type 1 is low in antral follicles of rat ovaries, unregulated during maturation, and highest in graffian follicles. Thereafter, expression of the enzyme in rat ovary decreases during luteinisation and is almost undetectable in corpus lutea (Ghersevich *et al.*, 1994). Expression of 17β-HSD type 1 and P450 Aromatase in granulosa cells is almost regulated in parallel. Both of the enzymes are under multihormonal regulation i.e., under the effects of pituitary gonadotropin and also modulated by estrogens, androgens and growth factors. During luteinisation, 17β HSD

type 1 expression may be down regulated earlier than P450 Aromatase and thus limit estradiol biosynthesis in luteinising granulosa cells.

1.4. Testicular steroidogenesis

In the testis, LH stimulates testosterone secretion and FSH is important in the initiation and maintenance of spermatogenesis. The secreted testicular androgen testosterone and its activated form dihydrotestosterone (DHT) act on numerous target end organs causing the development of male secondary sexual characteristics and inhibiting the pituitary secretion of LH and FSH. Peptide secretory products of the testis include inhibin, activin and follistatin which also regulate gonadotropin secretion. Sertoli cell products may serve as the mediators of interaction between germ cells, Leydig cells, peritubular myoid cells and the Sertoli cells of the testis. The development of the male germ cells in the seminiferous tubule essentially consists of three phases: spermatogonal clonal expansion, meiosis, and spermatogenesis. Spermatogenesis is a 73-day process by which a primitive stem cell, the type A spermatogonium, passes through a series of transformations to give rise to spermatozoa. In the seminiferous epithelium, cells in these developmental phases are arranged in defined in stages. Along the seminiferous tubules, these stages follow one another in a regular fashion, giving rise to the wave of the seminiferous epithelium.

Spermatogenesis is dependent on pituitary FSH and on intratesticular testosterone. FSH and androgens seem to have different preferential sites of action during spermatogenesis. Stages VII and VIII appear to be androgen-dependent, whereas maximal binding of FSH and activation of FSH-dependent enzymes occurs in Stages XIII to XV of the spermatogenic cycle. LH affects spermatogenesis by increasing intra-testicular testosterone levels. The levels of FSH required to initiate spermatogenesis in these patients are low. Thus, both FSH and LH are apparently required for the initiation and completion of spermatogenesis. Both FSH and LH are necessary to maintain quantitatively normal spermatogenesis in man. LH stimulates testicular

steroidogenesis by binding to LH receptors on Leydig cells. In addition to LH, FSH may indirectly affect Leydig cell function by action on Sertoli cells and spermatogenesis. In addition to LH, FSH and androgens many other peptides and growth factors (e.g., inhibin, activin, insulin-like growth factor 1, transforming growth factors) are secreted locally in the seminiferous tubular microenvironment.

1.5. Hepatic xenobiotic and steroid metabolism

Despite relatively simple chemical structure, steroids occur in a wide variety of biologically active forms. This variety is not only due to the large range of compounds secreted by steroid-synthesizing tissues, but also to the fact that circulating steroids are extensively metabolised peripherally, notably in the liver, and in their target tissues, where conversion to an active form is sometimes required before they can elicit their biological responses. Steroid metabolism is therefore important not only for the production of these hormones, but also for the regulation of their cellular and physiological actions. The plasma concentrations of SHBG are regulated; they may be increased 5-10-fold by estrogens and decreased twofold by testosterone (Griffin & Wilson, 1998). Thus, a 20-fold higher concentration of total testosterone in men than in women results in a 40-fold difference in free testosterone. Unliganded plasma SHBG binds to either steroid or to SHBG-receptor; SHBG must first bind to the receptor and then the steroid in order to act: SHBG that is liganded to steroid cannot bind to the receptor.

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Figure 13: Biotransformation of steroids in hypothalamus, pituitary and liver

Hypothalamus and pituitary

Progesterone ----------→ 5α-pregnane 3,20 dione (5α reductase)

5α-pregnane 3,20 dione ----------→ 3α-hydroxy 5α-pregnane 3,20 dione (3α HSDH)

Liver

Oxido reduction

Estrone + NADH + H^+ <==========> Estradiol + NAD

Glucoronide formation

Steroid-OH + UDPGA-------→ Steroid glucuronide

Sulphate formation

(1) SO_4^{--} + ATP --- -------→ APS + P-Pi (ATP sulphurylase)

(2) APS + ATP --- -------→ PAPS (ATP kinase)

(3) Steroid-OH + PAPS --- -------→ Steroid-O-SO^{---} + PAP + H+ (ATP sulphokinase)

The SHBG-receptor complex present on the membranes of target tissues may be responsible for the interaction between the steroid hormone and cAMP pathways (Rosner, 1991). These observations provide a mechanistic explanation for the finding that some estrogenic effects are rapid (milliseconds) and are possibly mediated in a non-genomic manner. The intracellular form of the SHBG protein may sequester or direct hormone to the target tissue. Estrogens are eliminated in faeces and urine. The principal metabolites found in urine are polyhydroxylated forms conjugated at C3 to glucuronic acid or sulfate. Elimination in bile is subject to enterohepatic circulation, and 20% of estrogens may be lost through faecal elimination. The terminal plasma half-life of estradiol after intravenous administration to

humans was 27 min; the volume of distribution was calculated to be 0.082 l/kg bw (White *et al.*, 1998).

Steroid hydroxylation accomplishes the same goal by stereo-selectively and regio-specifically attaching hydroxyl groups to the steroid, which also provides sites for subsequent conjugation reactions. Sex-specific differences in steroid hydroxylation, uridine diphosphate (UDP)-glucuronosyltransferase, and sulfotransferase enzymes (Fig. 13) have been investigated and well documented in mammals (Niwa *et al.*, 1995; Pampori and Shapiro, 1993). Oxido-reduction of testosterone to androstenedione, dihydrotestosterone, and/or androstanediols is another hepatic biotransformation pathway that influences circulating levels of testosterone.

1.6 Heavy metals-Lead and Cadmium: As Endocrine disruptors

An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)population (WHO, 2002). The homeostasis of sex steroids and the thyroid are the main targets of (Endocrine Disrupting Chemicals) EDC effects; hence, reproductive health, considered as a continuum from gamete production and fertilization through to intrauterine and post-natal development of progeny, is recognized as being especially vulnerable to endocrine disruption (Mantovani, 2002). They can mimic or block chemicals naturally found in the body, alter hormonal levels, and thus, affect functions that these hormones control. Less direct interferences involve alteration of the body's ability to produce hormones, interference with the ways hormones travel through the body, and changes in numbers of receptors.

Table 1: Main classes of EDCs present in food and in environment with major relevance for female reproductive system

Chemical(s)	Pathways of exposures	Mechanisms of action
POPs		
Polychlorobiphenyls (PCB)	Food chain (fat-rich food, e.g. milk and derivatives, fatty fish, etc.), living environment	Alteration steroid hormone metabolism/transport, ability to bind with the thyroxin transport protein, transthyretin (TTR), interaction with thyroid hormone receptors, neuroendocrine effects
Dioxins and 'dioxin-like' PCBs	Food chain (fat-rich food, e.g. milk and derivatives, fatty fish, etc.), living environment	Aril hydrocarbon Receptor interaction leading to altered steroid hormone metabolism and neuroendocrine effects including on thyroid
DDT and metabolites	Food chain (fat-rich food, e.g. milk and derivatives, fatty fish, etc.), living environment and workplaces (in developing countries)	Mainly estrogenic activity but also interaction with
Substances used in agricultural and farm animal production		
Organochlorine insecticides (e.g. Lindane)	Food chain (fat-rich food, e.g. milk and derivatives, fatty fish, etc.), living environment, workplaces (mainly in developing countries)	Homeostasis of steroid hormones (estrogenic and/ or anti-androgenic effects, interaction with PR)
Triazoles, Imidazoles	Food chain (agricultural and zootechnical fungicides), living environment and workplaces (agricultural areas)	Inhibition of steroid hormone biosynthesis
Triazines	Food chain (herbicides), living environment and workplaces (agricultural areas)	Effects on HHG axis
ETU (metabolite of ethylene bisdithiocarbammates, e.g. Maneb), Benzimidazoles	Food chain (agricultural and zootechnical fungicides), living environment and workplaces (agricultural areas)	Thyreostatic effects
Industrial products and daily-use products		
Nonyl-phenols and octyl-phenols	Detergent by-products: food chain (seafood) and consumer products	Estrogen agonists—ER alpha
Bisphenol A	Food chain (e.g. plastics in contact with food), consumer products (e.g. dental sealant, plastic additive, etc.)	Estrogen agonist—ER alpha
Several phthalates (di-2-hexyl-ethyl-, di-n-butyl-, etc.)	Food chain (e.g. plastics in contact with food), consumer products (e.g. PVC, deodorants, adhesives, etc.)	Agonists of PXR, effects on steroid hormone biosynthesis
Polybrominated flame retardants	Food chain (fat-rich food, e.g. milk and derivatives, fatty fish, etc.), living environment, workplaces, consumer products (e.g. electronic devices, etc.)	Interaction with PXR leading to altered steroid and thyroid hormone homeostasis
Organotins	Food chain (seafood), consumer products (e.g. antifouling agents)	Aromatase inhibition
Perfluorooctane sulphonate	Food chain (bioconcentration in animal tissues), consumer products (e.g. plastics, carpets, materials, etc.)	Alteration HHG axis
Parabens	Main cosmetic, toiletries and pharmaceutical preservatives	Estrogen agonist—ER alpha and beta
UV-screen (benzophenone 2, 4-methylbenzylidene camphor, etc.)	Mixture for protection against UV radiation	Estrogen agonist—ER alpha
Cadmium	Food chain (e.g. refined food as flour, rice, sugar; seafood), cigarette smoking	Estrogen agonist—ER alpha
Phytoestrogens		
Isoflavones, lignans, etc	Food chain (e.g. vegetables, soy-based food), consumer products (e.g. cosmetics)	SERMs, high affinity for ER beta

POPs, persistent organic pollutants; PR, progesteron receptor; ETU, ethylenethiourea; PXR, pregnane X receptor; HHG axis, hypothalamo-hypophysis-gonadal axis; ER, estrogen receptor; SERM, selective ER modulator.

EDCs can act at multiple sites via multiple mechanisms of action. Receptor-mediated mechanisms have received the most attention, but other mechanisms (e.g., hormone synthesis, transport, and metabolism) have been shown to be equally important. For most associations reported between exposure to EDCs and a variety of biologic outcomes, the mechanism(s) of action are poorly understood. This makes it difficult to distinguish between direct and indirect effects and primary versus secondary effects of exposure to EDCs. Exposure to EDCs during the period when "programming" of the endocrine system is in progress may result in a permanent change of function or sensitivity to stimulatory/inhibitory signals. Exposure in adulthood may be compensated for by normal homeostatic mechanisms and may therefore not result in any significant or detectable effects. Exposure to the same level of an endocrine signal during different life history stages or during different seasons may produce different effects.

The issue of dose–response relationships is perhaps the most controversial issue regarding EDCs. One of the reasons is that EDCs often act by mimicking or antagonizing the actions of naturally occurring hormones. These hormones are present at physiologically functional concentrations, so the dose–response considerations for EDCs are often different than for other environmental chemicals, which are not acting directly on the endocrine system. Reports of low-dose effects of EDCs are highly controversial and the subject of intense research. Dose–response relationships are likely to vary for different chemicals and endocrine mechanisms. Timing of exposure is absolutely critical to the understanding of dose–response relationships for EDCs. This is true for both wildlife and humans and for cancer as well as for developmental, reproductive, immunological, and neurological effects. Numerous examples exist in the literature where age at exposure is a known risk factor.

Lead and Cadmium

Lead and Cadmium are two non-essential metals, which are not required by the body for any physiological function. One of their striking features is that of easy entry into the cells where they might inhibit and interact with several organ systems that keep them alive (Beeby et al, 1993). The functional, morphological and biochemical effects of these elements manifest themselves at different levels; the organism, organs and tissues, the cell and even at the subcellular level. The biological properties of heavy metals are discussed in terms of three important characteristics: the ability to form irreversibly complexes and chelates with organic ligands, which influence greatly the dynamics of transport, distribution and excretion of several important metal cations; the properties to form organic-metallic bonds and the potential to undergo oxidation-reduction reactions. Field and laboratory studies indicated that bioaccumulation of heavy metals, occurs in primary and secondary consumers of the food web. Among all those, lead and cadmium have been shown to accumulate in various tissues such as kidney and the liver. Also their accumulation in other organs as the hypothalamus, pituitary or gonads was reported (Lafuente *et al.*, 1999; Lorenson *et al.*, 1983; Ronis *et al.*, 1998).

Lead exposure

Lead is a naturally occurring, bluish grey metal that is found in small quantities in earth's crust. It is a divalent heavy metal with atomic weight 207.19 and vapor pressure - 1.0 mm Hg at 980° C. Lead in the atmosphere comes from various natural, anthropogenic sources. All human beings have lead in their body, primarily as a result of exposure to manmade sources. The most important pathways are ingestion of chips from lead painted surfaces, inhalation of lead from automobile emissions, food from lead soldered cans, drinking water from lead soldered plumbing and medications in the form of folk remedies. Although inhalation of lead from gasoline is no longer

considered as a public problem, the lead from dusts in automobile emissions has been deposited in the soil.

Toxicokinetics (Absorption/Distribution/Metabolism/Excretion)

In the body, inorganic lead is not metabolized but is directly absorbed, distributed and excreted. The rate at which lead is absorbed depends upon its chemical and physical form and on the physiological status of the exposed person (e.g. nutritional status and age). Inhaled lead deposited in the lower respiratory tract is completely absorbed. The amount of lead absorbed from GIT of adults is around 10-15% and the rest 85%-90% is excreted in faeces. In pregnant women and children, the amount of lead absorbed can increase to as much as 50%. The quantity absorbed increases significantly under fasting condition and with iron or calcium deficiency. GIT absorption in children may be only 30% for lead present in dust and dirt and 17% for lead in paint chips, compared with 50% for lead in food and beverages.

The rate of absorption of different lead compounds may vary considerably. A study in rats showed that relative to lead acetate (100%), lead carbonate was absorbed 164%; lead thalliate 121%; lead sulfite, lead napthenate and lead octanate 62-67%; lead chromate 44% and metallic lead 14% (Barlthrop and Meek, 1975). The limited data available indicate that laboratory animals absorb lead from the respiratory tract as efficiently as humans and the absorption rate is not affected by any chemical form or concentration of lead in air (EPA, 1986).

Once in the blood, lead is distributed primarily among three compartments- blood, soft tissue (kidney, bone marrow, liver and brain) and mineralizing tissues- bones and teeth. Mineralizing tissue contains about 95% of the total body burden of lead in adults. In bone, there is both labile component, which readily exchanges lead with the blood and an inert pool (ASTDR, 1999). The lead in the inert pool poses a special risk because of a potential endogenous source of lead. When the body is under physiological stress such as

pregnancy, lactation or chronic disease, this inert pool can show mobilization, thus increasing blood lead level (ASTDR, 1999).

The blood lead is not retained as it is and is either excreted by the kidneys or through biliary clearance into the GIT. In single exposure study with adults, lead has a half-life of 25 days, 40 days in blood soft tissues and 25 years in bone. The blood distributes lead to various organs. Animal studies have shown that liver, lungs, kidneys have greater accumulation of lead concentrations after acute exposure (inhalation, oral, dermal, intravenous routes) (ASTDR, 1999). Selective accumulation of lead occurs in hippocampus region of the brain. This accumulation is more in children than in adults (EPA, 1986). Approximately 75% of inorganic lead absorbed into the body is excreted in the urine and less than 25 % in faeces. Lead is also excreted in breast milk and therefore, available for intake by infants (Jensen, 1991; EPA, 1986).

Signs and symptoms of lead toxicity

Symptoms of lead intoxication and their onset vary due to differences in the susceptibility and duration of exposure. In symptomatic lead intoxication, blood lead levels generally range from 35 to 50μ g/dl in children and 40- 60 μg/dl for adults. Severe toxicity is frequently associated with blood lead levels of 70 μg/dl or more in children and 100 μg/dl in adults.

Cadmium exposure

Cadmium (Cd) is an element that occurs naturally in the earth's crust. Pure cadmium is a soft silver-white metal. Cadmium is not present in the environment as a pure metal, but as a mineral combined with other elements such as oxygen (cadmium oxide), chlorine (cadmium chloride), or sulfur (cadmium sulfate, cadmium sulfide). Cadmium is most often present in nature as complex oxides, sulfides and carbonates in zinc, lead and copper ores. The chlorides, sulfides are easily soluble in water to varying degrees. Cadmium is used extensively for electroplating and galvanization processes, in the production of pigments, in batteries, as a chemical agent and in various

industrial processes (ATSDR, 1989). Cadmium compounds have varying degrees of solubility which affects their absorption and toxicity.

Table 2: Signs and symptoms associated with lead toxicity

Mild Toxicity	Moderate Toxicity	Severe Toxicity
Myalgia or paresthesia, Mild fatigue, Irritability Lethargy, Occasional abdominal discomfort	Arthralgia, General Fatigue, Difficulty in concentrating, muscle exhaustibility, tremor Headache,diffuse abdominalpain, vomiting, weight loss, constipation	Paralysis, Encephalopathy leading to seizures, changes in consciousness, coma and death, gingival tissue, intermittent or severe cramps

Cadmium is present in ambient air in the form of particles in which cadmium oxide is probably an important constituent. Cigarette smoking increases cadmium concentrations inside houses. The average daily exposure from cigarette smoking (20 cigarettes a day) is 2–4 μg of cadmium. Cadmium concentrations in unpolluted natural waters are usually below 1 μg/litre. Contamination of drinking-water may occur as a result of the presence of cadmium as an impurity in the zinc of galvanized pipes or cadmium-containing solders in fittings, water heaters, water coolers, and taps. Levels of cadmium could be higher in areas supplied with soft water of low pH, as this would tend to be more corrosive in plumbing systems containing cadmium.

Food is the main source of cadmium intake for non-occupationally exposed people. Crops grown in polluted soil or irrigated with polluted water may contain increased concentrations and meat from animals grazing on contaminated pastures (IARC, 1976). Animal kidneys and livers concentrate cadmium. Levels in fruit, meat, and vegetables are usually below 10 μg/kg, in liver 10–100 μg/kg, and in kidney 100–1000 μg/kg. In cereals, levels are about 25 μg/kg wet weights.

Toxicokinetics (Absorption/Distribution/Metabolism/Excretion)

Cadmium is more efficiently absorbed from the lungs than the GIT (ATSDR, 1999). Inhalation and absorption usually involves cadmium in a particulate matter form where absorption being a function of deposition, which in turn is dependent upon the particulate size (particles >= 10μm diameter) tend to be deposited in the upper respiratory tract and particles <=0.1μm diameter are deposited in the alveolar region.

Absorption through gastrointestinal tract appears to be a saturable process with the fraction absorbed decreasing at high doses. The absorption of cadmium through GIT has modified many physiological factors such as high fat or protein content in the diet. Dermal absorption of cadmium is generally low (0.2-0.8%).Absorbed cadmium is transported in the blood by RBC and albumin. Acceptable blood cadmium levels in adults are 1 μg/dl.

Although cadmium is widely distributed throughout the body, most of it (50% to70% of the body burden) gets accumulated in the kidneys and liver. Cadmium burden in the kidneys tends to increase in a linear fashion with age up to 50 to 60 years of age after which it remains somewhat constant or slightly declines (Goyer, 1991). During pregnancy, cadmium present in maternal body is almost impermeable through the placenta so that fetus is exposed to only small amounts of maternal cadmium (ATSDR, 1999).

Cadmium is not transformed into any other form but rather binds to various biological components, such as protein and non-protein sulfhydryl groups and anionic groups of various macromolecules. Major binding protein of cadmium is metallothionein. Metallothionein is very effective in binding with cadmium and some other metals and is instrumental in determining the disposition of cadmium in the body. It is a family of proteins with a molecular weight of 6.5 Kd, which is rich in cysteine residues. It contains 20 cysteine residues that remain invariant along the amino acid sequence. All cysteines are known to participate in the coordination of 7 mol of Cd or zinc (Zn) per mol of MT. Coordination of these cysteine residues results in a high binding

affinity for Zn (10^{-18}) and Cd (10^{-22}) (Kagi and Vallee, 1961). The seven atoms of bound Cd are arranged in two separate polynuclear metal clusters, one containing three and the other four metal ions (Figure 14). There are tremendous differences in the half-life of MT synthesized as a result of chemical induction of the MT gene. For example, the half-life of Zn-MT is approximately 18–20 h, whereas that of Cd-MT is about 3 days.

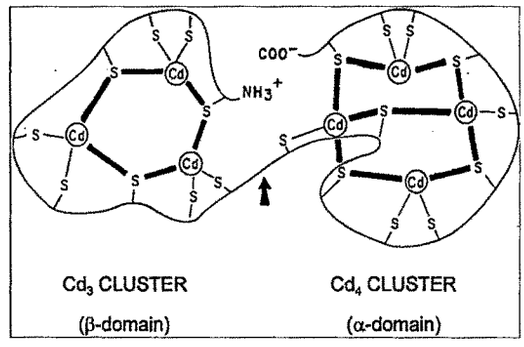

Figure 14: Metal clusters of metallothionein.

Concurrent titration studies indicated that at lysosomal pH, most of the Zn is released from MT whereas most of the Cd is not. This could be the reason Cd MT has a higher half-life *in vivo* than does Zn MT. There are four isoforms of metallothionein-MT^{-1}, MT^{-2} that are expressed in almost all tissues, MT^{-3} is present in the brain and MT^{-4} specifically expressed in keratinocytes. Several studies have shown MT protects the renal tubule cells from toxicity of cadmium. Cd not bound to MT induces denovo synthesis of MT in liver cells. In long term Cd exposure, there occurs a slow release of CdMT from the liver to the blood. Cadmium-MT is the form that is readily taken up into the kidney and transported into the lysosomes, where they are catabolised. However, the rate of influx of Cd-MT into renal tubular cells and rate of de novo synthesis of MT in the kidney regulates the pool of free intracellular cadmium ions that can interact with renal tubular cells.

After transportation, one of the mechanism by which Cd^{2+} affects cell function and gene expression were recently reviewed by Bhttacharyya *et al.*

(Bhattacharyya *et al.*, 2000). Cd^{2+} can easily enter into the cells through the L-type voltage Ca^{2+} channels and receptor-mediated Ca^{2+} channels (Blazka and Shaikh, 1991) because both cations have similar radii size and charge (Ca^{2+} = 0.97 A°, Cd^{2+} = 0.99 A°).

The principal route of excretion is via urine, with a daily average excretion of 2 to 3 μg for human beings (ATSDR, 1999). Typical daily excretion has been reported to be about 0.01% of the total body burden (ATSDR, 1999).

1.7 Endocrine disruption: *Mechanistic understanding*

1.7.1 Biochemical Basis of Endocrine Disruption

Both lead and cadmium are sulfhydryl reactive metals. The sulfhydryl reactive metals have three major properties, which mechanistically explain how they elicit a majority of their toxic effects (Fig. 15). First they are transition metals that promote hydrogen peroxide and enhance the subsequent iron and copper induced production of lipid peroxides and the highly reactive hydroxyl radical. Lipid peroxides alter membrane structure and are highly disruptive of mitochondrial function. The pro oxidant properties of the metals are exaculated by their inhibitory effect on antioxidant processes. Lead and cadmium have high affinities for glutathione (GSH), which is the primary intracellular antioxidant and conjugating agent (Quig, 1998). Importantly a single atom of lead or cadmium can bind to and cause the irreversible excretion of up to two GSH tripeptides. The metal GSH conjugation process can deplete the cellular GSH and thus decrease antioxidant capacity. These metal induced depletion of intracellular GSH and increased levels of malonaldehyde in brain and liver have been demonstrated in animal models (Bagchi *et al.*, 1996; Nigam *et al.*, 1999). They not only directly remove GSH from the cell but also inhibit the activation of two key enzymes involved in GSH metabolism; GSH synthase and GSH reductase (Daggeett, 1998; Hsu e al., 1998; Adonylo and Otaza, 1999a). Both lead and cadmium inhibit the activation of the free radical quenching enzymes

catalase, SOD and GPx (El-Maraghy *et al.*, 2001). The inhibition of GSH peroxidase has been attributed to the formation of a selenide complex. Se is an integral component of GPx. The selenium concentration is then insufficient to maintain both the optimal glutathione peroxidase activity and the detoxification of the metal. Decreased activities of SOD and GPx may increase their susceptibility to oxidative injury. Both lead and cadmium can readily displace Zn and Cu, which are cofactors for SOD causing a decrease in the enzyme activity. Phagocytic cells may be another important source of reactive oxygen species in response to metal ions. Furthermore, various studies have suggested that the ability to generate reactive oxygen species by redox cycling quinones and related compounds may require metal ions. Recent studies have suggested that metal ions may enhance the production of tumor necrosis factor-α (TNF-α) and activate protein kinase C, as well as induce the production of stress proteins. Thus, some mechanisms associated with the toxicities of metal ions are very similar to the effects produced by many organic xenobiotics.

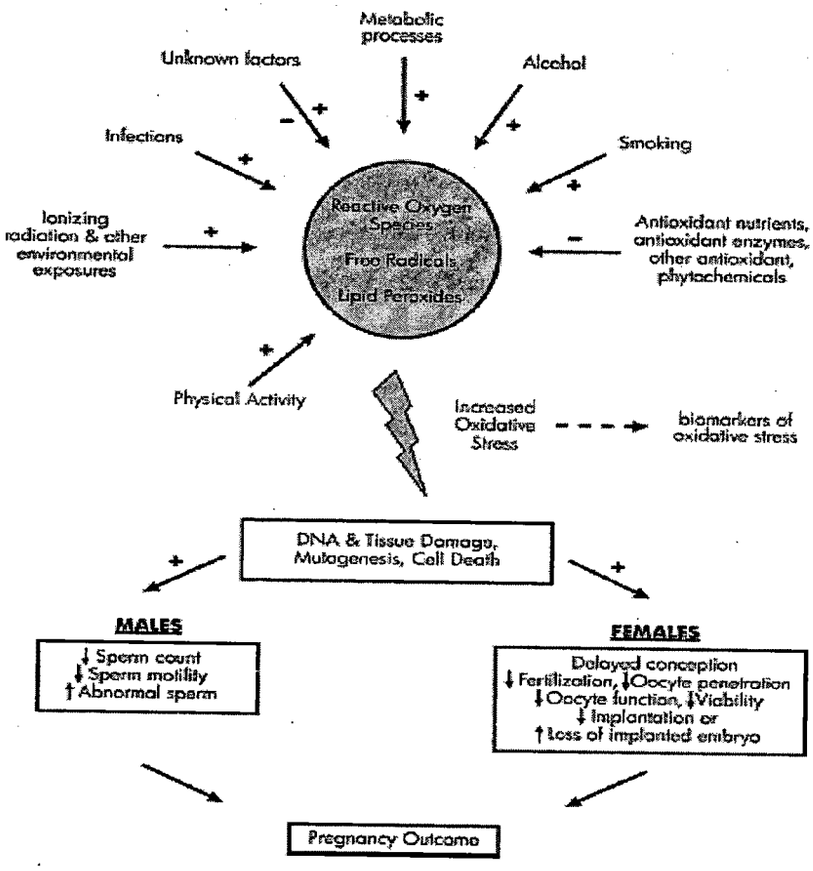

Figure 15: Role of oxidative stress in male and female fertility

1.7.2 Cellular Basis of Endocrine Disruption

There are many studies with "*in vitro*" cadmium exposure on ovarian cells, to understand the toxic effects. *In vitro* exposure of cadmium to whole ovary culture caused a decrease in production of progesterone but not in estradiol production (Piasek and Laskey, 1999). FSH stimulated granulosa cell cultures in presence of 5-40 μg/ml cadmium caused suppressed progesterone and estradiol production (Paksy *et al.*, 1992). Cadmium (8-64 μM) exposure to human granulosa cells caused morphological changes along with suppressed progesterone production, thereby indicating direct effect of cadmium on granulosa cells (Paksy et al,, 1997). Several "*in vitro*" studies have been performed to understand the mechanism of these toxicants. Male offspring treated with lead during pregnancy and lactation showed decreased

testicular FSH receptor binding (Wiebe *et al.*, 1982). They also reported that testicular cells in culture have fewer FSH binding sites and decreased adenylate cyclase activity when exposed to lead *in vitro. In vitro* exposure of lead caused a decrease in testosterone production, a diminution of the surface area of smooth endoplasmic reticulum and inner mitochondrial membranes in testicular leydig cells, (Zirkin *et al.*, 1982), suggesting leydig cells as a target for lead intoxication. also reported decreased testosterone production in crude interstitial testicular cells at a dose of 100 μM lead for 2-hour incubation period. Cadmium added *"in vitro"* (100 μM) to interstitial cells caused a decrease in viability (Ng and Liu, 1990). Laskey and Phelps (1991) added various metal ions *"in vitro"* including lead and cadmium, which caused inhibition of testosterone production, suggesting that inhibition by metal ions is at multiple sites of steroid biosynthesis pathway.

1.7.3 Molecular Basis of Endocrine Disruption

Although each metal activates its own unique set of signaling events, many metals also activate general ROS-mediated stress response pathways. DNA microarray technology has recently been used to examine the global effects of metals in several model systems. Many of the global effects of metals on transcription are thought to be controlled by the interactions between metal response elements (MREs) and MRE binding transcription factors such as MTF-1. Since PKC inhibitors can abrogate MT induction, it is thought that some metals induce signaling cascades through PKC activation, subsequent alteration of MTF-1 phosphorylation, and activation of MT transcription (Saydam *et al.*, 2002). Craft and Freedman examined the role of p53 activation in MT induction by Hg and Cd and found MT transcription was reduced in p53 −/− cells when compared to wild-type p53 cells. These results suggest that p53 may be part of a general mechanism in the cellular response to both Hg and Cd exposure. The Zinc finger is a major structural motif involved in protein-nucleic acid interactions and is present in the largest super family of transcription factors. Zinc ions coordinate this finger like structure through

bonds created with cysteine (Cys) and histidine (His) residues. Studies have also shown that transient exposure to endocrine disruptors during development leads to abnormal function in adult life and the endocrine effects in these cases have been attributed in part to changes in the pattern of DNA methylation that occurs in certain genes perhaps leading to the persistent expression of hormone-responsive genes (Anway *et al.*, 2005).

1.8 Developmental windows: *Susceptibility towards endocrine disruptions*

Approximately 60 chemicals have been categorized as endocrine disruptors, including both synthetic and natural compounds (Brevini *et al.*, 2005). Pesticides, persistent pollutants such as polychlorinated biphenyls (PCBs) and dioxins, additives used in the plastic industry, and flame retardants have all been shown to have hormonal activity (McLachlan *et al.*, 2006, Whitehead and Rice ,2006). Exposures to EDCs happen throughout the life cycle—from preconception through adult reproductive years. Many EDCs are stored in adipose tissue and have very long half-lives, so they can persist in tissues for decades. EDCs have been identified in follicular fluid, semen, amniotic fluid, fetal cord blood, breast milk, serum, and adipose tissue. In addition to direct toxicity to oocytes and spermatozoa, endocrine disruptors may also interfere with ovulation, fertilization, implantation, pregnancy, and embryonic, fetal, and pubertal development. A growing body of scientific evidence suggests that exposure to endocrine-disrupting chemicals (EDCs) early in life may alter development of the reproductive tract and hormonal responsiveness in adulthood (McLachlan *et al.*, 2006). Coupled with this evidence are a number of disturbing trends in some geographic regions, including a reduction in fertility, an increase in hormone-sensitive cancers, an earlier age of puberty in girls, and a decrease in the number of boys being born. Evidence from animal studies indicates that these conditions are likely to originate during the prenatal period. Exposures to environmental contaminants early in life are of particular importance, because the reproductive system is undergoing an intricately orchestrated process of

growth and differentiation. A fetus is vulnerable not only because of the rapid development and growth that are occurring, but also because it possesses immature and underdeveloped excretion pathways, low levels of chemical binding proteins, and an underdeveloped blood-brain barrier that is unable to protect the nervous system from toxic exposures (National Research Council Committee on Pesticides in the Diets of Infants and Children, 1993).

1.8.1 Gestational and lactational window

Several workers have reported that lead exposed industrial workers have higher blood lead level (Al-Neamy *et al.* 2002, Ruangkanchansetr *et al.*, 1999; Suplido and Ong, 2000; Milnerowicz *et al.*, 2000). A study by Needleman (1979) correlated increase in prenatal exposure of lead with increased risk for minor congenital abnormalities. It is reported that the children of parents who overt lead poisoning, could be at a greater risk for neurological development impairmentThere are only a small number of epidemiological studies, with no firm evidence on the effects on reproduction in humans (Poradovsky *et al.*, 1984; Laudanski *et al.*, 1991). A study on women living in lower Silesia showed a positive correlation with the cadmium concentration and myelomos in the myometrium (Pochwalowski *et al.*, 2001). Human studies had indicated that cadmium gets accumulated in human ovary (Vagra *et al.*, 1993), specifically in granulosa cells (Paksy *et al.*, 1997). There is a great concern that chemical substances or mixtures could produce effects from prenatal exposure that would not be detected from pubertal or adult exposure. Besides this, there is a lack of scientific evidence of known endocrine disruptors or reproductive toxicants that can affect the prenatal stage of development without affecting the adult or prematuration stages, and whether effective doses and affected endpoints may differ among the different life stages. In earlier studies, the relations between the decreased birth weight of newborn infants and increased Cd concentrations in maternal blood or the placenta due to smoking were pointed out (Kuhnert *et al.*, 1987). However, report on pregnant women living in an area close to a copper smelter, it was suggested that exposure to lead and cadmium could promote the development of

complications in pregnancy such as threatened spontaneous abortion, toxaemia, and anaemia, by increased lipid peroxidation (Tabacova *et al.*,1994). Laudanski *et al* found that the mean blood concentration of Cd in mothers delivered of preterm infants was higher than that of women who went to full term in an area with high amounts of lead and cadmium in the soil (Laudanski *et al.*, 1991). Moreover, breast milk is another route by which maternal exposure to Cd has an impact on neonatal infants, but no relation has been found between maternal exposure to Cd and the concentration of Cd in breast milk in previous studies (Radisch *et al.*, 1987) because of the low concentration of Cd in breast milk. Cd is transferred in part to the next generation through breast milk after birth is now well established (Nishijo *et al.*, 2002). Few studies exist that examine the effects of maternal body burden of Cd and the lactational transport of Cd on the next generation during pregnancy and lactation. Maternal exposure to Cd seems to increase early delivery, which leads to a lower birth weight.

Similarly, reports also shows that lead (Pb) easily crosses the placenta and enters the fetal brain where it interferes with normal development. Monkeys exposed to lead from birth, so that blood lead levels are maintained at about 15 microgms/dl, show increased distractibility , inappropriate responses to stimuli, and difficulty adjusting response strategies(Rice,1993). A review of animal studies reports deficits in performance, learning, and attention associated with low-level lead exposures (Rice, 1998). Several neurodevelopmental processes are altered by lead exposure, leading to abnormal brain development. Intrauterine neurodevelopmental effects of lead affect both the cellular structure of the brain and its chemistry (Silbergeld, 1992). Structural effects include altered cell proliferation, differentiation, synapse formation, and programmed cell death. Neurochemical effects include altered neurotransmitter levels (acetylcholine, dopamine, glutamate) and altered dopamine receptor density in various parts of the brain (Lucchi *et al.*, 1986). Lead is also a potent inhibitor of the NMDA (glutamate) receptor. The fetal brain may be particularly sensitive not only because unique

organizational processes are underway but also because of an immature blood-brain barrier. One study found greater uptake of lead in fetal brain during gestation than after birth in rats (Rossouw, 1987).

1.8.2 Pubertal window

Puberty is characterized by rapid physiological changes such as growth spurt and maturation of the gonads and the brain. It entails the individual's transition period from a non-reproductive to a reproductive state. The initiating event in the pubertal transition to adult reproductive capacity is activation of GnRH release from the hypothalamus, which stimulates the release of gonadotropins from the pituitary, which, in turn, activates the gonads. Furthermore, development of secondary sex characteristics results from increased levels of circulating sex steroid hormones. Sex steroids activate the sex specific brain morphology and neurochemistry organized during early postnatal life, as discussed above. Therefore, it is not surprising that this postnatal developmental transition is sensitive to EDCs. It is important to consider that perinatal exposures to EDCs may cause permanent changes that are manifested during the pubertal process, during which endogenous steroid hormone production increases and activational effects begin to develop. Moreover, puberty itself may be associated with further organizational or reorganizational changes in the nervous system and is beginning to be considered a second critical period for brain sexual differentiation (Sisk and Foster, 2004). Thus, EDC exposures during this life cycle phase may cause permanent alterations on adult reproductive endocrine functions.

This vulnerable period of transition into adulthood is fine tuned by endocrine-regulatory mechanisms. Endocrine-disrupting chemicals have been implicated in numerous physiological processes affecting normal reproductive health in human beings and animals. Monitoring changes in pubertal onset and development may function as early warning signs for reproductive capacity, both individually and at the population level. The physiological processes that regulate onset of puberty and transit through adolescence are not yet fully understood. Next to a genetic component,

environmental factors are influencing timing of puberty onset (Andrea *et al.*, 2006).

1.9 Ovulation Induction models: Reproductive toxicity studies

The advantages of using ovulation induction in immature rats through exogenous gonadotropins include minimal prior exposure of the ovaries to endogenous gonadotropins (Sekiguchi and Honma, 1998). Variability in follicular development and ovarian steroid secretion in different days of the estrus cycle characteristic of cycling adult rats is avoided. The time relationship of serum estradiol to endogenous gonadotropin surges that result in ovulation is difficult to control for. In addition, adult rat cycle length can vary unpredictably, thus adding to potential variability in reproductive and endocrine parameters (Greenwald, 1988). Among procedures for reproductive toxicity studies in rodent females, the vaginal smear test is generally used to check the daily estrous stage. Though, the smear test is useful for detecting abnormal conditions in reproductive physiology at an early stage, however, it is very laborious as routine work and a rather long observation period is required to detect disorder of the estrous cycle in individual females even if the disorder is obvious. Preovulatory dynamics of steroids has been demonstrated *in vivo* by ovarian follicles of cyclic and superovulated rat females (Kajta, 1998). Studies using TCDD have also clearly shown impaired ovulation in immature rats treated with equine chorionic gonadotropin (Ushmohama *et al.*, 2001).

1.10 Aims and Objectives

Environmental pollution by toxic metals is a global problem, resulting in an increase in heavy metals including cadmium and lead in air, water and food, above the recommended safety levels causing a deleterious effect on human health. Lead (Pb) and Cadmium (Cd) are two non-essential metals, which are not required by the body for any physiological function. Their solubility in water makes them partially harmful, allowing easy entry into the cells where they inhibit and interact with several vital functions. Lead and cadmium have been shown to accumulate in reproductive tissues. In this regard, our lab has also reported their accumulation in various organs including hypothalamus, pituitary, ovary and liver, which has led to disruptions in the function of Hypothalamic-Pituitary-Gonadal (HPG) axis in female rats (Pillai *et al.*, 2002; Nampoothiri *et al.* 2006; Pillai *et al.*, 2005).

In recent decades, the increasing incidence of infertility has been associated with exposure to endocrine disruptors like pesticides and industrial residues (Eertmans *et al.*, 2003; Carman, 2005). Many external causes of infertility are exposure to substances related to occupation such as pesticides, polychlorinated biphenyls (PCBs), dioxins , furans, ethanol, phenols and phthalates, and metals like cadmium, lead, mercury etc. (Eertmans *et al.*, 2003; Santamarta, 2001). Industrial cadmium exposure is extremely relevant, affecting more than 1,500,000 workers a year in the United States alone (Ragan and Mast, 1990). Reproductive dysfunction has been described in both women and men exposed to lead and cadmium at the workplace (Rom, 1976). In a study by the World Health Organization (WHO) on the effects of lead and cadmium in the blood of adult men, the overall results indicate that even low-level exposure to lead and cadmium can significantly reduce the quality of semen, although the study did not show conclusive evidence of male endocrine reproductive alterations (Telisman *et al.*, 2000).

Since reproductive physiology is under the regulation of endocrines, it is important to study their effects as endocrine disruptors. One major aspect

of heavy metal associated endocrine disruption is reproductive dysfunction. Various reports have suggested that these heavy metals act at all the level across the HPG axis (Klein *et al.*, 1994; Lafuente *et al.*, 1999). At hypothalamus, it affects the levels of various neurotransmitters, involved in the release of hormone (GnRH) required for the secretion of the pituitary hormone that is involved in the various reproductive activities at gonadal level. Since sex steroids control many reproductive functions, a possibility that changes in the synthesis or breakdown of these hormones may alter reproductive capacity in man and other animals exposed to these heavy metals cannot be excluded.

Studies in literature have proven lead and cadmium as reproductive teratogens. Data from literature also has suggested that Pb and Cd in isolation are known physiological disrupters. But in environment there is constant presence of one or more metals to which humans are exposed to, leading to synergistic, additive or antagonistic effects. This proves that there is a need to study the effects that are caused by such multiple exposures that too at low level. Earlier work from our lab has shown that simultaneous exposure of Pb and Cd affect the hypothalamic-pituitary-ovarian function at each organ level in pregnant and non pregnant rats (Nampoothiri and Gupta, 2008; Nampoothiri *et al.*, 2007; Nampoothiri and Gupta, 2006; Pillai and Gupta, 2005).

It is clear from literature that incidence of female and male infertility increases due to Pb and Cd exposure. For all studies of developmental reproductive toxicity, it is crucial to define the exposure that produces the effect. Hence, it was of great interest to study the effects caused by co-exposure of Pb and Cd on hypothalamic-pituitary-gonadal-hepatic axis after prenatal and early postnatal exposure with special emphasis on biochemical and molecular basis of endocrine disruptions. Thus, to understand biochemical, cellular and molecular mechanism of lead and cadmium mediated endocrine disruption across HPGH axis, current study was performed using rat as a model system.

In light of the literature reviews cited above the objectives of the present study are

I. To study the sexual-dimorphic pattern of endocrine disruptions after gestational and lactational co-exposure to lead and cadmium on Hypothalamic-Pituitary- Gonadal-Hepatic (HPGH) Axis in F1 generation Post natal day (PND) 56 rats.

II. To study the biochemical and molecular mechanism of endocrine disruptions after pubertal co-exposure to lead and cadmium on HPGH axis in PND 56 female rats.

III. To study the biochemical and molecular mechanism of cellular toxicity by lead and cadmium in luteinized granulose cells: *"in vivo" and "in vitro" studies*