Chapter 1
Introduction

CHAPTER-I

INTRODUCTION

Concern about the constantly increasing environmental levels of heavy metals due to increase in urbanization and industrialization has stimulated interest in the study of toxic substances and its consequences on life systems. **Lead** (Pb) and **Cadmium** (Cd) are main two heavy metal pollutants.

Lead and Cadmium are two non-essential metals, which are not required by the body for any physiological function. One main feature of these heavy metals, that make them particularly harmful is their solubility in water. This allows them to enter into the cells easily where they might inhibit and interact with several organ systems that keep them alive (Beeby et al, 1993).

LEAD

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 and anthropogenic sources. Because of many industrial activities, that have brought about its wide distribution, lead is ubiquitous in the environment today. All humans have lead in their bodies, primarily as a result of manmade exposure.

ROUTES OF LEAD EXPOSURE

AIR: Most of the lead in ambient air is in the form of sub micron particles. Some 30%-50% of these inhaled particles are retained in the respiratory system and as a result most of the lead is absorbed into the body. All particles that are deposited in the lungs are further absorbed.

Drinking water

Lead concentrations in drinking water and ground water vary from 1μg/l to 60 μg/l. In a study, it was reported lead pipes contribute 30 % blood lead levels (Kramer et al., 1994). In areas, where lead pipes are common, the contribution of lead in drinking water to total lead intake may be even more pronounced (Elwood et al., 1984).

FOOD: Large population is exposed to lead via food intake. Most of the lead enters food during storage and manufacture i.e., canned foods and in alcoholic drinks. The most important pathway through which atmospheric lead enters the food chain is thought to be foliar contamination of plants. This contamination depends on the rate of fallout of lead in the districts where food is grown. It tends to be higher in heavily industrialized areas. Several studies have reported average lead intakes in the range of 100-500 μg/day for adults, with individual diets.

Other routes of exposure

Other routes of exposure include high traffic density, high lead levels in dust, soil in residential areas near smelters or refineries, consumption of vegetables grown on highly lead soluble soils.

Table1: Lead concentrations in various media

Medium	Rural	Urban	Near point source
Ambient air (μg / m^3)	0.1	0.1-0.3	0.3-3.0
Indoor air (μg / m^3)	0.03-0.08	0.03-0.2	0.2-2.4
Soil (ppm)	5-30	30-4,500	150-15000
Street Dust (ppm)	80-130	100-5000	25,000
House Dust (ppm)	50-500	50-3,000	100-20,000
Diet (ppm)	0.002-0.08	0.002-0.08	0.002-0.08
Drinking water (μg/L)	5-75	5-75	5-75
Paint (mg/cm^2)	<1 --- >5	<1 --- >5	<1 --- >5

Ref: EPA, 1989.

Table 2: Sources of lead exposure

Occupational exposure	***Environmental Exposure***	***Hobbies and related activities exposure***	***Exposure through Daily used substances***
Plumbers, Lead miners, lead smelters and refiners, Auto repairs, Ship builders, Traffic police officers Steel welders, Firing range instructors	Lead containing paint,Soil/ Dust near lead industries, Plumbing leachate, Ceramic ware, Leaded gasoline	Glazed pottery making, Target shooting, Lead soldering, Painting Stained glass making, Home remodeling	Folk Remedies, "Health Foods", Moonshine Whiskey,Cosmetics Gasoline Huffing.

Ref: *(ASTDR- Case studies in Environmental Medicine: Lead toxicity, 1999)*

BIOLOGICAL FATE OF LEAD

Absorption

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). It has been shown that lead may be released from the bone after menopause (Silbergeld, 1991) and clearly higher blood lead levels are seen in postmenopausal than in premenopausal women (Silbergeld et al., 1989).

Of the blood lead, 99 % is associated with RBC; the remaining 1 % is available for transport to tissues (Desilva, 1981; EPA 1986). 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 studies with adults, lead has a half-life in blood is of 25 days, 40 days in 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).

Excretion

Approximately 75% of inorganic lead absorbed into the body are 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, 1983; EPA, 1989).

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 ranges 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.

Table 3:Continuum 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 abdominal pain, vomiting, weight loss, constipation	Paralysis, Encephalopathy leading to seizures, changes in consciousness, coma and death, gingival tissue, intermittent or severe cramps

LABORATORY EVALUATION OF LEAD TOXICITY

Laboratory tests suggested for lead intoxication include

- ***Complete Blood Count with peripheral smear***: In Pb poisoned patient haematocrit; hemoglobin values are slightly to moderately low. Total WBC counts are usually normal. Basophilic stippling is seen in-patients who have significantly poisoned for a prolonged time.
- ***Blood lead level***: A blood lead level is the most useful screening and diagnostic test for lead exposure. A blood lead level reflects lead's dynamic equilibrium between absorption, excretion and deposition in soft and hard tissue compartments. Blood lead

levels respond relatively to abrupt or intermittent changes in lead intake and within a limited range, bear relationship to those intake levels.

- ***Erythrocyte protophorphyrin level***: An elevated level of protoporphyrin in blood is a result of accumulation secondary to enzyme dysfunction in erythrocytes. It reaches steady state in the blood only after the entire population of circulating erythrocytes has turned over, about 120 days. It serves as an indirect measure of long time lead exposure. Major disadvantage is that it is not sensitive at lower levels of lead. Detection of erythrocyte zinc protophorphyrin is probably the most reliable indicator of lead exposure, because it is a measure due to mobilization fraction of bone lead. In lead intoxicated individuals; zinc is inserted into heme instead of iron. The threshold for detecting elevated zinc protophorphyrin levels is 25-30 μg/dl of blood in adult males, 15-20 μg/dl in adult females and 15 μg/dl in children.
- ***δ-Amino Levulinic Acid Dehydratase (ALAD) activity and δ-AminoLevulinic Acid (ALA) levels :*** Erythrocyte δ-ALAD activity measurement is a sensitive measure of lead toxicity. It is known that erythrocyte ALAD activity is inhibited by lead which results in stimulation of Amino Levulinic Acid Synthatase activity (ALAS), leading to an elevated ALA levels in blood and urine. The threshold for detecting elevated ALAS activity and blood and urinary ALA is 40 μg/dl in both adults and children, but evidence indicates it may be as low as 15-20 μg/dl. Inhibition of erythrocyte ALAD is being noted at very low lead levels , even at 10 μg/ dl in lead exposed populations (WHO, 1996).

TREATMENT OF LEAD TOXICITY

Table 4 below shows the guidelines of therapy according to blood lead level. Education, aggressive environmental intervention and in extreme cases, chelation therapy is recommended depending on blood lead level.

Table 4: Guidance for treatment action according to blood lead level

Blood lead level μg/dL)	**Treatment actions**
10-19	▪ Provide lead education and referrals Provide diagnostic testing within 3 months and follow up every 3 month
20-44	▪ Provide lead education and referrals ▪ Provide coordination care ▪ Provide clinical evaluation and management ▪ Provide diagnostic testing within 1 month to 1 week and follow up 1month ▪ Perform aggressive environment intervention
45-69	▪ Provide lead education and referrals ▪ Provide coordination care within 48 hours ▪ Provide diagnostic testing within 24-48 hours and follow up ▪ Perform aggressive environment intervention ▪ Provide appropriate chelation therapy
> 70	▪ Situation of medical emergency ▪ Perform diagnostic testing immediately as an emergency ▪ Hospitalize and begin chelation therapy immediately

HEALTH EFFECTS

Lead is known to affect all systems of the body. The toxicity of lead may be largely explained by its interference with different enzyme systems: lead inactivates several enzymes by binding to –SH, amine, phosphate, carboxyl groups of its proteins or by displacing other essential metal ions (ASTDR, 1999). For this reason, effect of lead on several organs and organ systems has been well documented.

Hematological effects

Lead can induce two types of anemia. Acute high-level lead poisoning causes hemolytic anemia while in chronic lead poisoning, lead can induce anemia by both interfering with erythropoises and by diminishing red blood survival. This is evident when blood lead level is significantly elevated for prolonged periods.

The key enzymes involved in the synthesis of heme are - δ AminoLevulinic Acid Synthatase (ALAS), a mitochondrial enzyme that catalyses the formation of – δ AminoLevulinic Acid (ALA), and ALA Dehydratase (δ-ALAD), a cytosolic enzyme that catalyses the formation of porphobilinogen. Through a series of steps, coporphyrin and protophorphyrin are formed from porphobilinogen and finally, the mitochondrial enzyme ferrochelatase catalyses the insertion of iron into protophorphyrin to form heme.

Table 5: Summary of lowest observed adverse effect levels (LOAELs) for lead induced health effects in adults and in children.

LOAEL at given blood lead level (μg/l)	Heme synthesis, hematological and other effects	
	In adults	**In children**
800-1000	Frank Anemia	--
700		Frank Anemia
500	Reduced hemoglobin synthesis	
400	Increased urinary ALA and elevated coproporphyrin	Increased urinary ALA and elevated coproporphyrin
200-300	FEP elevation in males	Reduced hemoglobin synthesis , FEP elevation
150-200	FEP elevation in females	
100-150		Vitamin D3 reduction
100		ALAD inhibition
50-80	Anemia, reduced heme synthesis	
40-70		Anemia, reduced heme synthesis

The activity of another erythrocyte enzyme, pyrimidine –5' nucleotidase, is significantly reduced in lead exposed females at blood lead levels of 30 μg/dl and reduced activity has been detected at levels below 5μg/dl below threshold limit. The

consequence of reduced activity is thought to be accumulation of cellular nucleotides, reduced erythrocyte stability and survival, and reduced mRNA and protein synthesis related to globulin chain. This decreased activity has serious implications regarding health hazards of very low level of lead in blood (Mohammed-Brahmin et al., 1985).

It was observed that school children who were exposed to manmade sources had increased blood lead level and Free Erythrocyte Protophorphyrin (FEP) levels (Ho et al.,), with a reduced Mean Corpuscular Volume (MCV) and Mean Corpuscular Heamoglobin (MCH) levels (Jacob et al., 2000). Several reports have shown that lead exposure caused a marked decreased activity of erythrocyte ALAD activity in occupational workers (Costa et al., 1997; Tomokuni and Ogata, 1980). Lead administration to male and female rats through drinking water (0-5000ppm) for 3 and 6 weeks, caused an inhibition of kidney δ-ALAD activity (Lauweys, 1995), with an increased urinary ALA and FEP levels (Buchet et al., 1976).

Effects on nervous system

Most sensitive target of lead poisoning is the nervous system. Developing nervous system in children can be affected adversely at blood lead levels less than 10 μg/dl (CDC. 1997). Irreversible severe brain damage (overt encephalopathic symptoms) occurs after acute exposure to high concentrations of lead. Children suffer from neuropsychologic effects and decrement in intelligence after lead exposure. Several studies have shown that for every 10 μg/dl increase in blood lead level, children's IQ dropped by 4 to 7 points (Yule et al., 1981; Schroeder et al., 1985; Winneke et al., 1990). Increase in blood lead level may also impair hearing and peripheral nervous system (ATSDR, 1999). The study

in Boston has shown that prenatal exposure caused an adverse effect on postnatal development (Bellinger et al, 1985).

Table 6: Summary of lowest observed adverse effect levels (LOAELs) for lead induced nervous effects in adults and in children.

LOAEL at given blood lead level (μg/l)	Effects on nervous system	
	In adults	**In children**
1000-1200	Encephalopathic signs and symptoms	
800-1000		Encephalopathic signs and symptoms
500	Overt subencephalopathic neurological symptoms, cognition impairment	
200-300	Peripheral nerve dysfunction (slowed nerve conduction velocities)	
100-150		Cognitive impairment
100		Hearing impairment
40-120	Forgetfulness, irritability, lethargy, impaired concentration, impotence	

Other effects of lead

Lead induces both acute and chronic nephropathy. Acute lead nephropathy is characterized by proximal tube dysfunctioning, with development of Fanconi-type

syndrome, alterations in mitochondrial structure, development of cytosolic and nuclear inclusion bodies. Chronic lead nephropathy caused by chronic high exposure to lead over many years (BPb levels > 500 µg /L), which is irreversible and typically accompanied by interstitial fibrosis, both hyperplasia and atrophy of tubules, glomerulonephritis, tubular absorption of uric acid and finally leading to renal failure. This increase in uric acid levels also leads to gout (Skerfving et al., 1995; Levin and Goldberg, 2000). Correlation between blood lead level in the range of 20-725 µg /l with measures of glomerular impairment in a large population has been reported (Stassen et al., 1992). Some population based studies had shown increased serum creatinine level or decrement in creatinine clearance at Blood lead level of 60 µg /dl (Kim et al., 1996; Payton et al., 1994). Lead interacts with renal membranes and enzymes and disrupts energy production, calcium metabolism, and glucose homeostasis, ion transport processes and renin-angiotensin system. Vicery et al. (1982) reported that rats exposed to 0-500 ppm of lead through drinking water continuously *in utero* and after birth by giving their mothers, showed a dose dependent decrease in renal rennin level. A study where rats were fed with 0.5 mg/ml Pb through drinking water for 6 months caused a change in renal sodium secretion and renin angiotensin system (Fleischer et al., 1980). It is also demonstrated that acute lead exposure causes an elevation in both plasma renin activity and aldosterone concentration in rabbits (Meredith et al., 1985). Stevenson et al. (1976) showed that chronic ingestion of lead caused a decrease in activities of renal Pyruvate carboxylase, phosphophenolpyruvate carboxykinase, fructose 1,6- diphosphatase and glucose 6- Phosphatase and increase in activities of Adenylcyclase-cAMP system, thus affecting glucose homeostasis.

Gastrointestinal disturbance is a sign of acute intoxication, generally at blood lead levels of 100-200 μg/dl in adults, but may also occur at levels of 40-60 μg/dl in children. Gastrointestinal symptoms include abdominal pain, constipation, cramps, nausea, vomiting, anorexia and weight loss.

There are studies to suggest that there exist inverse relationship between blood lead levels and vitamin D levels . Lead inhibits the formation of 1, 25-dihydroxyvitamin D3, which maintains the extracellular and intracellular homeostasis of calcium ions. Depletion of 1, 25-dihydroxyvitamin D3 impairs cell growth, maturation, tooth and bone development, which are seen at blood lead levels > 62 μg/dl.

Literature has shown that lead exposure for longer period of time causes cell hyperplasia, cytomegaly and cellular dysplasia in rodents (Goyer, 1985). Gene mutations in mammalian cell culture are also seen with *in vitro* lead exposure.

Blood pressure elevation is known to occur on lead exposure. Many studies have shown that both diastolic and systolic blood pressures may be affected by blood lead concentrations as low as 100-200 μg /l in both males and females (Goyer, 1996; Levin and Goldberg, 2000). There are studies indicating that doubling of blood lead levels may increase the systolic pressure by 1 mm of Hg and diastolic pressure by 0.6 mm Hg (Hertz-Picciotto and Croft, 1993; Staessen et al., 1996).

Reports have suggested that lead can induce DNA strand breaks (Fracasso et al., 2002) and down regulate Protein Kinase C (PKC alpha) expression and thus acting as a tumor promoter. Report by Lutz et al. (1999) suggested that blood lead levels ranging from 1 to 45 μg /dl in 279 children showed an increased levels of Immunoglobulin E, indicating allergic type of response. It has been demonstrated that lead exposed male

workers have decreased CD 3^+ $CD45RA^+$ cells with an increased blood lead concentrations (Sata et al., 1998). Lead in *vitro* enhances alloantigen specific T-cell proliferation (McCabe et al., 2001). It is also reported that lead can also disrupt the regulatory mechanism of cytokine release like IL-1 alpha, TNF alpha, IL-6 and also enhance the production of Th1 type of cytokines (Krocova et al., 2000) and facilitate the T-cell- B-cell interaction dependent proliferation of lymphocytes (Razani-Boroujerdi et al., 1999).

CADMIUM

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.

ROUTES OF EXPOSURE

Air

In air, Cd exists as fine particles (<10 μm), in the major form as cadmium oxide. These fine particles can remain airborne for days to weeks and travel long distances. Cigarette smoking in houses increases cadmium concentrations inside the houses. The

average daily exposure from cigarette smoking (20 cigarettes a day) is 2-4 μg of cadmium (Ros and Sloof, 1987).

Water and soil

Cadmium exists as dissolved or a part of insoluble complexes in water. Solubility is promoted by acidic conditions. Soil with high organic matter promotes formation of organic complexes that are poorly soluble. Cd in soils may leach into water, which is enhanced by acidic conditions. Cd in soil can be taken up and retained by aquatic and terrestrial plants and bioconcentrated in aquatic animals. In mainly in the kidney and liver.

Food

Food is the main source for cadmium exposure in non-occupational people. Levels in fruits, 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 weight. Shellfish fish has 200-1000 μg/ kg (Galal-Gorchev, 1991).

SOURCES OF CADMIUM EXPOSURE

- Food-lichens, mosses
- Cigarette smoke
- Smelting, Refining of metals, electroplating, Galvanization Processes, Soldering and welding
- Cadmium-Nickel Batteries, Reactor control rods

(Ref: ATDSR, 1989)

BIOLOGICAL FATE OF CADMIUM

Absorption

Cadmium is more efficiently absorbed from the lungs than the GIT (ATSDR, 1999). The absorption efficiency is a function of solubility of the specific cadmium compound as well as its exposure concentration and route. 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 tree and particles <=0.1μm diameter are deposited in the alveolar region. Alveolar deposition efficiency in animal models ranges from 5% to 20% (Barrett et al, 1947; Boisset et al., 1978) and in humans, it is estimated to be up to 50% for small particles (Nordberg et al., 1985). Actual cadmium absorption via inhalation exposure has been estimated to be 30% to 60% in humans (Elinder et al., 1976).

Absorption through gastrointestinal tract appears to be a saturable process with the fraction absorbed decreasing at high doses (Nordberg et al., 1985). The absorption of cadmium through GIT has modified many physiological factors such as high fat or protein content in the diet. Shaikh and Smith (1980) reported a mean retention time of 2.8% (1.1% to 7 %) for 12 human subjects given at a single oral dose of radiolabelled cadmium chloride. Cadmium absorption is decreased by coabsorption of divalent and trivalent cations like Zinc, Chromium, and Magnesium and increased by iron and calcium deficiencies (Goyer. 1991). Dermal absorption of cadmium is generally low (0.2-0.8%).

Absorbed cadmium is transported in the blood by RBC and high molecular proteins such as albumin (Goyer, 1991). Normal 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 (Goyer, 1991). Cadmium burden in the kidneys, tends to increase in a linear fashion with age up to 50 to 60 years of age after which remain somewhat constant or slightly decline (Goyer, 1991). During pregnancy, cadmium present in maternal body is almost impermeable through the placenta and so that fetus is exposed to only small amounts of maternal cadmium (ATSDR, 1993).

Cadmium is not transformed into any other form but rather binds to various biological components, such as protein and non-protein sulfahydryl groups and anionic groups of various macromolecules (ATSDR, 1993). 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 and with 7 metals distributed in two domains, the alpha and beta clusters. 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. Cadmium in blood plasma is mainly bound to albumin and other larger proteins immediately after uptake from GIT or the lungs. The liver takes up cadmium bound to albumin. 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.

Excretion

The principal route of excretion is via urine, with a daily average excretion of 2 to 3 µg for human beings (ATSDR, 1999). Daily excretion represents only a small percentage of the total body burden, which accounts for 17 to >30 years half-life of cadmium in the body (Friberg et al., 1974). Unabsorbed cadmium is removed from GIT by fecal excretion. Typical daily excretion has been reported to be about 0.01% of the total body burden (ATSDR, 1999).

Table 7: Signs and symptoms of cadmium toxicity

Acute Intoxication	*Chronic Intoxication*
Metal fume fever, Pulmonary effects, Headache, Chills, Muscle aches, Nausea, Vomiting, Diarrhea	Renal effects- proximal tubular dysfunction, with excretion of low molecular weight proteins; Fanconi syndrome (amino aciduria, glycosuria, phosphaturia, renal tubular acidosis); Pulmonary effects; Bone effects – osteomalacia

LABORATORY EVALUATION OF CADMIUM TOXICITY

Diagnoses for cadmium intoxication are:

- Case history
- Search for proteinurea, in order to screen for a beginning renal impairment like
- Beta2 microglobulin (low molecular weight protein)
- The Retinol binding protein (a low molecular weight protein)
- Alpha-1- microglobulin (a low molecular weight protein)

- Albumin (a high molecular weight protein)
- Measurement of Cd in biological tissues.

Table 8: Biological-monitoring tests for populations exposed to Cd in the industry and the environment.

Parameter	Tissue	Normal value	Max. Allowable Concentration	Significance
Cd	Blood	<0.5 µg/L cadmium	5 µg/L	Recent Cd exposure
Cd	Urine	<2µg/g creatinine	<5µg/g creatinine	Moderate exposure
β2microglobulin	Urine	<300µg/g creatinine		Tubular impairment
Retinol binding protein	Urine	<300µg/g creatinine		Tubular impairment
Albumin	Urine	<20mg/g creatinine		Glomelular impairment

TREATMENT: Disodium or calcium EDTA may be used in the treatment of the acute intoxication but one must be very careful in presence of renal impairment. No chelating agent can be proposed for chronic treatment.

HEALTH EFFECTS

Deleterious effects of cadmium on the body are due to larger half-life, therefore being exposed for a larger period of time. The major system where cadmium exerts its biological effect is the renal system.

Effects on renal system

Cadmium accumulation occurs mainly in the kidneys when exposed for longer periods. This accumulation is due to metallothionein, which is present in greater concentration in the kidneys. Cadmium affects resorption in proximal convoluted tubules, thereby increasing urinary excretion of low molecular weight proteins, known as tubular proteinuria. More severe cadmium damage includes aminoaciduria, glucosuria and phosphatauria. The critical concentration of cadmium in kidney is 200 μg/g of wet weight (Kjellstrom et al., 1977, 1984; Roels et al. 1983). Disturbances in renal homeostasis would lead to disturbances in renal handling of phosphorus and calcium leading to mineral resorption from bones, leading to renal stones and osteomalacia. A population study in Belgium revealed that cadmium exposure was positively correlated with the changes in serum alkaline phosphatase activity and urinary secretion of cadmium, along with changes in calcium metabolism (Stassen and Lauwreys, 1993). Also, blood cadmium level was correlated with renal tubular proteinuria, in a population (age group of 16-81 year), who were exposed to cadmium either by environment or by occupational activities (Alfven et al., 2002).

Animal and cell models are used to understand the mechanism of nephrotoxicity caused by cadmium. Cadmium is shown to disrupt the H+ antiport system (Endo et al., 1998) and also cause a decrease in the number of Na-Pi cotransport system (Park et al., 1997; Ahn et al., 1995). It has also been demonstrated that there is a inhibition of renal Na+K+ ATPase, leading to polyuria, glucosuria (Kim et al., 1988). Blumenthal et al. (1990) showed that Cd 2+ disrupts the normal absorptive functions of renal tubule epithelium using a model system of mouse cortical tubule cells. He (1997) later reported

that cadmium exposure *in vitro* impairs the Pi transport capacity. Cadmium is reported to have a direct effect on renal tubular cells, where alphamethyl D-glucoside (alphaMG) uptake was reduced, outflow of potassium was increased, cAMP content was decreased and Na+K+ ATPase activity was inhibited (Jiang et al., 1996). Both " *in vivo*" (Tanimoto et al., 1993) and "*in vitro*" (Ishido et al., 1995) cadmium treatment leads to apoptosis and DNA fragmentation (Hamada et al., 1996) in human kidney.

Hematological effects

A well-known disease "Itai-Itai" caused by cadmium is known to produce several deficiencies in hemopeoitic system. Long-term exposure is known to decrease the production of erythropoietin (EPO). A report by Horiguchi et al. (1994) revealed that patients of Itai-Itai disease had decreased hemoglobin level and decreased erythropiotein levels. He (1996) later reported that rats fed with cadmium for 6 to 9 months have low levels of plasma EPO, along with hypoinduction of EPO mRNA in the kidneys. Several animal studies have indicated that cadmium administration causes anemia, decrease in RBC count (Kostic et al., 1993; Hiratsuka et al. 1996) and poikilocytosis of RBC Hamada et al., 1996).

Other health effects of cadmium

Cadmium is known to cause both behavioral and neurological effects (WHO, 1994; Vataev et al., 1994). Cadmium can be accumulated in the areas of brain (Clark et al., 1985), but not in uniform manner. Administration of low level cadmium continued from conception to the12th week of age, caused functional alterations in the central and peripheral nervous system without any signs of clinical intoxication (Frery et al., 1993) Vataev et al. (1994) reported significant changes of EEG recorded from somatosensory,

visual and auditory cortical areas and from hippocampus upon cadmium exposure by i.p injections in rats. It has been shown that combined exposure of lead (300 mg/l) and cadmium (10 mg/l) through drinking water to mothers during pregnancy and lactation caused an increase in DOPA, 5-HT contents in the cerebellum of brain (Antonio et al. (2002). Male rats which were exposed to cadmium (272.7 micromol/ L) for one month through drinking water had decreased glutamine, glutamate and aspartate levels in anterior hypothalamus region, along with a decrease content of glutamate, aspartate and taurine in prefrontal cortex area of the brain (Lafuente and Esquifino, 2002).

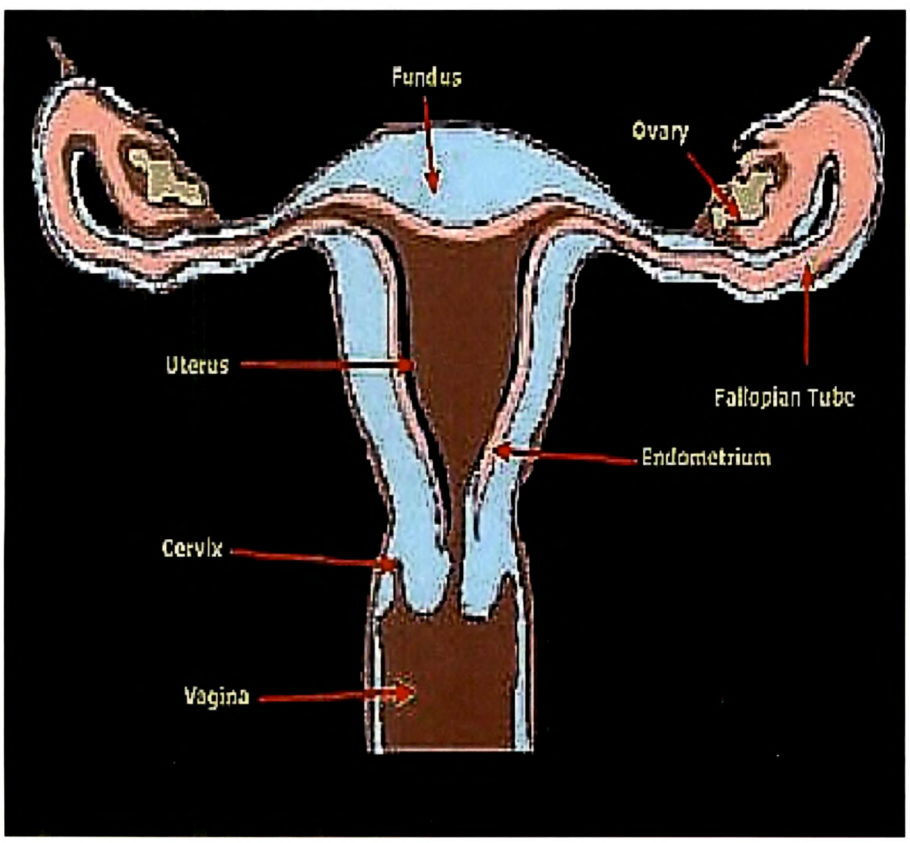
Several studies have indicated that cadmium interacts with the DNA (Hossain and Huq, 2002; Hartwig and Schwerdtle, 2002). Report by Misra et al. (2002) has shown that micromolar concentrations of cadmium significantly increased cell division and increased free cytosolic Ca2+ ion concentration. Administration of cadmium chloride (1.9-7.6 mg/ kg.b.wt, single i.p) causes an increase in the frequency of sister chromatid exchange at higher doses (5.7, 7.6 mg/kg.b.wt) with no change at lower doses, while *in vitro* exposure of CdCl2 (10, 15 and 20 mg/ml) to mouse bone marrow cells for 24 hours, caused a induction of chromosomal aberrations (Fahmy and Aly, 2000).

Lead and cadmium, also demonstrates their harmful effects on reproductive system and known as major reproductive toxicants.

REPRODUCTIVE SYSTEM

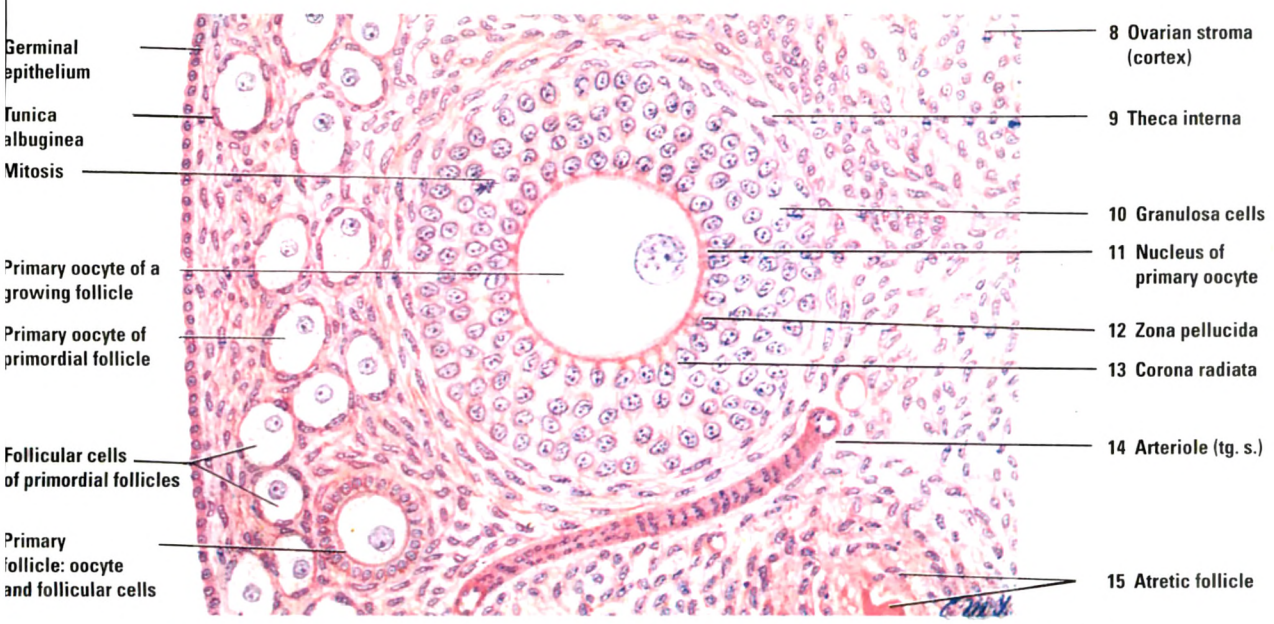
Female reproductive system consists of internal organs located in the pelvis and the external genitalia. The internal organs are the ovaries, oviducts, uterus and the vagina (Fig. 1).

Figure 1: Female reproductive system

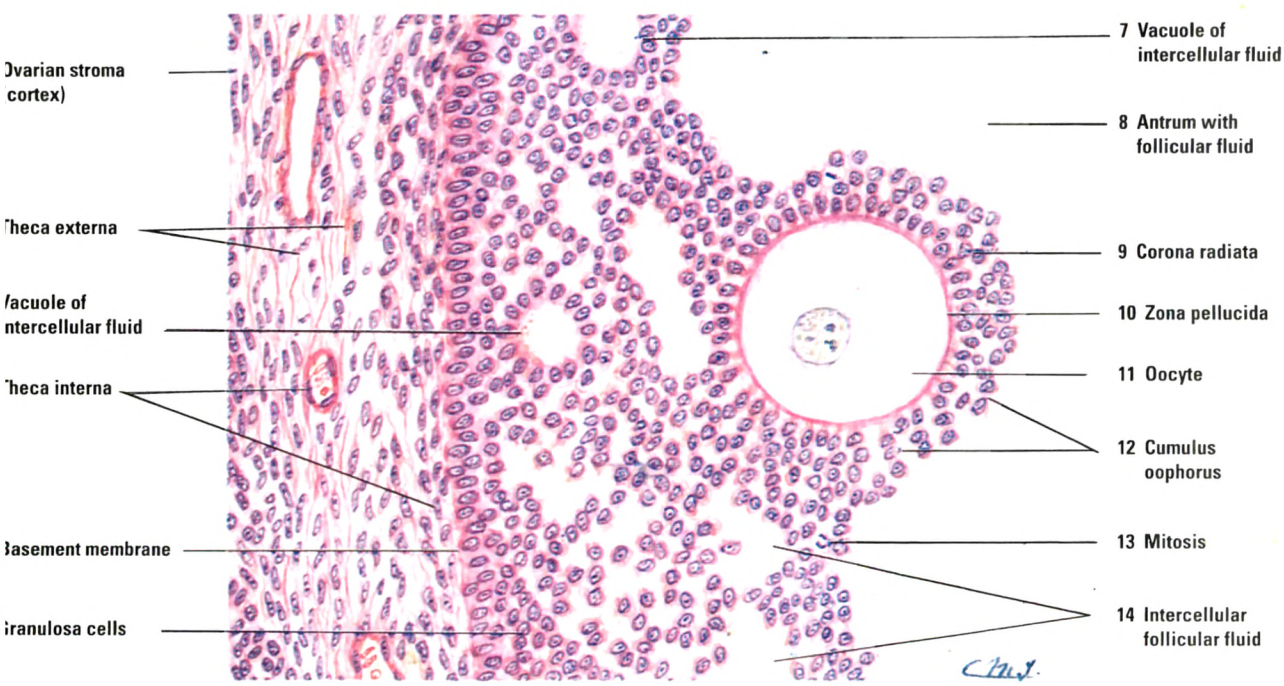


Structure of ovary

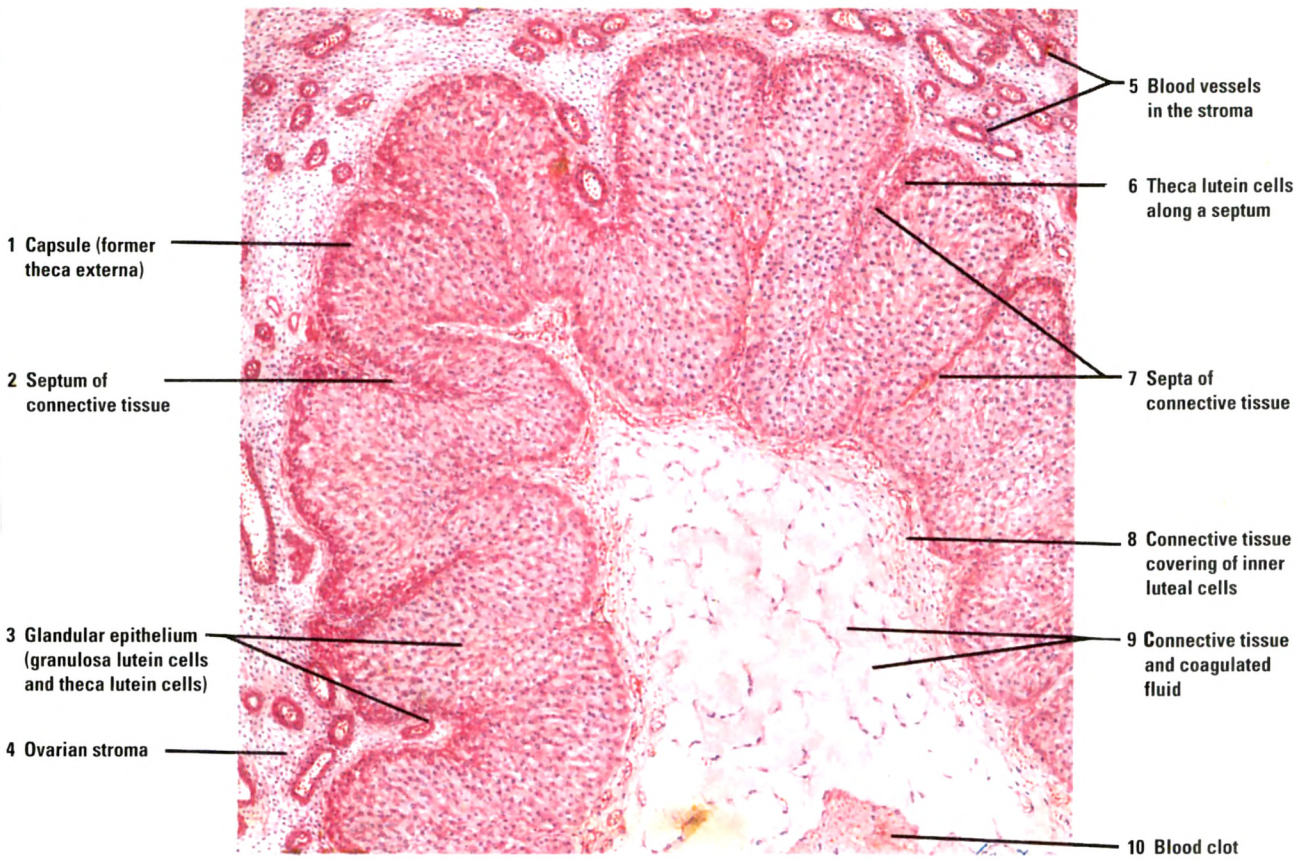
The maturation process of oocyte occurs in the ovarian follicle. An egg is considered to be in follicle, when a definite layer of cells becomes arranged around the egg in a uniform capsule of follicular epithelium, which are derived from embryonic stroma. Such follicle with a single layer of simple squamous epithelium around the egg is called as primordial follicle or primary follicles (Fig 2a and 2b). The simple squamous epithelium enlarges to form columnar epithelium, which multiplies, first forming a double layer and finally a stratified cuboidal follicular epithelium. At this stage, the epithelial cells are recognized as granulosa cells and the follicles as secondary follicles. Histologically, granulosa cells (GC) are small flat epitheliod in shape, with a frothy cytoplasm, indistinct cell borders and small, round to oval hyperchromatic nuclei. These cells surround small cavities called Call-Exner bodies, which contain material consisting of excess of basal lamina. Multilayered secondary follicles transform into vesicular follicles, with the formation of multiple fluid filled intracellular spaces. These spaces eventually enlarge and coalesce to form the cavity called the antrum. This structure is now called tertiary follicles, which on preovulatory growth is called graffian follicle. The oocyte, together with its attached granulosa cells that once occupied the center of the secondary follicle, now becomes acentric-forming hillock, cumulus oophhurus. The enlargement of the granulosa cell layer is accompanied by the development of an outer encapsulating sheath derived from the stroma. This constitutes the theca, divided as theca interna and theca externa. After the ovulation, both granulosa and thecal cells get transformed into corpus luteum, which gets regressed at the end of the estrus cycle and this process is called atresia. This is followed by the beginning of the next estrus cycle.



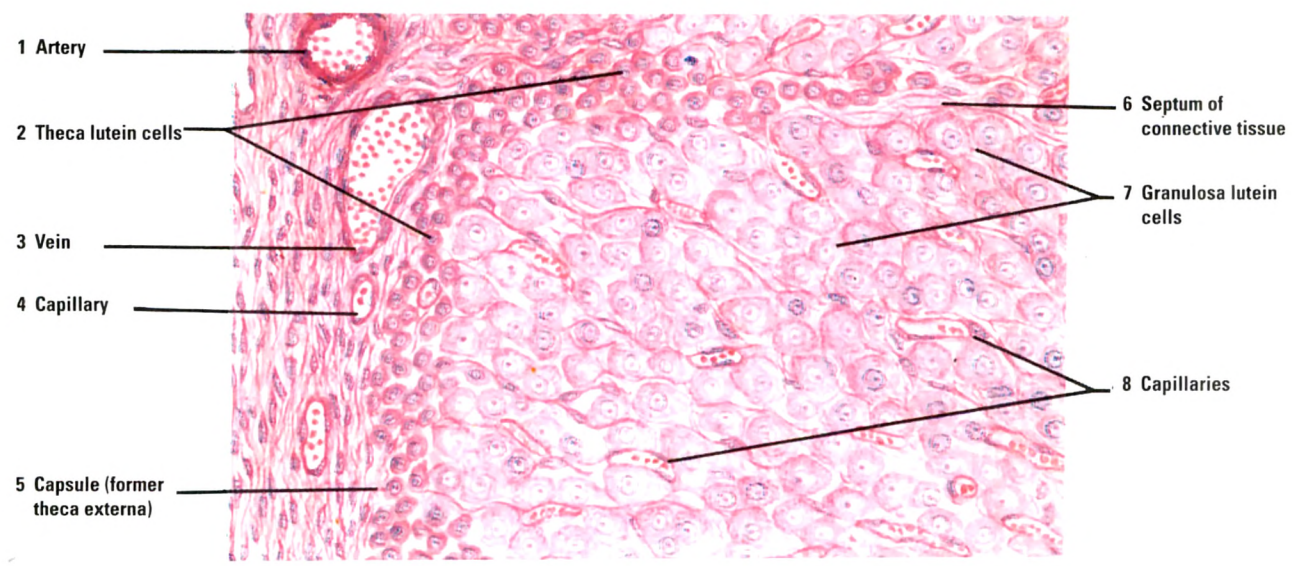
Ovary: Ovarian Cortex, Primary and Growing Follicles. Stain: hematoxylin-eosin. High magnification.



Corpus Luteum



Corpus Luteum (panoramic view). Stain: hematoxylin-eosin. Medium magnification.



Structure of Uterus

Uterus is an integral part of female reproductive system, which receives the rapidly developing morula from the oviduct. Uterus is divided into two regions, the body and the cervix, by a constriction on its surface, which corresponds to a narrowing of its cavity. The large upper portion is the body; the upper rounded part is the fundus. The narrower lower portion is the cervix. The lumen of the cervix, the endocervical canal; has a constricted opening, or *os*, at each end. Uterine wall is composed of a) an external layer, the serosa or perimerium; b) a middle internal layer, the myometrium and c) an inner layer, the endometrium as seen in fig 3a and b.

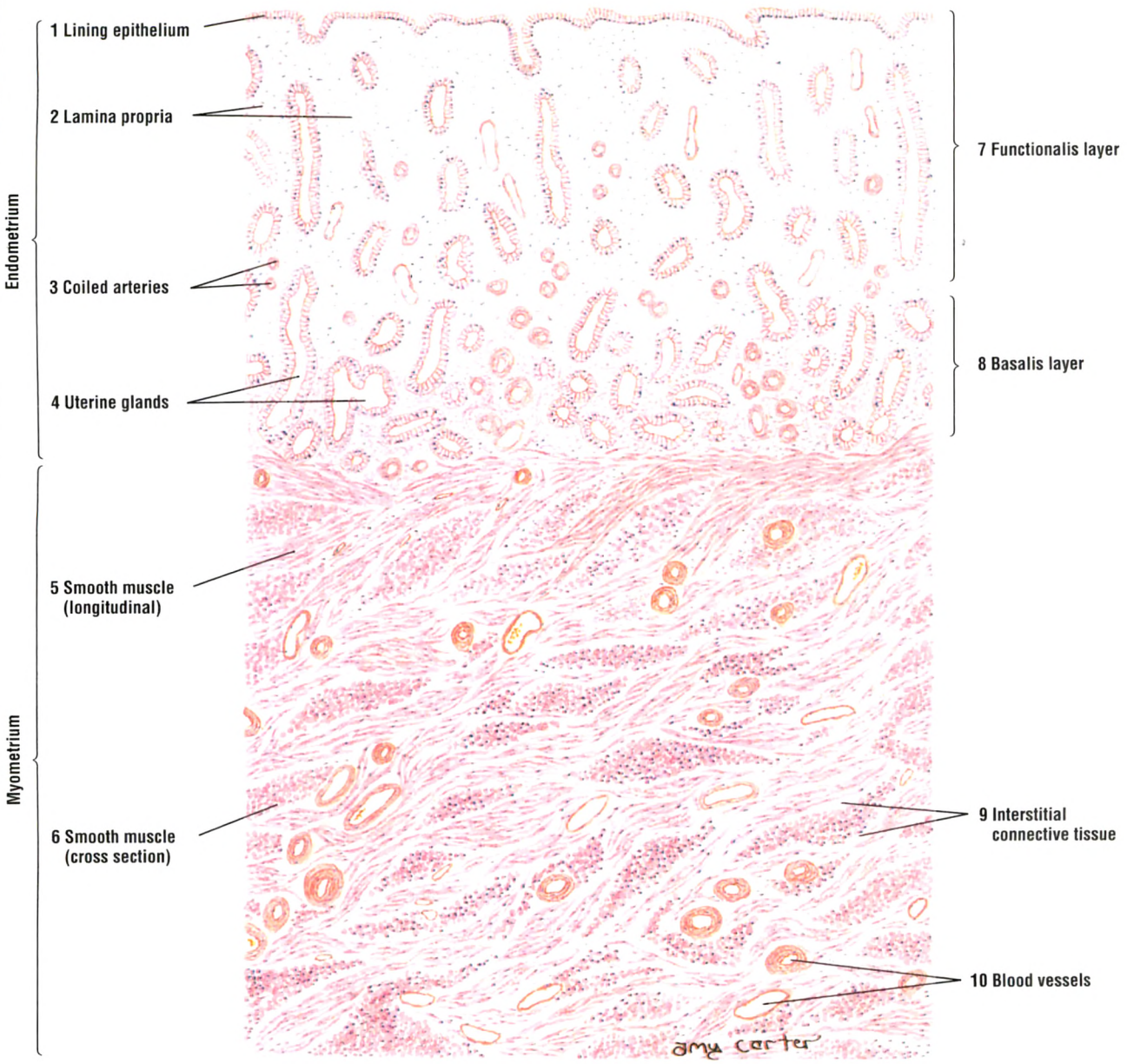
Myometrium is the thickest layer of the uterine wall, which consists of three indistinct layer of smooth muscle fibers. In non-pregnant uterus, the smooth muscle cells are comparatively less as compared to the pregnant uterus, where uterus shows enormous eniargement.

Uterus has a special epithelial lining of cells called the endometrium. It consists of a surface epithelium made up of the glands and stroma. The endometrium can be further divided into two zones: a superficial termed as functional layer, and a deeper one termed the basal layer that lies adjacent to the myometrium.

Reproductive cycle

Rat reproductive performance is mainly controlled in form of estrus cycle. The word " estrus" means gadfly sting or frenzy. The estrus cycle is a cascade of hormonal and behavioral events that are progressive, synchronized and repetitive. The main ovarian events that take in estrous cycle are growth of the follicle, ovulation of the eggs

Uterus: Proliferative (Follicular) Phase



Uterus: Secretory (Luteal) Phase

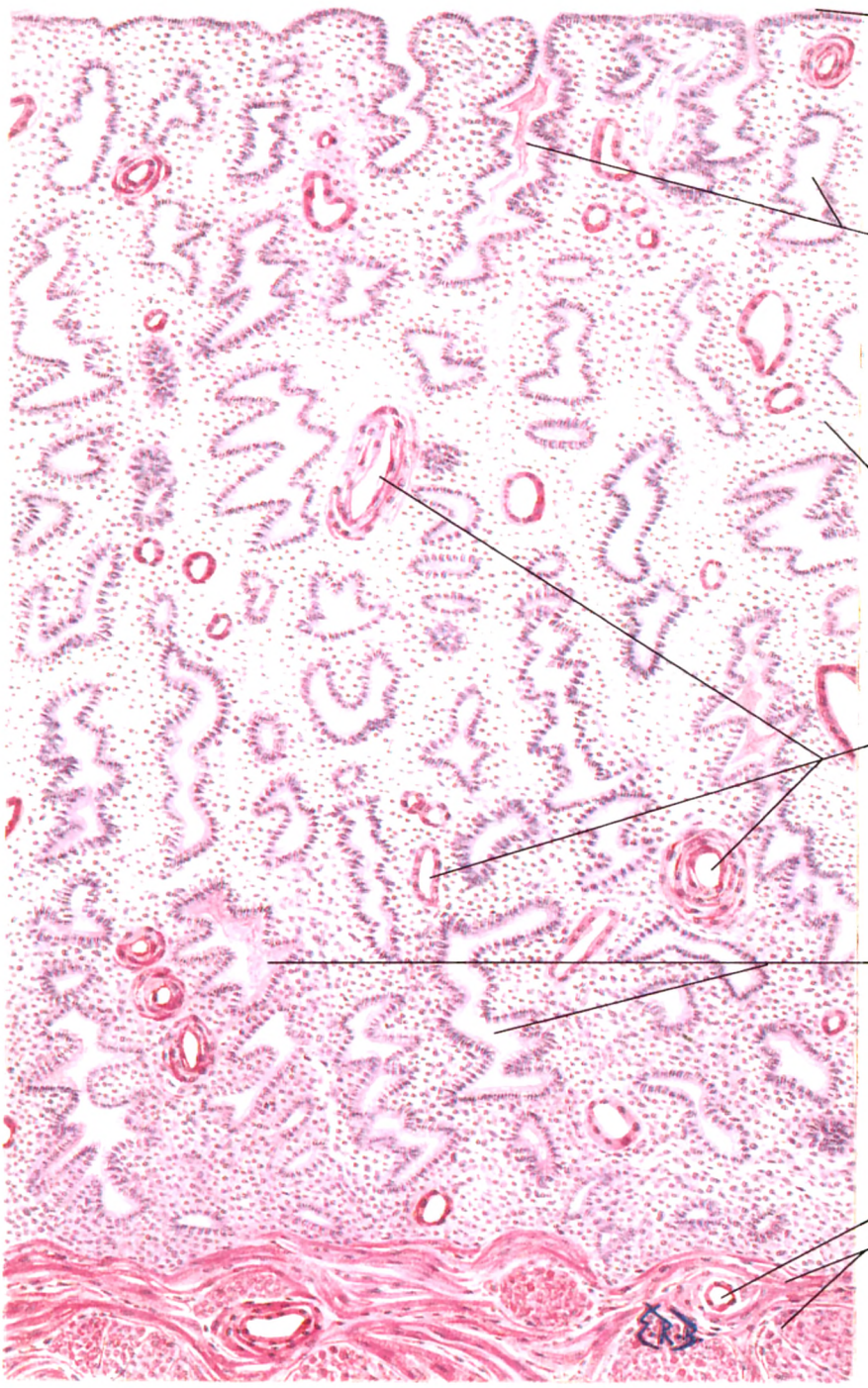


Figure 4: Rat Estrus Cycle

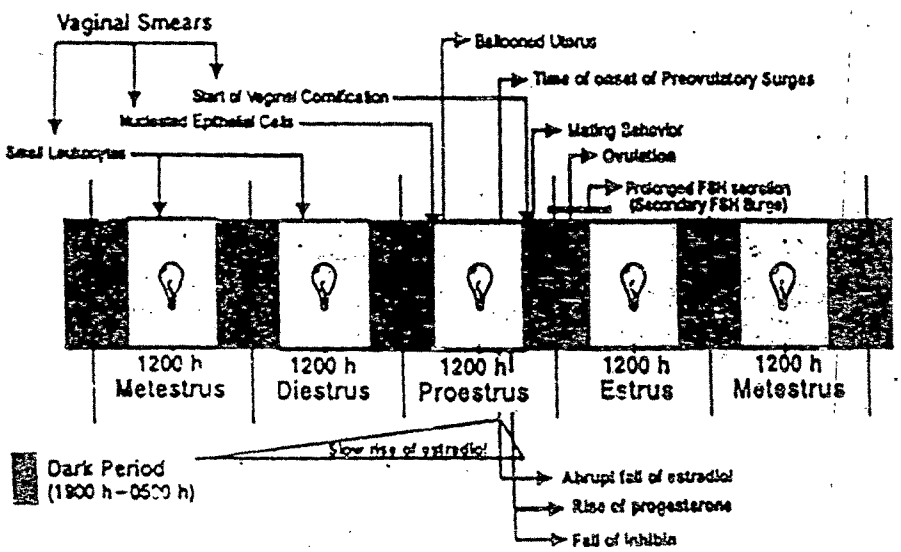


Table 9: Features of rat estrus cycle.

	Diestrus	Proestrous	Estrus	Metaestrus
Ovary	Small follicles are only present, with large corpus lutea from the previous ovulation, which secrete for very short time unless pregnancy intervenes.	Some follicles grow rapidly.	Ovulation is spontaneous and occurs about 10 hours after the beginning of estrus.	Many corpus lutea that secretes only for short time.
Uterus	Small, anemic, low motility, lumens small and slit like. Cells of the uterine mucosa is columnar; polymorphonuclear leukocytes in the stroma; endometrial glands collapsed, atrophic.	Become more vascular, water content increases and organ distends. Contractility more pronounced. Epithelial cells become higher. Leucocytes disappear from mucosa. Endometrial glands hypertrophy.	Gains maximum vascularisation. Epithelial cells reach maximum development. No leucocytes.	Epithelium continues vacuolar degeneration and replacement. Leukocytes in stroma. Decreases in size and vascularity.
Vagina	Epithelium thins. Leucocytes abundant in stroma migrate through the epithelium into vaginal lumen.	Epithelium thickens; numerous mitoses and inner layers. Old layers of epithelium line the lumen. Leucocytes no longer migrate through the epithelium.	Outer layer of epithelial cells becomes cornified and sloughed into the lumen. In early estrus, these cells retain their nuclei, but in later stages no nuclei visible and these cells are irregular, flat, cornified plates.	Deeper layers of the estrous epithelium line the lumen, superficial layers having become cornified and sloughed off. Reduction of mitotic activity in epithelium.
Smear	Stringy mucous in which entangled, many leucocytes and a few nucleated epithelial cells.	Largely small, round, nucleated epithelial cells. None to few leucocytes.	Large number of large cornified cells with degenerated nuclei. Smear is cheesy.	Many leucocytes and few cornified cells.

Ovarian changes during estrus cycle

Folliculogenesis is the major event that occurs in the proestrous of the ovarian cycle. The presence of distinct germinal vesicle or nucleus, the faint appearance of diploid number of chromosomes and distinct nucleolus characterize this stage. The oocyte remains in this stage until " ***selected***" for maturation. The timing of resumption of maturation has been reported with respect to onset of sexual receptivity, where ovulation occurs inducing the release of LH. In the adult, the final maturational step of oocyte begins within the follicle and is known as first meiotic division. This process is a reduction division and controlled by preovulatory surge of LH, that is, the chromosome number is reduced from diploid content of 42 to haploid number of 21. The first signal that meiosis I have been reinitiated is breakdown of germinal vesicle, disappearance of nuclear membrane and chromosome thickening. This occurs between 5 to 6 p.m. on proestrous, 2-3 hours after beginning of ovulation, release of LH and 7 to 9 hours before time of ovulation (Tsafriri and Kraicer, 1972).

Major component of the growing ovary arc the granulosa cells. There are abundant ultrastructural evidence that active protein synthesis mainly steroid biosynthesis are absent until shortly prior to ovulation in the granulosa cells. Theca interna cells rapidly enlarge and assume a polygonal shape, which vacuolated cytoplasm and vesicular nuclei. These cells are enmeshed in a reticular and fibrous network and contain a plexus of capillaries and lymphatics. This layer hypertrophies as proestrous approaches and is considered as one of the major site of estrogen production during the cycle. The outermost follicular layer theca externa contains contractile tissue that is thought to play a role in ovulation.

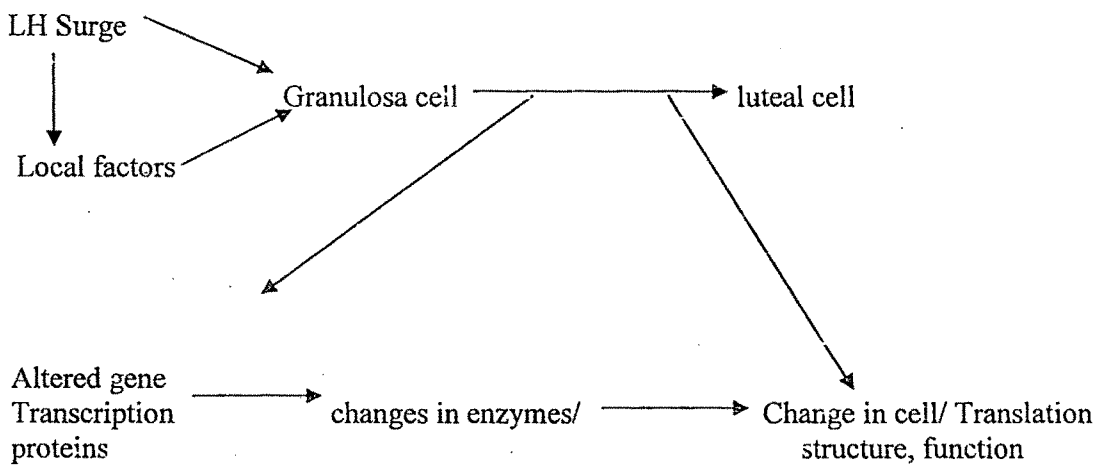
Following folliculogenesis, ovulation occurs in late proestrous or in the early estrus stage. The ovulatory follicle bulges out from the ovarian surface and appears turgid and highly vascular. Within a minute, stimulation by gonadotropin –Luteinizing hormone (LH) occurs from the stigma and forms the apex of the preovulatory follicle. This is the first sign of ovulatory process. As the follicular rupture approaches, the superficial germinal epithelial cells covering the stigma are breaking into small clumps. The underlying stroma thins, and a delicate vesicle that causes the release of the viscous follicular fluid. There occurs actual escape of the ovum following breakdown of the stigma. First granulosa cells begin to ooze through the plugged orifice, followed very quickly by remaining egg mass. Once the egg is extruded a great burst of follicular fluid follows, then cumulus and oocyte would be extruded subsequently into the stream. Oocyte then moves into the oviduct by ciliary movement. In case of the oocyte that is not fertilized, this oocyte gets regressed to form the corpus luteum. This generally occurs in the metaestrus stage. Corpus luteum has dual origin – both from granulosa cell and thecal cells of preovulatory follicle. This steroidogenic organ maintains the pregnancy. Formation of corpus luteum is dependent on the conversion of granulosa cells into luteal cells, a differential process called luteinization (Fig. 5). At the time of antrum formation in the follicle, LH receptors appear on these cells. These LH receptors are found only in granulosa cells from large preovulatory follicles. During luteinisation, proliferation of granulosa cells decline and increase in enzymes involved in progesterone synthesis occurs.

By diestrus stage, newly formed corpus luteum attains maximum size, which is maintained through metaestrus of the following cycle. By diestrus of the following cycle,

corpus luteum abruptly regresses. This process is called as atresia. During this stage, alterations in the granulosa cells occurs in two patterns: (1) prominent necrotic changes in the oocyte with the secondary alterations in the granulosa cells or (2) degenerative changes in the granulosa with an almost unchanged oocyte.

The degeneration of granulosa cells has all characteristics of apoptotic cell death. Cells demonstrate nuclear condensation and marked dilation of cytoplasmic cell organelles. This regression also coincides with the closure of the blood vessels, appearance of areas of degeneration, leukocyte infiltration, and increased cholesterol content. Such corpus luteum if present in unmated animal, are referred as " non functional" corpus luteum which do not secrete sufficient progesterone to support a decidual reaction induced by mechanical or chemical trauma of uterine endometrium. In absence of leuteotropic support initiated by mating, secretion of progesterone decreases through diestrus and a new ovulation takes place thereafter. Corpus luteum of unmated rats secretes lower level of progesterone for only 1 or 2 days, which serves as a basis for, short cycle in rodents.

Figure 5: Biochemical and morphological aspects of luteinisation



Uterine changes during estrus cycle

During the cycle, which is controlled by ovarian hormones, the endometrium undergoes changes in its secretory activity and structure, which is correlated with the cyclic growth and maturation of ovarian follicles. The endometrium passes through a sequence of morphological and functional changes every estrus cycle. Two main phases are a) Proliferative Phase and b) Secretory Phase.

The proliferative phase occurs concurrently with follicular maturation and is influenced by the estrogens being produced. The secretory phase coincides with the functional activity of corpus luteum and is primarily influenced by the progesterone being produced.

At the end of estrus cycle, the endometrium consists of a thin band of connective tissue, about 1mm thick, containing the basal portion of the uterine glands and the lower portion of the arteries. Under the influence of estrogen, the stromal and the epithelial cells in the stratum basale begins to proliferate by numerous mitotic divisions and the following changes can be seen: a) the epithelial cells in the basal portion of the glands proliferate, reconstituting the glands and migrating to cover the endometrial surface, b) the stromal cells proliferate and ground substances accumulates and c) the spiral arteries lengthen as the endometrium is replenished, but these arteries are only slightly coiled.

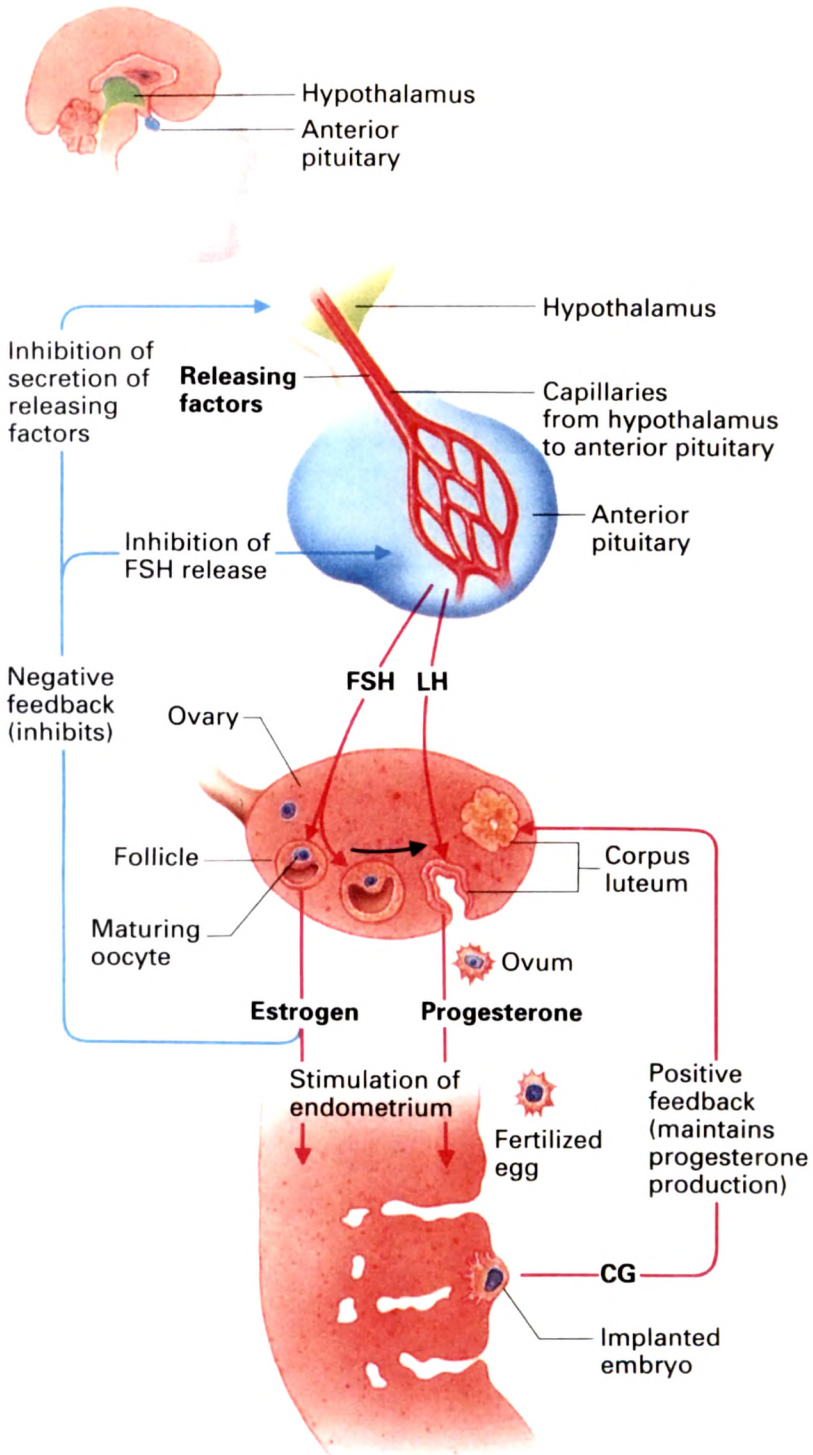
Under the influence of progesterone, several changes occur in the stratum functionale of the endometrium, beginning a day or two after ovulation. The glands enlarge and become corkscrew shaped and their lumens become sacculated as they fill with the secretory products from the epithelial cells. The mucoid fluid being produced by these cells are rich in nutrients, particularly glycogen, required to support development if

implantation occurs. Mitoses are rare since the growth seen at this stage results from hypertrophy of the epithelial cells and an increase in vascularity and edema. The spiral arteries lengthen and become more coiled during this phase.

In endometrium, two enzymes are known to convert estradiol to its inactive metabolite estrone: microsomal 17 β hydroxysteroid dehydrogenase type2 (17β HSD-1) and peroxisomal 17β HSD-4. Bassett et al. (1988) showed that growth and differentiation of the uterine endometrium during implantation or decidualisation is associated with decreased endometrial conversion from estradiol to estrone. This decrease in conversion could help in maintenance of endometrial estradiol concentration sufficient to permit receptor interaction.

Neuroendocrine control of estrus cycle

The estrus cycle is under control of central nervous system. External signals that are perceived by neurons are transmitted to the cells in the hypothalamus and where the decapeptide, GnRH is released and reaches the anterior pituitary through the portal vein. The gonadotrophs in response to GnRH release peptide gonadotropins, which induce all ovarian events. The ovarian steroids exert feedback effects on the host target tissues, including brain, pituitary where estradiol receptors are present. Estradiol maintains a suppression of GnRH by negative feedback system. There exists positive feedback system, which induces ovulation in the morning of estrus. Peak level of estrogen primes the reproductive axis, reaches a critical threshold level, indicating ovarian readiness for ovulation and allows the neural signal, to be transmitted to hypothalamus to release GnRH (figure 6).



Feedback control of estrogen and progesterone levels in the blood of female mammals. FSH = follicle-stimulating hormone; LH = luteinizing hormone; CG = chorionic gonadotropin.

GnRH is released in a pulsatile pattern of rhythmic secretory bursts, whose frequency and amplitude varies according to stage of the estrus cycle. The highest GnRH release triggers the preovulatory surge of gonadotropins on the afternoon of proestrous. GnRH neurosecretory activity depends upon two major inputs in both internal and external environment, which include the ovarian steroid feedback and neural integration consisting of neuropeptides, neurotransmitters, and neuroactive amino acids.

Gonadotropins

Hormones secreted from anterior pituitary serve as a initiator for all the cellular events, which takes place during estrus cycle. The key hormone from the pituitary is Follicle Stimulating Hormone (FSH). Basal levels of FSH are secreted on estrus through metaestrus, diestrus and midday of the proestrous. Then, circulating levels of FSH increase rapidly and reach the peak at late proestrous stage (Fig. 7). FSH levels begin to decline to the baseline after this time and early morning of estrus a secondary rise of FSH begins and peak shortly thereafter. Circulating levels of FSH then begin to decline and reach baseline by early evening.

Secretion of progesterone during the cycle potentiates the inhibitory effects of estrogen upon the secretion of FSH. Granulosa cells of the ovary secrete a hormone called inhibin that suppresses FSH secretion. There is an inverse relationship of FSH release and inhibin concentration throughout the estrous cycle. During basal secretion of FSH, the concentration of inhibin in the blood is very high, and during proestrous-estrous surge of FSH the activity decreases. Estradiol secreted through diestrus stimulates hypothalamic neurons to secrete GnRH into the portal vein during early afternoon of

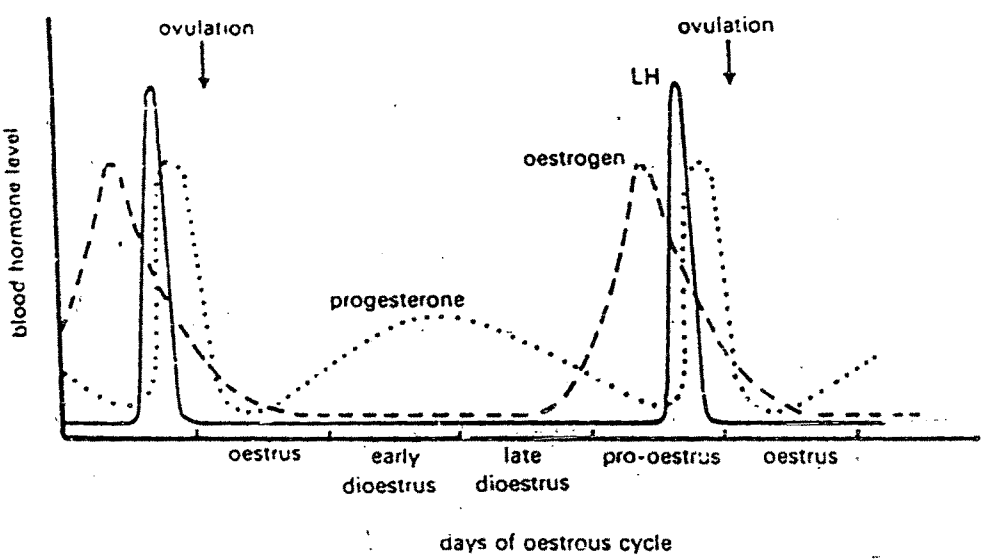
proestrous and GnRH in turn stimulates pituitary to the release of FSH. Follicular progesterone released due to LH stimulation enhances the secretion of FSH in proestrous stage. The secondary rise of FSH in the estrus stage is due to consequence of primary FSH surge in proestrous. The following resumption of inhibition secretion by late estrus causes a decrease of FSH thereafter. Follistatin is other polypeptide purified from side fractions obtained during purification of inhibin, on the basis to inhibit FSH secretion. This protein also is an activin binding protein, which neutralizes bioactivity of activin. In rat ovary, follistatin mRNA expression is limited to the granulosa cells and early luteal cells, with the intensity of signal increasing throughout follicular maturation and declining in the preovulatory graffian follicle. Following ovulation, the signal declines further with advancing luteinisation and disappears during luteolysis. The signal also declines sharply following atresia in nondominant follicles.

FSH receptor gene is exclusively expressed in the granulosa cells, where FSH acts directly to induce FSH receptors, cytoproliferation and differentiation via cAMP/ Protein Kinase A mediated post receptor binding. When FSH recruits follicles for preovulatory development, LH receptors on granulosa cells develop. The earliest binding of FSH during the ontogeny of GC's has been demonstrated about the primary follicle stage, when oocyte ceases to grow (Midley Jr, 1973; Richard and Midley Jr, 1976). FSH increases the concentration of its own receptor that estrogens synergies the effect (Hseuh et al., 1984). An Autoradiographic study using I^{125} has shown that GC's of preovulatory follicle has a uniform distribution of FSH receptors (Midley Jr, 1973). The secondary messenger system i.e., cAMP dependent protein kinase A induces biochemical activity along with the morphological changes (Amsterdam et al., 1981) in Granulosa cells.

Exposure of immature GC's to FSH causes flattened epitheliod shape to assume spherical shape, which is correlated with transformation of flattened to cubiod GC's during folliculogenesis (Mossmann and Duke, 1973; Baker and Franchi, 1967). Enhancement of steroidogenesis after induction of aromatases and cytochrome side chain cleavage enzyme (SCC) by FSH is correlated with the change in intracellular organelles of GC's. A gradual shift towards the development of tubular smooth endoplasmic reticulam (SER) occurs as steroidogenesis progresses (Crisp and Denys, 1975; Rigby et al., 1986). FSH stimulated cells generally have highly complex tubular type of cristae as compared to lamellar type seen in immature GC's (Crisp and Denys, 1975; Rigby et al., 1986). Immunoblotting and pulse chase experiments have shown that *de novo* synthesis of all components of SCC enzyme complex occurs after FSH and cAMP stimulation (Trzeciak et al., 1986; Voutilainen et al., 1986). FSH induces the antrum formation of the follicle, and early studies have shown that antrum formation can be correlated with the production of proteoglycans (Zachariae and Jensen, 1958; Zachariae, 1959). FSH also seems to induce several membranes related phenomenon. FSH increases membrane receptors for LH, Prolactin, EGF and FSH itself, by cAMP phenomenon.

Another pituitary gonadotropin, Luteininsing Hormone (LH) plays an important role in the estrus cycle. Serum levels of LH are lowest from the morning of estrus, after ovulation, through metaestrus, diestrus, and midday on proestrous. On the afternoon of proestrous circulating levels of the LH begin to increase rapidly and ultimately reach the peak. This rapid increase is called as "LH Surge ", which induces follicular rupture and ovulation, which is followed by decrease in LH level. The stimulus for ovulation inducing surge of LH is the ovarian estradiol secreted on diestrus and the primary

Figure 7: Hormonal Profile during Estrus cycle



Hormonal fluctuations in the course of the rat oestrous cycle. (From Short in Austin and Short 1972)

stimulus for increased secretion of estrogen is the tonic pattern of LH secretion from metaestrus through diestrus. Some reports (Banks and Freeman, 1978; Freeman et al., 1976) have shown that LH surge is limited only to proestrous stage, by heightened secretion of progesterone. The increase in progesterone from metaestrus through diestrus is secreted from newly formed corpus luteum and appears to be autonomous of pituitary support.

Ovarian receptors for LH exist in the theca cells, granulosa cells, and the corpus luteum. Theca cells contain nearly constant number of LH receptors, which are primary involved in androgen synthesis. Action of estradiol along with stimulatory action of FSH determines the number of receptors on granulosa cell membrane. LH receptors on granulosa cells appear at the time of antrum formation. Several studies, both in *vitro* and in *vivo* has shown that FSH has the ability to increase LH receptor on GC's (Hseuh et al., 1984; Amsterdam and Linder, 1984; Amsterdam et al., 1981). This process seems to be mediated by cAMP, as it can be induced by cAMP analogs (Amsterdam et al., 1981; Adashi et al., 1984); Prostaglandin E_2 (PGE_2) (Erickson et al., 1982); Choleratoxin (Knecht et al., 1981) and forskolin (Adashi and Resnick, 1984). Concomitant presence of estrogens (Richards, 1980; Hseuh et al., 1984), progestins (Rani et al., 1981), androgens (Rani et al., 1981), insulin (May et al., 1980), IGF-1 (Adashi et al.,1985) along with FSH induces LH receptors. Presence of EGF or GnRH agonists in GC culture was shown to inhibit the FSH induced LH receptor formation. Autoradiographic studies using I^{125}- hCG have suggested that receptors exists in theca cells, mural GC's and the number of receptor sites in peripheral GC's was 7 to 10 times greater than cumulus (Amsterdam and Linder, 1984). The average LH receptor content of preovulatory GC's of rat is about 20,

000 binding sites per GC (Amsterdam and Linder, 1984, Dufau and Catt, 1978). LH receptor present on the cell membrane may exist in monomer form (Luborsky and Behrman, 1979) as well as small clusters (Anderson et al., 1979; Amsterdam et al.,1980) as seen by binding of radiolabelled or ferritin labelled hCG . The size of LH cluster is related to the phenomenon of desensitization. This is due to limitation of receptor mobility after clustering (Schlessinger et al., 1978), and leading to interference in adenylate cyclase coupling.

The biochemical aspects of luteinisation characterize the cellular response to LH receptor stimulation. LH enhances the synthesis of progesterone by alterations in cholesterol metabolism and by inducing the enzyme cholesterol P450 SCC enzyme, primed by FSH (Funkenstein et al., 1984).

Inhibition of LH release occurs by negative feedback provided by estrogen and progesterone secreted by the ovaries. This occurs by modulating the frequency of pulsatile release of GnRH at the hypothalamic level. GnRH receptors are also present on cell membrane of GC. Studies have indicated that GnRH receptors are homogeneously distributed between GC in the intact follicle and Corpus luteum (Seguin et al., 1982; Pelletier et al., 1982). Several studies have shown that GnRH prevent the FSH induced LH receptor formation and significantly decrease cAMP, progesterone production in GC's of diethylstilbestrol (DES) treated immature rats (Hsueh et al., 1984; Amsterdam et al., 1981). It is also seen that GnRH will stimulate progesterone production in GC's of preovulatory follicle (Hsueh et al., 1984). Thereby, it seems that GnRH interferes with FSH stimulated processes only in the early phases of differentiation of the follicle while in mature follicles; it facilitates steroidogenesis (Hillensjo and LeMarie, 1980).

Pituitary peptide hormone potentiates the release of ovarian steroids. Growth of ovarian follicles and a concomitant enhanced secretion of estrogens characterize the preovulatory period of estrous cycle. The secretion rate of estradiol into ovarian venous plasma is low on estrous, begins to rise significantly by late metaestrus through morning of diestrus and reaches the peak concentration by afternoon of proestrous. That is, in 4-day cycling rats, peripheral plasma levels of estradiol are basal through estrous, late metaestrus and begin to rise through early diestrus. The increase continues through diestrus and early proestrous to reach peak level and plateau by mid proestrous and then level falls. In adult rats, the dominant progestin secreted into ovarian venous blood during estrous cycle is 20 α hydroxy preg-4-en-3-one, also called as 20 α Hydroxy Progesterone. This progestin is a metabolite of progesterone and its synthesis is catalyzed by 20 α Hydroxy Steroid Dehydrogenase. There are 2 peaks of both 20 α hydroxy progesterone and progesterone secretion into ovarian vein blood during the cycle. The first peak occurs during the afternoon of metaestrus and both steroids rise from newly formed corpus luteum. Second peak occurs at late afternoon of proestrous, which might arises from granulosa cell of preovulatory follicles. This increase occurs nearly simultaneously with major ovulation inducing the release of LH secretion. Thus, different ovarian steroids and pituitary hormones control the estrus cycle.

Several neuropeptides and neurotransmitters are also known modulate the secretion of gonadotropins. Neuropeptide Y, exerts positive effect on GnRH induced FSH and LH secretion following estrogen priming. Also, Neuropeptide Y inhibits FSH secretion in absence of priming. Galanin is known to stimulate the LH release. Substance

P, may inhibit LH release at pituitary but shown to variable effects on LH and FSH release when delivered to hypothalamus. Glutamic acid, the excitatory amino acid increases circulating LH whereas GABA is a potent inhibitor of LH secretion. Opiod peptides can inhibit ovulation and their antagonists raise LH secretion. Besides, there are several peptides like β -endorphin, inhibits the release of gonadotropins and its concentration is inversely correlated with preovulatory LH release on proestrous rats (Lindheimer and Katz, 1972).

Biogenesis of steroid hormones

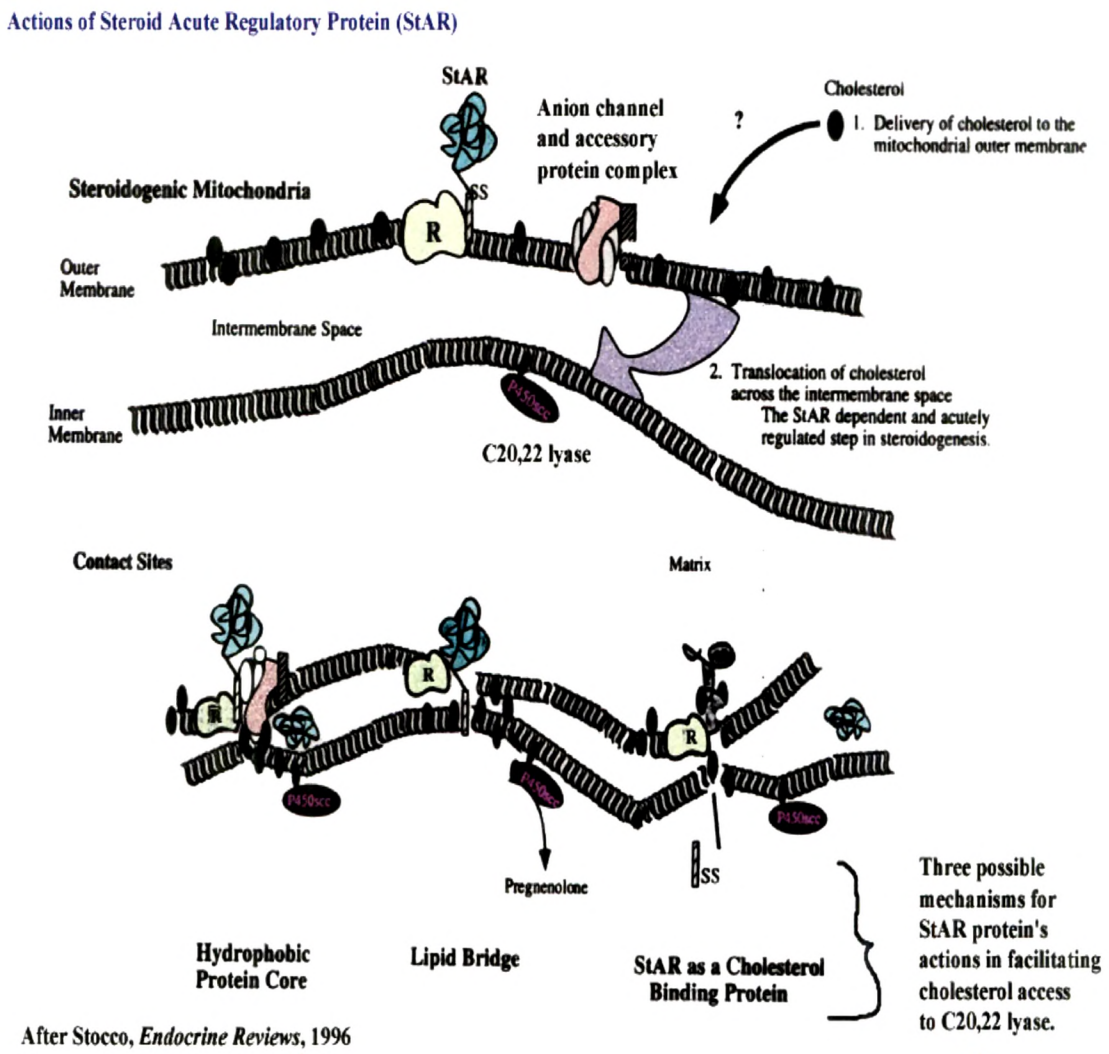
Steroid hormone biosynthesis is mainly regulated by events that ultimately affect steroid production through four parameters or processes: 1) Steroidogenic enzyme level as determined by transcription, stability and translation of the mRNAs encoding the enzymes, 2) Steroidogenic enzyme activity, determined 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 synthesised denovo within the ovary from 2C units 3) cholesterol liberated from cholesterol esters stored within the lipid droplets

In ovary, LH stimulates activity of Adenyl 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 (Gwyne and Strauss, 1982; Golos et al., 1987). 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. 8). Other domain is N-terminal domain which has mitochondrial targeting sequence that directs the StAR protein to the mitochondria (Strauss et al., 1999). 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. Conversion of cholesterol to pregnenolone is rate-limiting step in ovarian steroidogenesis; it is catalyzed by cholesterol side chain cleavage enzyme complex, consisting of cytochrome P450 side chain cleavage enzyme (CYP11A), flavoprotein. (Waterman and Simpson, 1985). Apart from its normal stimulation of the Adenyl cyclase-cAMP pathway (Fig 9)., the preovulation surge also activates phospholipase C-Protein Kinase C in granulosa cells.

Figure 8 : Actions of Steroidogenic Acute regulatory protein (StAR)



After Stocco, *Endocrine Reviews*, 1996

Figure 9 showing FSH signal transduction pathway in granulosa cell of a dominant follicle.

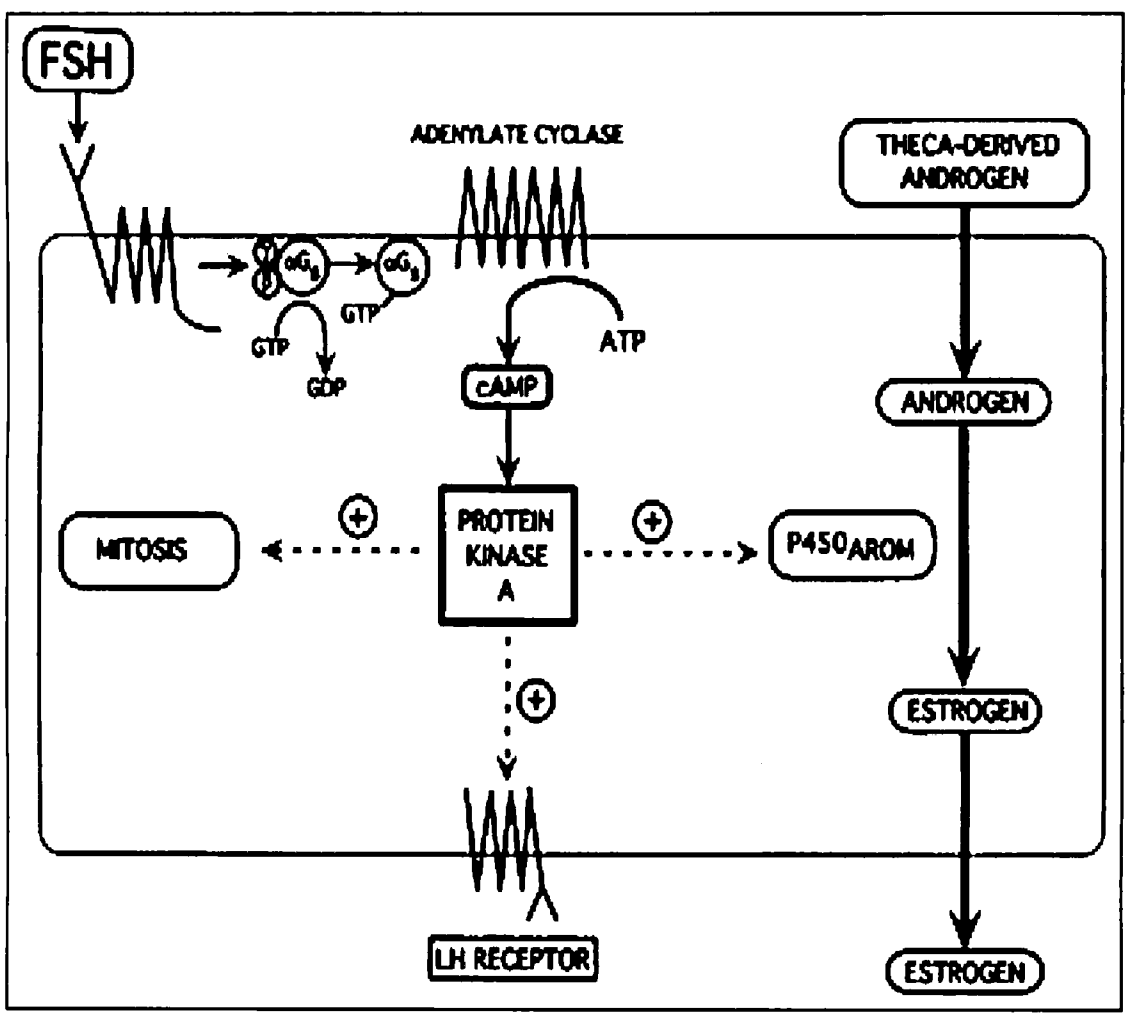


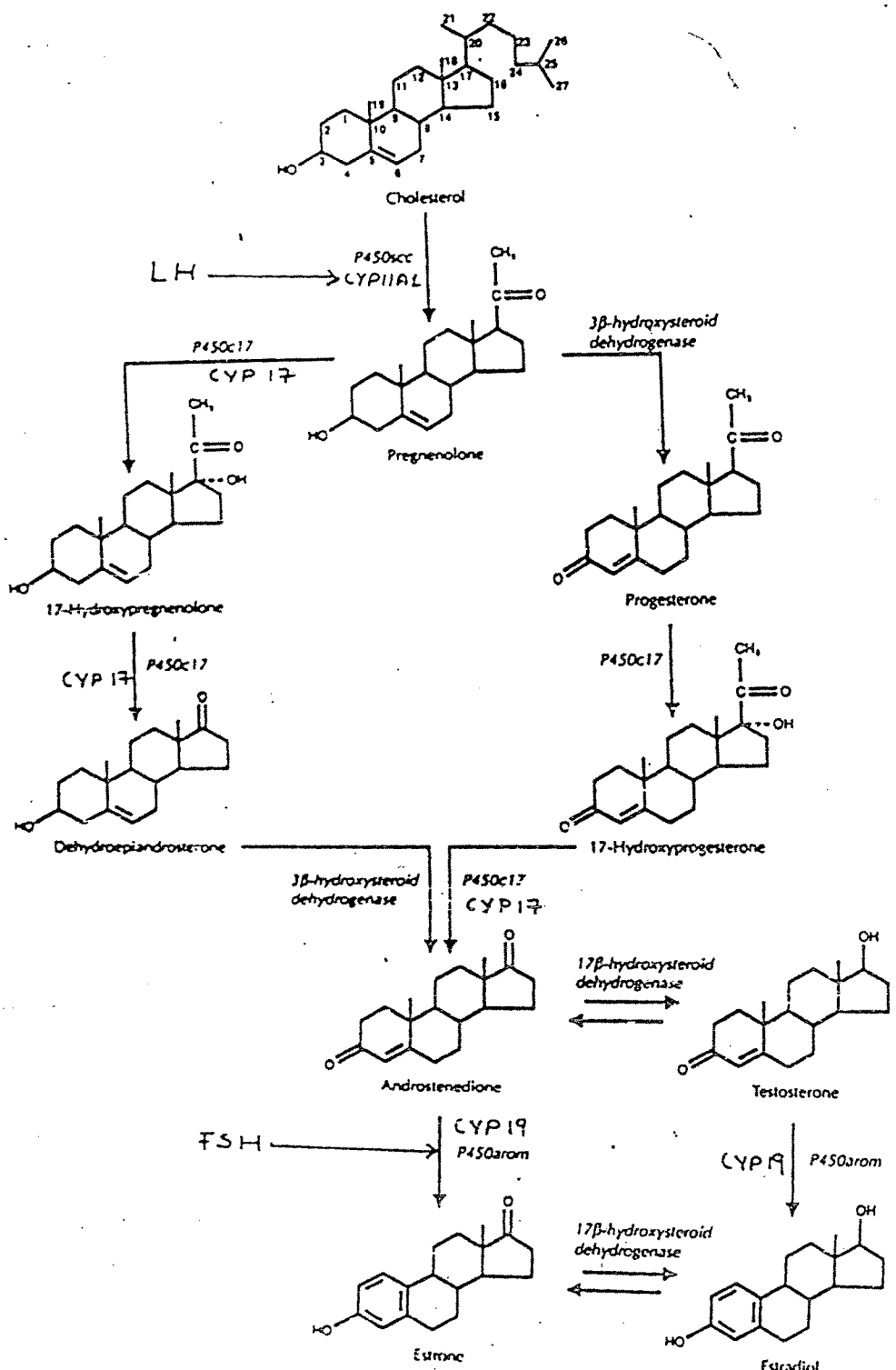
Table 10: Localization of steroidogenic enzymes in the ovary

Enzymes [A]	Theca Interna	Granulosa (Preantral follicle)	Granulosa (Preovulatory follicle)	Luteinized Theca (Corpus luteum)	Luteinized Granulosa (Corpus luteum)
P450scc	+	-	-	++[a]	++
3β-HSD	+	+	-	++	++
P450arom	-	+	+	-	+
17β-HSD	-	+	+	-	-

[A]- P450 scc- P450 Side Chain Cleavage; 3βHSD- 3β Hydroxy Steroid Dehydrogenase; P 450arom- P450 Aromatase; 17βHSD-17β Hydroxy Steroid Dehydrogenase

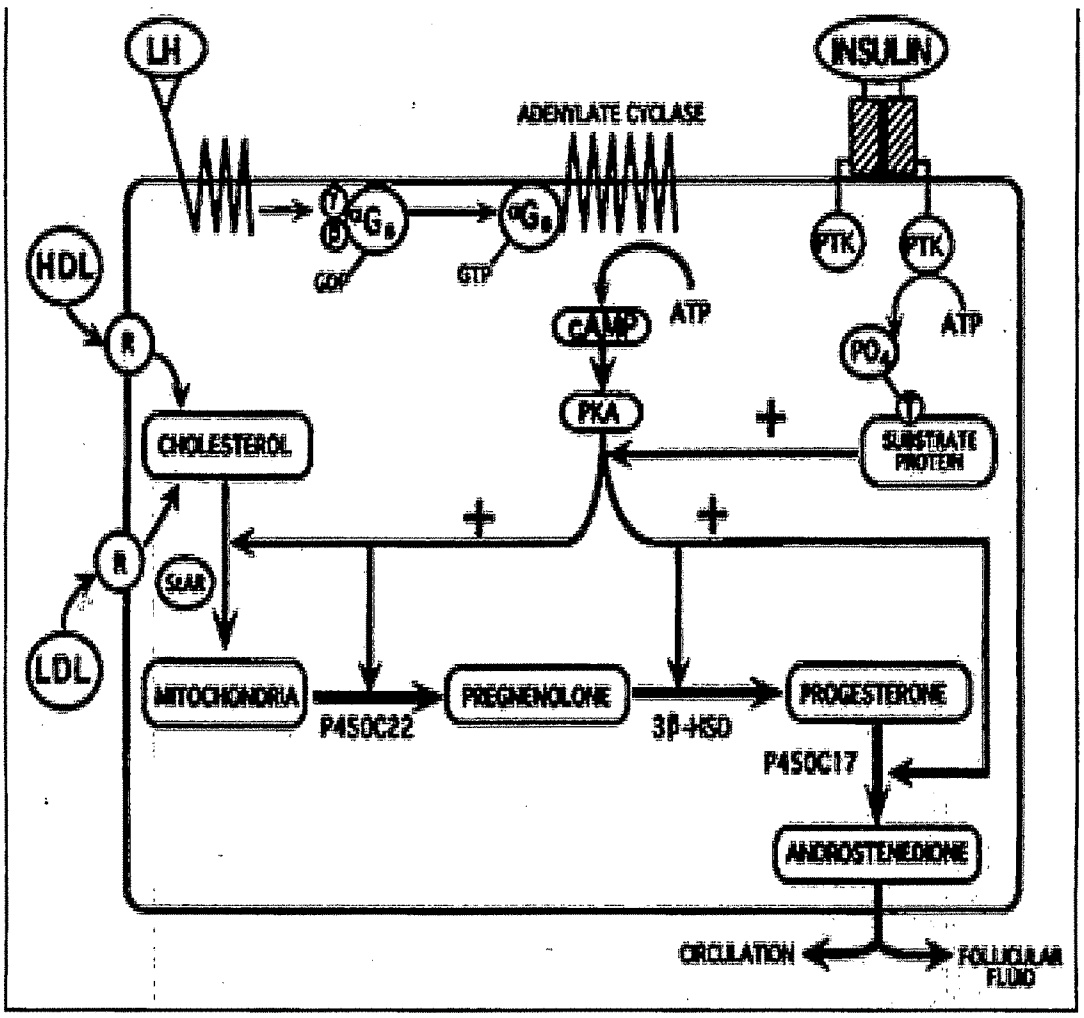
Principal steroid producing cells of the ovary – Granulosa cell, Theca and Corpus Luteum, which possess complete enzymatic complement required for steroid hormone synthesis(Figure 10). The main pathway of steroid synthesis involves conversion of pregnenolone to progesterone. In ovarian follicle, Δ^5 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.

Figure 10: Steroidogenesis in ovary



Pathways of ovarian follicular steroidogenesis [reproduced with permission from *Clinical Gynecologic Endocrinology and Infertility* (Speroff, Glass, and Kase, Eds.), 5th ed.].

Figure 11 showing the regulatory mechanisms of androgen production by theca interstitial cells by Luteinising Hormone (LH).



(Ref: Erickson, GF. Normal regulation of ovarian androgen production. Semin Reprod Endocrinol 11:307, 1993. Reproduced with permission from Thieme Medical Publishers.)

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. LH regulates first step in steroid hormone biosynthesis by controlling conversions (Fig 11).

Table 11: Characteristics of the genes and mRNAs of cytochrome P450 and their electron transfer proteins.

Enzyme	Species	Gene (N)	Chromosome	Gene length (kb)	mRNA length (kb)	Protein length (a.a)
Mitochondrial						
P450scc	Man	1	15	>20	2	482 (521) 481 (520) 490 (526)
	Cow	1	15	> 20	2	
	Rat	1	15	>20	2	
Microsomal						
P450c17	Man	1	10	6.6	1.9	508 509 507 507
	Cow	1	10	6.6	1.9	
	Rat	1	10	6.6	1.9	
	Mouse	1	19	6.6	1.9	
	Chicken	1	19	6.6	1.9	508 503 508 503 507
P450arom	Man	1	15	>70	2.9, 3.4	
	Rat	1	15	>70	2.9, 3.4	
	Mouse	1	9	>70	2.1, 2.5	
	Chicken	1	9	>70	4	
	Trout	1	9	>70	2.6	522

P450 Side Chain Cleavage (P 450scc) - This enzyme catalyzes the first and rate-limiting step in the biosynthesis of steroid hormones (Hanukoglu and Jefcoate, 1980; 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 bond).

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; Goldring et al., 1987). After ovulation, the theca and granulosa cells become the progenitors of small and large luteal cells of the corpus luteum, while this cell lineage continues to be reflected in the functioning of the two types of luteal cells (Keyes and Wiltbank, 1988; Alila et al., 1988; Nelson et al., 1992). 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. In the rat preovulatory follicle, the concentration of LH receptors shows a steep gradient decreasing from the periphery of the follicle to its center (Amsterdam et al., 1975; Lawrence et al., 1980). Immunohistochemical and *insitu* hybridization studies revealed that LH receptors are expressed only in granulosa cells of preovulatory follicles, but not in small follicles (Meduri et al., 1991; Camp et al., 1991). Thus, the expression of P450scc in granulosa cells is closely associated with the induction of LH receptors in these cells.

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 (de Launoit et al., 1992).

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). Immunohistochemical studies reveal localization of 3β-HSD in the same steroidogenic cells as P450scc. Immunostaining failed to detect 3β-HSD in rat granulosa cells (Dupont et al., 1990), even though these cells display high 3β-HSD activity converting pregnenolone to progesterone even before the induction of P450scc (Goldring and Orly, 1985). 3β HSD mRNA is seen in follicles but increase is highly seen in corpus luteum, which is consistent with the secretion of progesterone in luteal phase.In contrast to steroidogenic P450s, 3β-HSD activities is present in a wide range of tissues (Labrie et al., 1992).

P450 C_{17-20} Lyase (P450c17): This enzyme catalyzes two key reactions: a) 17α-hydroxylation of C21 steroids, and b) cleavage of the C17-C20 bond of C21 steroids. In the ovary, P450c17 is expressed in theca interna cells (Hedin et al., 1987; Sasano et al., 1989). Antibody or cDNA probes show very low expression of P450c17 in granulosa cells of humans and rats (Hedin et al., 1987; Sasano et al., 1989). Thus, theca interna cells

can synthesize androgens, but granulosa cells, which produce estrogens, are dependent on androgen precursor supply from theca interna (Adhashi, 1991). This process is called the two-cell hypothesis of follicular estrogen production (Adhashi, 1991). P450c17 from all species examined can hydroxylate C-17 of both pregnenolone and progesterone; although the K_m for these two substrates can differ 10 fold in some species (Namiki et al., 1988; Takemori and Kominami, 1991; Kitamura et al., 1991; Barnes et al., 1991). Whereas, the rat P450c17 can convert both 17-OH-pregnenolone and 17-OH-progesterone into DHEA and androstenedione, respectively (Takemori and Kominami, 1991; Kitamura et al., 1991), the human and bovine P450c17 can cleave the C17-C20 bond of 17-OH-pregnenolone but not of 17-OH-progesterone (Takemori and Kominami, 1991; Barnes et al., 1991; Perrin et al., 1991). Thus, the substrate specificity of P450c17 determines whether androgen biosynthesis proceeds mainly through pregnenolone or progesterone. The pattern of CYP17 mRNA is similar in corpus luteum and in follicles. CYP 17 mRNA is seen in theca interna of follicle and theca-lutein cells of Corpus luteum, it is absent in granulosa and granulosa-lutein cells (Doody et al., 1990). The ovulatory surge of LH is associated with the disappearance of P450c17 expression in bovine (Rodgers et al., 1987) and rat (Hedin et al., 1987), but not in human corpora lutea. The factor(s) that mediate this suppression may be different from LH, because in cultured luteinizing rat follicles the activity of P450c17 is maintained with or without LH (Hedin et al., 1987; Richards and Hedin, 1988).

Aromatase (P450arom): P450arom catalyzes conversion of testosterone into 17β-estradiol. In the ovary P450arom is expressed in granulosa cells which is the major site of estrogen production in females (Richards and Hedin, 1988; Sasano et al., 1989).

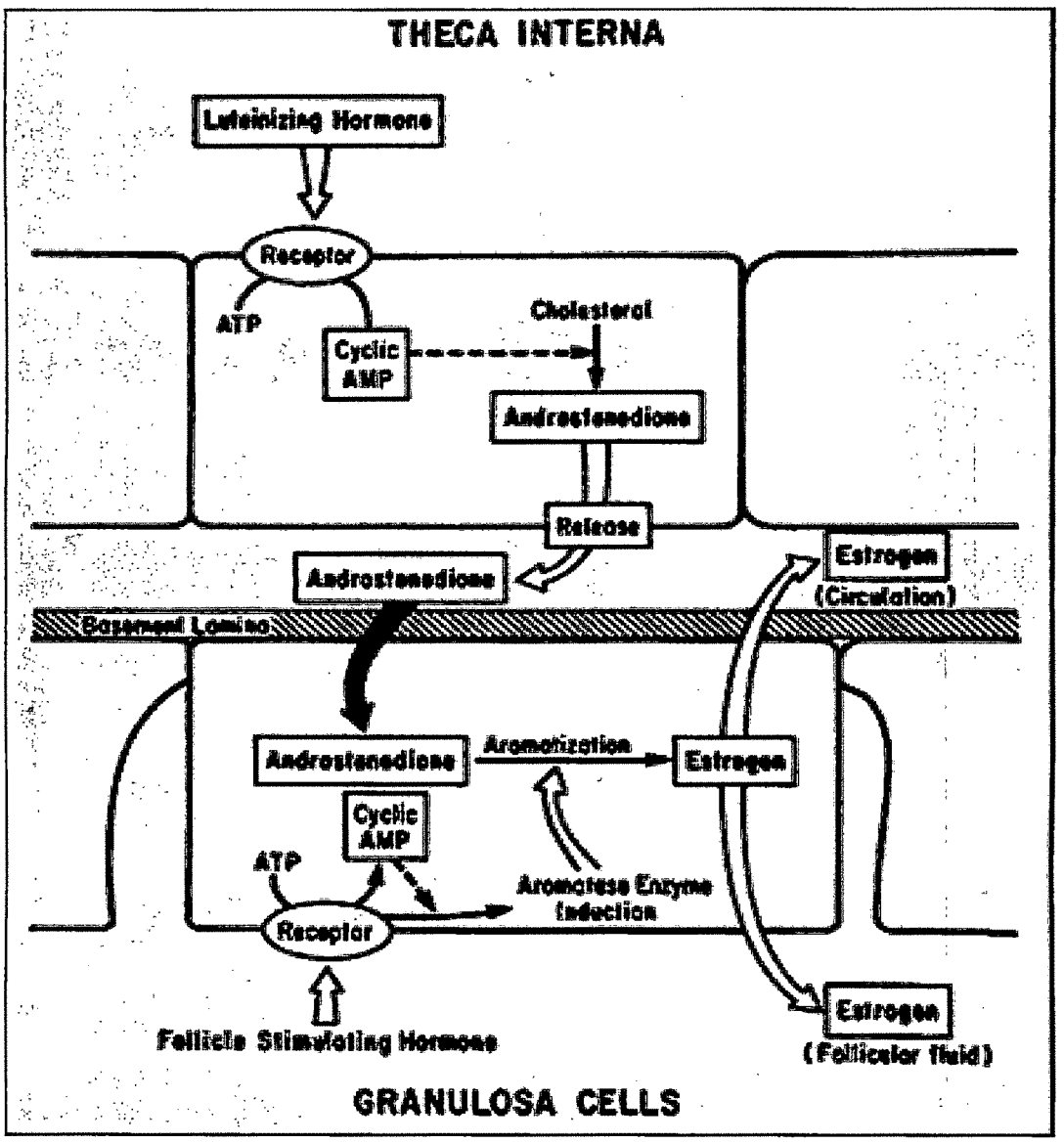
However, this enzyme is widely expressed in many tissues besides gonads, e.g. adipocytes, breast, central nervous system, skin and placenta (Santen, 1987; Simpson et al., 1992). The gene encoding P450arom is the longest one among steroidogenic P450 genes. This gene is also unique among P450 genes in 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, that 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 (Inano et al., 1990; Luu-The et al., 1990; Martel et al., 1992).

There are multiple 17β-HSD isozymes with androgen or estrogen specificity (Inano et al., 1990; Luu-The et al., 1990; Martel et al., 1992) . The isozyme from the porcine testis also displays 20α-hydroxy-steroid dehydrogenase activity at about a tenth of the 17β-HSD activity (Inano and Tamaoki, 1986). For the conversion between androstenedione and testosterone, the porcine testicular 17β-HSD prefers NADPH rather than NADH (K_m=11 μM vs. 177 μM) (Inano and Tamaoki , 1986).

Figure 12 showing the "Two Gonadotropin-Two Cell Concept" of follicle estrogen production

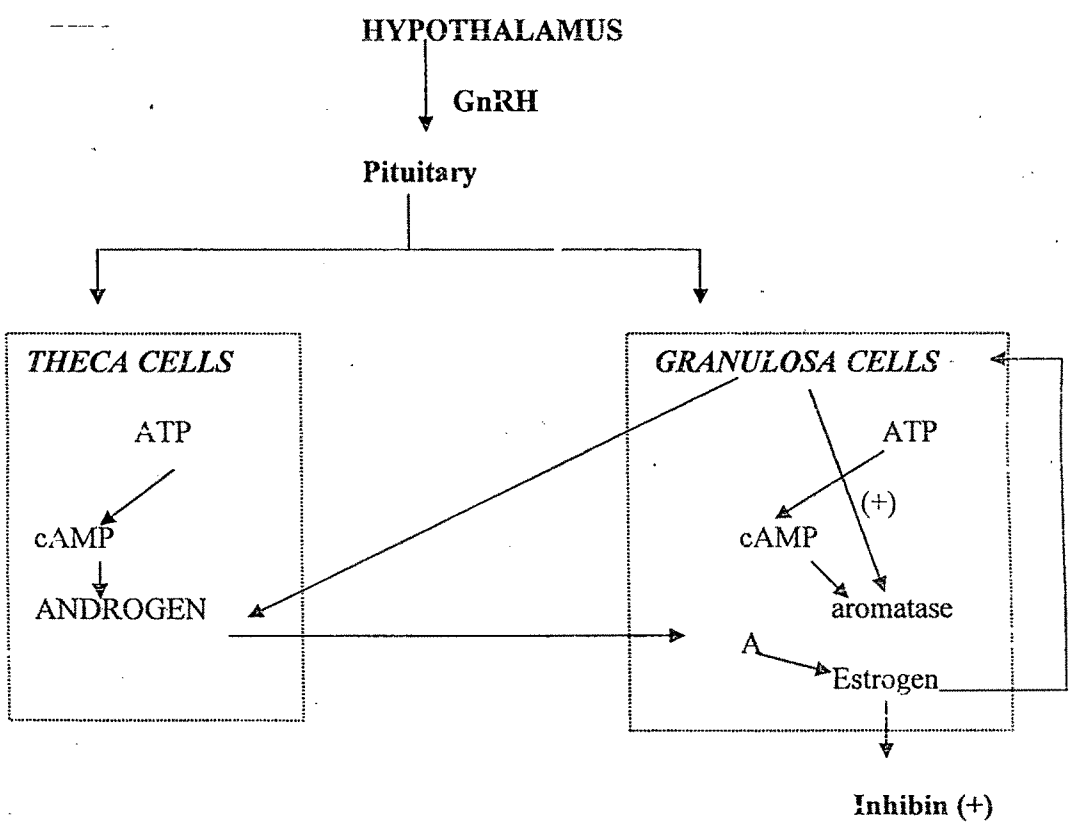


(Ref:: Erickson, GF: Normal ovarian function. Clin Obstet Gynecol 21:31, 1978).

A 17β-HSD has been purified from placenta, and a cDNA and two homologous genes have been cloned and sequenced (Luu-The et al., 1990a, b) . This enzyme catalyzes the interconversion of 17β-estradiol and estrone, and can also use androgen substrates (Luu-The et al., 1990). Its K_m for 17β-estradiol is 10 μM versus 250 μM for testosterone (Luu-The et al., 1990). Thus, this enzyme is specific for estrogens and not androgens.17 βHSDH, 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 3βHSDH 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 HSDH 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 HSDH 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 (Ghersevich et al., 1994a). The expression of 17β HSDH 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., 1994a). Expression of 17β HSDH 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 HSDH type 1 expression may be down regulated earlier than P450 Aromatase and thus limit estradiol biosynthesis in luteinising granulosa cells. Epidermal Growth Factor (EGF) suppresses the FSH induced expression of 17 HSDH type 1 (Ghersevich et al., 1994b) and P450 Aromatase (Hseuch et al., 1981).

Figure 13: Generalized action of gonadotropins in the ovary.



Gonadotropin and peptide transmitters as possible regulators of steroidogenesis

Both FSH and LH are required for estrogen synthesis and the amount of estrogen produced depends on relative exposure to each gonadotropin, after a minimum effective dose of gonadotropins has been achieved (Hsuech et al., 1984). Increased secretion of estradiol causes further proliferation of granulosa cells and follicular growth and an increase in number of estradiol receptors (Hseueh et al., 1984; Tilly et al., 1992).

In mature follicles, FSH is in concert with estradiol, causes an increase in LH receptors on granulosa cells. LH acts on granulosa cells through these receptors to augment progesterone secretion, which then increases FSH release at mid cycle. LH increases FSH release at mid cycle. LH increases progesterone receptors in granulosa cells. Following atresia associated with loss of receptors.

In granulosa cells, leptin is known to cause inhibition of insulin induced aromatase activity in undifferentiated granulosa cells (Agarwal et al., 1999), which implies that leptin receptors in granulosa cells decrease as the follicle grows and develop, thereby matured follicles do not show much response to leptin. Several studies have shown that leptin inhibited Insulin like Growth Factor-I (IGF-1) and Transforming growth factor alpha (TGFα) induced estradiol production in cultured granulosa cells and FSH induced progesterone secretion (Spicer, 2001; Barkan et al., 1999). Guo et al. (2001) showed *in vitro* addition of leptin (10-30 ng / ml) to cultured granulosa cells, caused a dose dependent inhibition of estradiol production, but progesterone showed no change. In theca cells, leptin directly inhibits steroidogenesis in cattle thecal cells (Spicer and Fransisco., 1998). Several *in vitro* studies have shown that leptin may exert inhibitory effect on granulosa and thecal steroidogenesis (Barash et al., 1996; Spicer and Fransisco,

1997). Ghizzoni et al. (2001) suggested that leptin may play a minor , but regulatory role on unstimulated human ovarian steroidogenesis by interfering with the either translational or post translational steps of baseline CYP17 and or aromatase synthesis of the enzymes.

The ovary is responsive to exogenous estrogens. Estrogen increases the number of granulosa cells of ovary and also increases the FSH receptors. Estrogen and FSH in concert increase the LH receptor levels of granulosa cells and sensitize these cells to the ovulatory effect of LH.

Regulation of gonadal function by growth factors

Growth promoting substances, which are produced by the specific cell types that have paracrine and autocrine effects. The main growth factors are Insulin like Growth Factor (IGF), Epidermal Growth factor (EGF), Transforming Growth factor (TGF), Fibroblast Growth factor (FGF).

There are evidences that ovary is site of IGF-1 gene expression (Hernandez et al., 1989; Carlsson et al., 1992; Wang et al., 1997). Granulosa cells are the major ovarian cells concerned with IGF-1 gene expression (Wathes et al., 1995; Leeunberg et al., 1996). IGF- type 1 receptor is expressed in the granulosa cells (Zhou et al., 1991; Zhou & Bondy,1993). Both *in vitro* and *in vivo* studies have clearly documented the ability of FSH to effect dose dependent increment in granulosa cell IGF-1 binding. There are studies which indicate that IGF-1 receptor mRNA are highest in healthy growing and dominant follicle granulosa cells and reduced to undetectable levels in atretic follicles regardless of follicular size, suggesting the loss of IGF effect due to receptor down regulation (Mognet et al., 1993; Zhou et al., 1996).

It is known that IGF-1 acts at the level of granulosa cells to amplify gonadotropin hormone action in rat (Jia et al., 1986; Zhiwen et al., 1987). Monniaux et al.(1992) based on their experiment in ovine granulosa cells, proposed that IGF-1, in synergy with FSH, clearly increases the percentage of cell expressing P450 side chain cleavage enzyme and cell proliferation. Mingeshi et al. (2000) showed that IGF-1 could enhance the FSH receptor expression in granulosa cells. Several reports have suggested that the future dominant follicles contain increased IGF-1, highest estradiol, lowest IGF binding proteins especially type 4 (Yuan et al., 1998; Mihn et al., 2000; Ginther et al., 2000). In contrast, future atretic follicles have low IGF-1, High IGF binding proteins (IGFBP's), low aromatase activity and low estradiol synthesis (Adashi et al., 1986). Several reports have suggested that FSH in the early and late follicular phase may contribute to increased concentration of bioavailable IGFs in dominant follicle by regulating synthesis and proteolysis of IGFBP's (Zhou et al., 1996; Bernard et al., 1996). Some workers have shown the theca-interstitial cells as the IGF-1 action site (Cara & Rosenfield, 1988; Magoffin et al., 1990).

Theca cells of the ovary produce EGF. In granulosa cells, EGF stimulates growth, increases FSH binding and inhibits induction of LH receptors. A study has shown that EGF suppresses FSH dependent LH receptor production in serum free cultures (Mondschein and Schomberg, 1981). EGF is also known to stimulate progesterone synthesis (Jones et al., 1982). Few studies have indicated that LH and FSH addition increases total EGF receptor of the ovary (Jones et al., 1982; St-Arnaud et al., 1983). St-Arnaud et al. (1983) also showed that ovarian EGF receptor is high in proestrous stage and low during diestrous stage. Grasselli et al. (2002) showed that in vitro exposure of

vascular endothethial growth factor (VEGF) to cultured porcine granulosa cells caused a change in progesterone and estrogen, which is dependent on follicular size.

TGF family includes TGF, TGF-β and inhibin related peptides. TGF-β is produced by both theca and granulosa cells. Basini et al. (2002) showed that human TNF- alpha inhibits progesterone production in granulosa cells and stimulates estradiol from small follicles; it does not affect proliferation but stimulates granulosa cell apoptosis. TGF-β promotes growth, cAMP production, production of both estradiol, progesterone and enhances FSH induced increase in aromatase and LH receptors in granulosa cells.

FGF family has two polypeptides: FGFa, FGFb. FGFa is produced by the granulosa cells and inhibits steroidogenesis, induction of LH receptors.

Follistatin is high affinity protein for both inhibin and activin. These compounds play an important role in follicular development by both autocrine and paracrine mechanism. Activin production in granulosa cells decrease after follicular growth whereas inhibin and follistatin production is maximum in matured follicle. Inhibin acts on theca cells to enhance androgen production, whereas activin may play an autocrine role in enhancing granulosa cell proliferation and aromatase expression.

Interleukin- 1(IL-1) is a cytokine produced by activated macrophages that can arrest folliculogenesis. Specifically, IL-1 has been shown to have both FSH- and hCG-stimulated estradiol productions in granulosa cells.

Another important cytokine in ovarian function is Tumor Necrosis Factor-β. TNF-β is shown to attenuate the differentiation of rat cultured granulosa cells. TNF-β is

also known to inhibit hCG-induced progesterone production and also inhibit FSH induced estradiol production.

Another intraovarian regulator is Nitric Oxide (NO), which is produced by Nitric Oxide Synthase (NOS). Two forms of NOS –inducible NOS (iNOS), endothelial NOS (eNOS) are localized in the rat ovary. iNOS mRNA is highest in small secondary follicles and levels decrease in larger preantral and antral follicles while eNOS mRNA is stimulated by gonadotropin secretion and peaks at the ovulation. Van Voorhis et al. (1994) reported that NO production in human granulosa cells on treatment with Sodium NitroPrusside (SNP), indicating there is an expression of e-NOS. Various studies indicate that rat granulosa cells from primary, secondary and small antral follicles (Van Voorhis et al., 1995; Matsumi et al., 1998) and rat stroma, thecal, and luteal cells (Zackrisson et al., 1996) also express iNOS. The expression of both iNOS and eNOS is regulated by gonadotropins (Jablonka-Shariff and Olson, 1997) since both Porcine Mare Serum Gonadotropin (PMSG) and Human Chorionic Gonadotropin (hCG) have been shown to influence eNOS and iNOS concentrations, thus confirming that both isoforms participate in the ovarian functions. Nitric oxide production in the rat granulosa cells is actively stimulated by interleukin-1, as well as by several proinflammatory cytokines (Ellman et al., 1993; Ben-shlomo et al., 1994; Matsumi et al., 1998). NO generation increases with both estradiol levels in human follicular fluid and with the follicular size (Rosselli et al., 1994; Anteby et al., 1996). These observations suggest a possible relationship between these characteristics, even though an inverse relationship between estradiol and nitrate concentrations has been observed in swine (Grasseli et al., 1998).

Reports suggest that NO plays a role in granulosa cells steroidogenesis. NO is known to causes a negative effect on steroidogenesis, probably by inhibiting androstenedione production (Dunnam et al., 1999). A further mechanism through which NO may be involved in the control of follicular development is its effects on apoptosis, the programmed cell death by which majority of ovarian follicles are lost during postnatal life (Kiess and Gallaher, 1998; Li et al., 1998). High NO levels have been shown to reduce apoptosis in both swine (Ponderato et al., 2000) and bovine (Basini et al., 1998) granulosa cells, whereas an opposite effect has been induced by low NO levels in more differentiated granulosa cells (from large follicles). A protective NO effect has been also observed in rat granulosa cells from immature (Matsumi et al., 1998) and preovulatory follicles (Yoon et al., 2002). In addition, the IL-1 induced anti-apoptotic effects have also been reported to be NO mediated (Chun et al., 1995).

Transport of ovarian steroid hormones in plasma

Most of steroids are bound to plasma proteins and carried to target tissues, where the hormone dissociate and pass through diffusion into the cell. Bulk of estradiol (60%) is bound to serum albumin but 38% is bound to TeBG (Testosterone Binding Globulin) and 2-3% is free (Rossner, 1996). It is usually assumed that protein bound hormone is inactive and that only free hormone is directly available to enter the target tissue, but the transport of steroid hormones may be actually be more complex (Mendel, 1992; Ekins et al., 1986). Progesterone is also mainly bound to cortisol binding globulin (CBG) and some amount is also to TeBG. During pregnancy, the concentration of these binding globulins are increased.

Targets and mechanism of action of steroid hormones

Steroid hormones have low molecular weight and readily enter into the cell by diffusion; although carrier mediated transport may occur. The major target organs include uterus, fallopian tubes, breasts and bone. Affinity and specificity of steroid receptors in cells of these organs allows low concentration of steroid hormones to produce biological responses. The lipid nature of steroid hormone allows them pass through the lipid bilayer easily by simple diffusion and so that it binds to its receptors that are present in the nucleus. The gonadal steroids binds to its receptor that are part of nuclear hormone receptor gene family, which encodes structurally related proteins that can have cis-regulating actions on the transcription of genes. The nuclear receptors have specific modular domains namely – a highly conserved DNA binding domain that directs the receptors to bind specific DNA sequences, ligand binding domain responds to the binding of the cognate hormone, this domain and the amino terminus then binds to several transcription factors. A ligand domain confers specificity for ligand binding and performs several functions like receptor release from Hsps, receptor translocation to the nucleus, homodimerization, and transcriptional activation. The DNA binding domain contain zinc finger motifs, one helix that determines specificity and other directs subunit interaction.

The conserved Hormone Responsive Elements (HRE) is a consensus sequence on the nuclear DNA, to which nuclear receptor protein bind. HRE's can effectively bind the activated homodimer steroid receptor complex. HRE's of gonadal steroids are arranged as palindromic repeats of six nucleotide bases with 3-nucleotide spacing. Binding of nuclear receptors to DNA elements can strongly activate or repress transcription of cis – gene. Adjacent DNA sequences can affect the activity of HRE.

Concentration of nuclear estrogen receptor in the uterus correlates with the level of estrogen in the blood of the rat (Clark, 1972). The number of nuclear complexes is at minimum during estrus and metaestrus, increases between metaestrus and diestrus and maximum at proestrous. The level of progesterone receptor varies during the estrous cycle. During the follicular phase, the level of cytosolic progesterone receptor is relatively low, and as the estrogen blood levels increase, the amount of progesterone receptor increase, an increase that is probably a requisite for progesterone action during pregnancy.

Major function of ovarian steroids

Ovarian steroids are major structural determinants of female reproductive system as it performs these functions: a) maturation of primordial cells, b) providing hormonal milieu for ovulation, c) developing tissues for implantation and d) establishing milieu for the maintenance of pregnancy. Estrogens increase the rate of synthesis of proteins, rRNA, tRNA, mRNA and DNA. Estrogens cause the proliferation of cells of uterine endometrium and vaginal epithelium. It also helps in rhythmic motility of the myometrium. Estradiol has anabolic effects on bone and cartilage and promotes growth. Progestin reduces the proliferative activity of estrogens on the vaginal epithelium, convert uterine epithelium to secretory phase by increasing secretory glands and glycogen content. Progestin decreases the peripheral blood flow, thereby decreasing body heat loss and increasing the body temperature in luteal phase.

If the egg produced in the follicular phase is fertilized with sperm, it results in a physiological state called the pregnancy. There has been speculation that the hormonal secretion rate by the oocyte-corona complex that directs different signals to transport

fertilized ovum to the uterus. Once fertilized ovum reaches the uterus, it undergoes a crucial process called implantation. Nidation can be initiated when the embryo and the endometrium reach the stage of synchronization. Along with the hormonal dependent changes leading to the development of the receptive endometrium, blastocyst must be hatched from the zona pellicuda. In rat, progesterone and estrogen is obligatory requirement for intrauterine implantation. Main events that take place in rat are: 1) priming the uterus with the estrogen in proestrous stage, 2) conditioning of uterus with progesterone during first 4 days of the pregnancy, 3) a prenidatory peak of estrogen on day 4 of pregnancy, the day before implantation occurs. An increasing level of progesterone is essential for embryonic development to proceed and for subsequent implantation to occur. The nidatory estrogen peak induces a secretory endometrium and a state of receptivity in the uterus towards the decidualisation stimulus of the embryo. This includes inhibition of endocytosis in the luminal epithelium and release of serum and non-serum proteins into uterine lumen, which is maximal at the day of implantation.

In rat, estrogen is a requirement for implantation. There are studies indicating the presence of 17 β hydroxy steroid dehydrogenase activity in preimplantation embryos (Wu and Matsumoto, 1985). It is seen that estrogen treatment in the uterus causes rapid elevations in cAMP level (Daughaday and Trivedi, 1987). Estrogen treatment *in vivo* and *in vitro* in rat uterus have been shown to increase cGMP accumulation, a response that appears to depend on RNA and protein synthesis (Flandroy et al., 1976). Uterine growth may be promoted by estrogen induced histamine release that causes increased capillary permeability (Szego and Lawson, 1977). A study showed that when rat embryos incubated in salt solution containing estradiol initiated a local increase in endometrial

vascular permeability when transferred to the uterus of pseudopregnant rats on day 5, whereas transfer of embryos incubated in estradiol free medium failed to show the response. This indicating that rat blastocysts require estrogen for the initiation of implantation (Dickmann, 1977). Progesterone modifies the metabolic activity of the uterus. Progesterone further inhibits further endometrial proliferation induced by estrogens and converts endometrium into secretory type. This happens when endometrium is ready to implant the blastocyst in the uterus. The functions of progesterone require prior increase in receptor levels by estrogen. Preimplanted rat embryos can interconvert steroid, thus allowing several events like uterine secretory activity, uterine blood flow, nutrient transport to the conceptus, which are crucial for pregnancy.

Embryonic signals along with maternal recognition are also important in process of implantation. During the preimplantation stage, several pregnancy-related proteins, steroids, hormones of the blastocyst interact with maternal endometrium to ensure the receptivity of the embryo. Once receptivity is obtained, several biochemical events like stimulation of vasodilation, angiogenesis in order to increase uterine blood flow, stimulate nutrients into the uterine lumen and protects the fetal allograft from immunological rejection and also maintain luteal function.

There are several biochemical signals that play an important role in recognition. Prostaglandin's plays an important role in mediating the local endometrial vascular response and also in the process transforming stromal cells to decidual cells. A study has shown that blastocyst, as a result of their interaction with endometrial epithelium,

stimulate the production of prostaglandin, which diffuses into endometrial stroma, to bring about vascular and decidual responses (Kennedy, 1980).

The amount of cytosolic progesterone receptor gradually increases during pregnancy to high levels and decreases before parturition. The ability of the rat uterus to form 5α pregnane3, 20 dione and 3α hydroxy 5αpregnan-20-one increases between days 11 and 21 pregnancy, so even though blood levels of progesterone are high at these times. This metabolic sequence is necessary for producing cellular environment wherein tissue level of progesterone is gradually lowered during pregnancy, and hence fewer estrogen-receptor complexes are formed. Elevated levels of cytoplasmic progesterone receptor complex during last few days of pregnancy in the rat could be due to increased levels of estrogen at this time. The decrease in nuclear levels of progesterone receptor before birth, is probably an important mechanism for decreasing the control of the uterus by progesterone and increasing its sensitivity to estrogen and may contribute to the onset of parturition.

Structure of placenta

Development of specific structure occurs, once the embryo gets implanted in the uterus, called the placenta. This physiological barrier serves as an interface between mother and the fetus

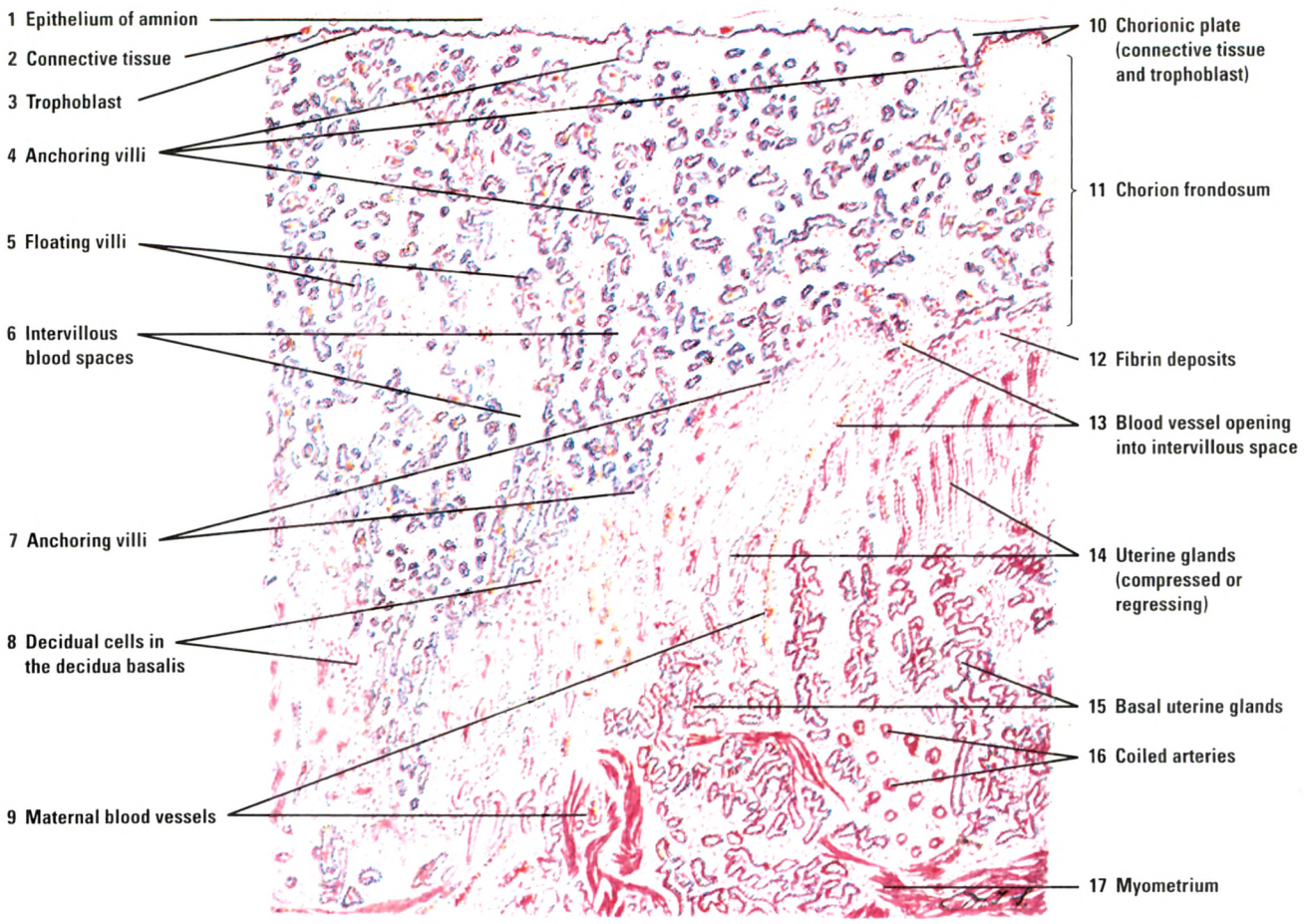
In rat, hemochorial placenta is seen where fetal trophoblast forms the cellular-synctia. The advantage of having such a kind of placenta to fetus is direct access to maternal nutrients and easy disposal of fetal wastes into maternal circulation. Placenta contains of a fetal portion, formed by the *chorion* and a maternal portion formed by the *decidua basalis.* These two parts are involved in exchange of substances between maternal and fetal circulation. Cytotrophoblast cells proliferate rapidly, sending cords or

masses of cells, called primary chorionic villi, into the synchytiotrophoblast. Shortly, after forming, these villi begin to branch as chorionic mesoderm invades their bases, forming central core of loose connective tissue in which embryonic blood vessels will gradually form secondary villi. These secondary villi together grow out to form tertiary villi. As tertiary villi are forming, cytotrophoblastic cells in the villi continue to grow out through the synchytiotrophoblast. Neighboring cells grow laterally and meet similar processes growing villi and form a thin layer of cytotrophoblastic cells called the trophoblastic shell. The villi adjacent to the decidua basalis rapidly increase in size and number and become highly branched. This region of the chorion, which is the fetal component of the placenta, is called the *villous chorion* (Figure 14).

There are several pregnancy-related proteins that are necessary for the maintenance of pregnancy. SP1 is one such glycoprotein with a molecular weight of 90 kDa, which is produced by synchytiotrophoblast and secreted into maternal circulation. Physiological role of SP1 is not clearly understood. It has been suggested that it may act as an immunosuppressant that prevents rejection of developing embryo. PAPP-A I a large glycoprotein, that is seen in ovarian follicular fluid. PAPP-A is a potent and specific leukocyte inhibitor. It seems that PAPP-A is a placenta derived protease inhibitor that can serve as a barrier to maternal proteolytic attack.

There is a luteotrophin identified in the rat placenta called rat Chorionic Mammoluteotropin (RCM), which has molecular weight between 25 kDa to 50 kDa, with a trophic effect on the corpus lutea. The concentration is highest on 12 day of pregnancy and originates from synchytiotrophoblast.

Placenta


1 Epithelium of amnion
2 Connective tissue
3 Trophoblast
4 Anchoring villi
5 Floating villi
6 Intervillous blood spaces
7 Anchoring villi
8 Decidual cells in the decidua basalis
9 Maternal blood vessels
10 Chorionic plate (connective tissue and trophoblast)
11 Chorion frondosum
12 Fibrin deposits
13 Blood vessel opening into intervillous space
14 Uterine glands (compressed or regressing)
15 Basal uterine glands
16 Coiled arteries
17 Myometrium

Rat placenta also produces two lactogenic hormones, namely Placental lactogen- I (rPL-I), which is present at middle of gestation and Placental lactogen-II (rPL-II), whose concentration increases after midpregnancy. Soares et al. (1985) showed that the giant cells of the trophoblast produce these hormones. Robertson et al. (1982) showed that rPL-I was seen on gestation day 8 and its concentration rose rapidly on day 12 and then declined almost being undetectable on day 15 of pregnancy. The work by Robertson and his group (1981, 1984) revealed that rPL-II was present in maternal circulation from day 12 of pregnancy until parturition, with its concentration increases as pregnancy progresses. The concentration rPL-II is highly correlated with the number of conceptuses, an effect that is probably due to a greater placental mass of rats carrying large litters. The total placental lactogen activity of the maternal blood is proportional to the number of conceeptus (Voogt et al., 1982); it is stimulated by the fetus (Tonokowicz et al., 1984; 1983) and inhibited by the pituitary (Voogt et al., 1985; Blank and Dufau, 1983). Several studies have indicated that both rPL-I and rPL-II have luteotrophic function in placenta (Ray et al., 1955; Robertson et al., 1982).

There are studies indicating that LH like activity has been detected in placenta (Haour et al., 1976; Wide and Hobson, 1980), which increases by day 8 of pregnancy and reaches its peak by day 14 and then decreases slightly until day 16, but then increased again steadily until term. The molecular size of the substance is around 15 Kd by size exclusion chromatography (Okker-Reitsma and Wilson, 1980).

Several studies have reported that there is GnRH like compound present in placenta (Tan and Rousseau, 1982, Siler-Khodr et al., 1986). Placental GnRH like protein is known to regulate placental steroid and prostaglandin production. It is also reported

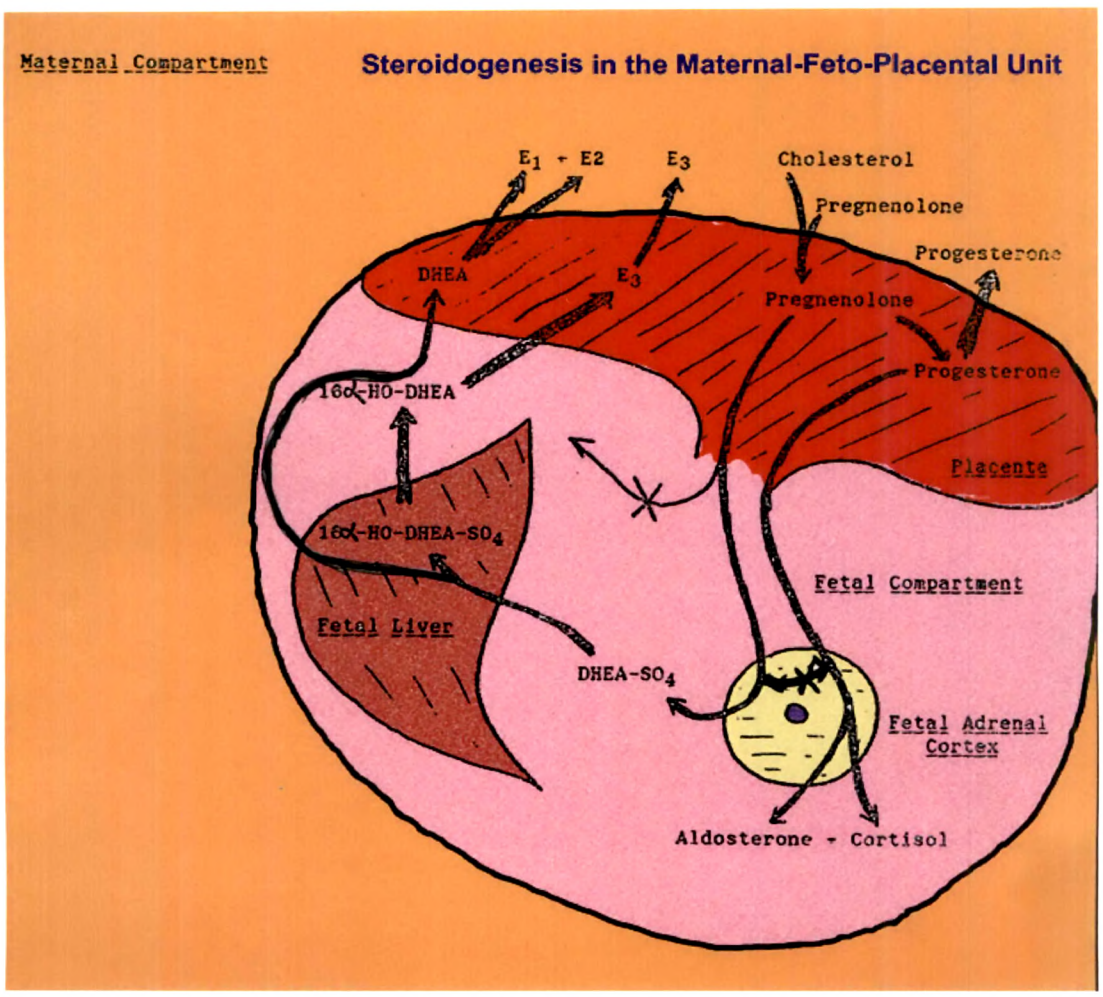
that GnRH like protein can stimulate (Siler-Khodr et al., 1986) and inhibit (Wilson and Jawad, 1980) the release of estrogen and progesterone by placental explants and cells *in vitro.* In human placenta, GnRH like peptide has been localized in cytotrophoblasts of the placenta.

Placenta functions as both endocrine and exocrine organ to maintain the growing fetus. There are several transport systems through which energy, nutrients and fetal waste disposal occurs thereby functioning as an exocrine organ.

Transport systems in placenta

Placental nutrient transfer is a process by which there is a net transfer of molecules, which is necessary for the growth and development of the fetus. Also, there is a transfer of waste products of metabolic process from fetus to mother through the placenta. The main nutrient –glucose is transported across the placenta by facilitated diffusion. The glucose transporters have been isolated from the microvillous plasma membrane (Bissonnette et al., 1982). Glucose transport is complicated by the metabolism of glucose by the placenta, as the glucose, which is transported in the umbilical cord to fetus is around only 40%. Lactic acid is also transported by facilitated diffusion. Along with lactate ions, there is evidence that H+ ions are also cotransported along with it (Moll et al., 1980). It is shown that in rats, L-alanine is the precursor of lactate (Palacin et al., 1985). The fetuses utilize the lactate supplied. Amino acids are actively transported across the placenta, which occurs only in the plasma membrane of the trophoblast. Subsequently, the amino acids diffuse across the fetal facing membrane into fetal plasma. The amino acid transfer requires the ATP. (Yudilevich and Sweiry, 1985). Three amino acid transport systems namely- A, L and ASC transport neutral amino acids. In Human

Figure 15: Steroidogenesis during pregnancy



four stages of iron transport are: a) iron in maternal circulation, bound to maternal transferrin attaches briefly to the specific transferrin receptors present on placental cell membrane, b) iron is released to the cytosol, where it again attaches to small molecular weight proteins like placental trasferrin and ferritin, c) iron moves across the trophoblast cytosol bound to these proteins and d) iron is released across the basal membrane and fetal capillary endothelium, where it binds to fetal transferrin. The transport for iron in rats increases between days 14 to 17 of gestation and further increases at day 20 (Mc.Ardle et al., 1985). The increase in transport system is associated with an increase in specific binding of transferrin receptors, as well as maturation of the mechanisms by which transferrin releases iron to the placenta.

Heavy metals such as lead and cadmium are highly reactive with thiol groups of proteins, can substitute for zinc in certain enzymes (Vallee and Ulmer, 1972; Hsu and gyo, 2002). Dainelson and Denker (1984) reported that Zn transport in placenta is disturbed by cadmium administration, suggesting cadmium uses same zinc transporter. There are reports suggesting that these metals enter the cells through sulfahydryl sensitive group (Gerson and Shaikh, 1982; Chao et al., 1984). It is also demonstrated that cadmium exposure in *vitro* affects the calcium active transport in trophoblastic cell line (Lin et al., 1997). Evans et al. (2003) reported that lead enters Rcho-1 trophoblastic cells by calcium transport mechanisms and complexes with cytosolic calcium-binding proteins. There are reports that demonstrate that both lead and cadmium can enter through calcium dependent channels (Hinkle et al., 1987; Tomsig and Suszkiw, 1991).

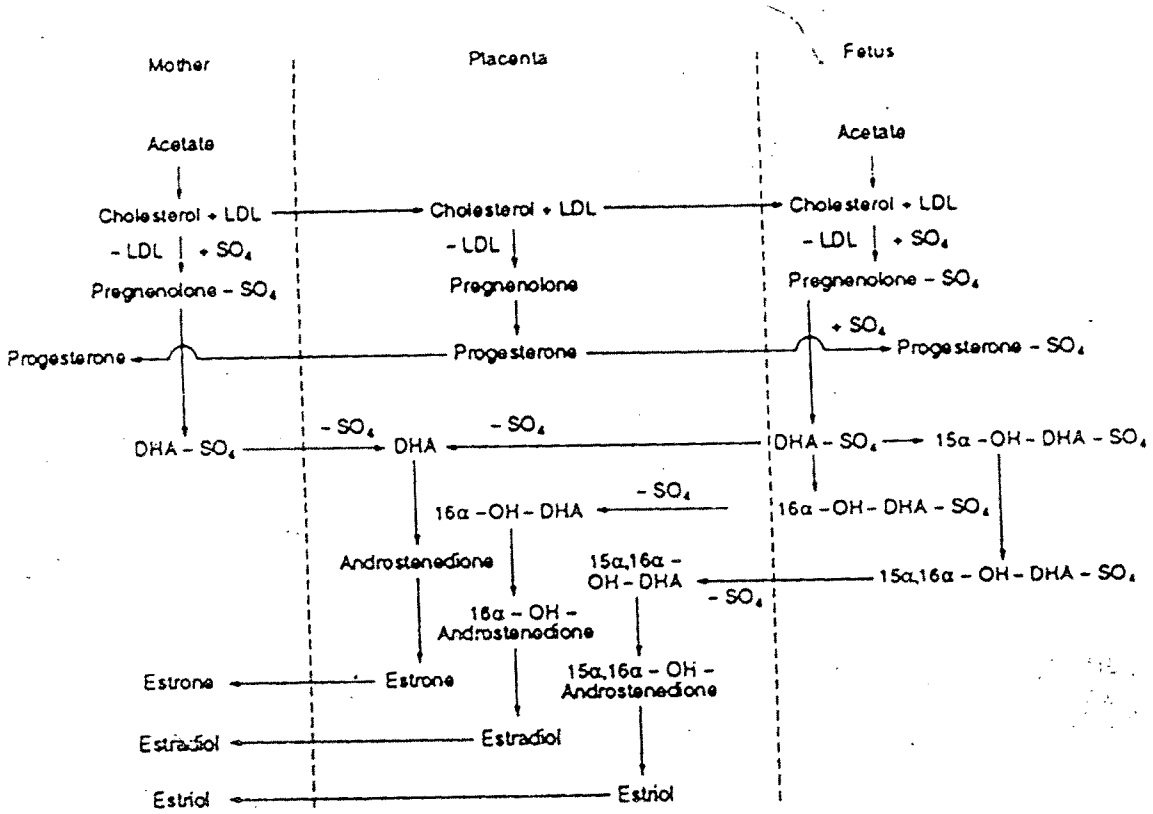
Placental steroidogenesis

Placental steroidogenesis is a process by which placenta form the steroid hormones, estrogen and progesterone (figures 16,17). Estrogen synthesis requires functional communication between the fetal adrenal gland and the placenta. The principal site of estrogen and progesterone synthesis is synchytiotrophoblast of the placenta that helps in preparation of uterus for implantation and maintenance of pregnancy thereafter (Pepe and Albercht, 1985). Progesterone has many functions like anti-inflammatory and immunosuppressive properties, which protects the conceptus from immunologic rejection by the mother. Estrogen and progesterone along with other hormones like prolactin are known to play an important function in mammary gland development for lactation. Estrogen increases blood flow within the uterus and placenta and regulates key components of maternal cardiovascular system to meet the physiological demands of the developing fetus.

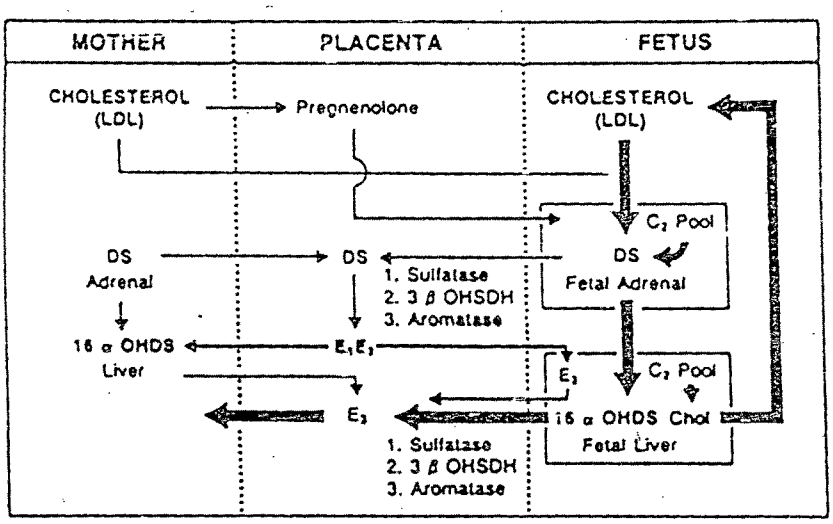
Biosynthesis of estrogen during pregnancy

The three main estrogens playing an important role in gestation are: estrone, estradiol and estriol. The steroid pathway is represented in figure. The rat placenta contains high amount of 3β hydroxy steroid dehydrogenase activity (3β HSD) (Wiener, 1974). Chan and Leathem (1975) showed that placenta homogenate contained androgen and testosterone; thereby implying that rat placenta can convert C_{19} steroids and serves as a source of estrogen production. It is also known that Dihydroepiandrosterone Sulfate (DHEAS) is a precursor for estrogen biosynthesis which is provided by fetal adrenal. Major enzymes involved in steroid biosynthesis are Δ^5 3βHydroxy steroid Dehydrogenase/ Δ^{5-4} isomerase (3 βHSD); P450 Aromatase, 17 β Hydroxy steroid

Figure 15b:



The steroidogenic pathways of the maternal–placental–fetal unit.



Sources of estrogen biosynthesis in the maternal–fetal–placental unit. LDL, low-density lipoprotein; Chol, cholesterol; pool, carbon–carbon unit; DS, dehydroepiandrosterone sulfate; E_1, estrone; E_2, estradiol-17β; E_3, estriol lapted from B. R. Carr and N. F. Gant, The endocrinology of pregnancy-induced hypertension, *Clin. Perinatol.* 10, 737, 1983).

Dehydrogenase (17β HSD); DHEA-sulfatase, 16 α Hydroxylase. DHEA-sulfatase acts on DHEA-sulfate to give free DHEA, which is passed from maternal and fetal unit. The DHEA can also be synthesized *denovo* in the placenta. DHEA is then acted upon by the 3βHSD and aromatase to yield estrone, which is then converted into estradiol by 17 βHSD. DHEAS is also hydroxylated by 16 α Hydroxylase to form 16-hydroxy DHEAS in the fetal liver, which is transported into the placenta and then catalyzed by the 3βHSD, aromatase and 17β HSD to yield estriol. Estrone and estriol is extensively interconverted from estradiol in the placenta. There exists an unequal distribution of placental secretion of estrogens in fetal- maternal unit. Estradiol is principally seen in maternal secretion whereas estrone is seen in the feral circulation. Aromatase is enzyme complex belonging to class of cytochrome P-450 enzymes. Enzyme complex of aromatase has cytochrome P-450 reductase, ubiquitous protein that transfers reducing equivalents from NADPH to the P450arom. In the process of aromatization, 1 mol of C_{19}, 3 molecules of molecular oxygen and 3 molecules of NADPH are utilized. The gene encoding P450 arom is CYP19. The gene contains nine exons and two polyadenylation sites in the last coding exon down stream from terminating stop codon, which give rise to two 3.4, 2.9 Kb transcripts. The gene utilizes two alternate promoters in the regulation of tissue specific expression within the placenta and the ovary. Some *in vivo* and short term studies have shown that rat placenta has the capacity to produce androgen from day 11 of pregnancy and the level of placental secretion progressively rises until the day 18 and it declines before parturition (Matt and Macdonald, 1984; Warshaw et al., 1985).

Biosynthesis of progesterone during pregnancy

The site of progesterone synthesis in the placenta is the basal zone. Placenta can produce the progesterone, as there is abundant expression of P450 side chain cleavage enzyme (P450scc) and 3β HSD enzymes. There is no fetus requirement for progesterone synthesis during gestation. Placenta has a limited capacity of synthesizing progesterone form cholesterol. In primary cultures of human trophoblasts, it has been shown that cells can take up LDL cholesterol via saturable process, and LDL degradation occurs as a consequence of LDL internalization by LDL receptors. Progesterone production requires the key enzyme P450scc, which is located in the inner mitochondrial membrane. This enzyme functions as a terminal oxidase in mitochondrial ETC. Electrons from the NADPH are accepted by an adrenodoxin reductase flavoprotein, which then donates it to adrenodoxin and finally transported to cytochrome P450 scc. The other key enzyme is 3 β HSD, which converts pregnenolone to progesterone, DHEA to androstenedione belongs to class of short chain dehydrogenase. Placental 3β HSD is of 41kDa protein with both dehydrogenase and isomerase activities that are inseparable. The enzymes -P450scc and P450 17α appear to be differentially regulated by LH. Placenta of rat does not express both P450 scc and P450 17 during the first part of pregnancy (Warshaw et al., 1985; Matt and Macdonald, 1984; Sridaran et al., 1981) and when circulating LH are elevated (Bogovich et al., 1981; Morshige et al., 1973). Progesterone levels in circulation are elevated between days 12 to 18 of pregnancy and decrease thereafter (Morshige et al., 1973). Richards and Kershey (1979) showed that aromatizing capacity of non-luteal cells or the granulosa cells is depressed until day 18 of pregnancy and increases dramatically after day 19. Progesterone is responsible for both inhibition of estrogen synthesis within

the follicle and the inhibition of follicular development between days 12 to 19 of pregnancy. There are also studies (Gibori et al., 1981; Gibori et al., 1978) indicating that only aromatizable androgens have a stimulatory effect on the corpus luteum, indicating that androgen must be first converted into estrogen by corpus lutea in order to maintain lutea cell function. Therefore, availability of an aromatase system in the luteal cell is crucial for the corpus luteum to secrete progesterone and maintain the live fetuses. Theca cell and the interstitial cells lack the aromatase enzyme systems during pregnancy, thereby indicating aromatase activity found in the non-luteal tissue is due to presence of aromatase in granulosa cells (Sridaran and Gibori, 1981). Gibori et al. (1982) showed that nonluteal cells are capable of producing more androgenic substance than luteal cells until the gestation day 19, where the aromatase enzyme system in corpus lutea is much more active than that in the non luteal tissue.

REPRODUCTIVE TOXICITY

Reproduction is a major aspect of human life. Reproductive toxicity can be defined as " The occurrence of biologically adverse effects on the reproductive system that may result from exposure to environmental agents". Effects on reproductive health due to occupational and environmental exposure are not well understood. Scientific epidemiological and toxicological data concerning the reproductive and developmental health risks of many chemicals, physical agents and environmental agents are limited. Toxicity may be expressed as alterations in reproductive organs, related endocrine system or pregnancy outcomes. The manifestation of such toxicity may include but not to be limited to adverse effects on onset of puberty, gamete production, transport, reproductive system normality, sexual behavior, gestation, parturition, lactation, development toxicity,

premature reproductive senescence or modification in other function that are dependent on integrity of the reproductive system. Developmental toxicity can be defined as the "occurrence of adverse effects on the developing organism that may result from exposure before conception during prenatal development, or postnatal till the time of sexual maturation ". Reproductive toxic effects have unique characteristics, which are not limited to single organ system and the target of toxicity may include either parent or offspring and manifested in different forms: a) exposure to reproductive toxicant in male or female may show its effect on the fetus, or b) Infertility may be inevident until children reach their puberty, or c) Normal reproductive function is only expressed intermittently.

REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF LEAD AND CADMIUM

Male reproductive system

Epidemiological studies

Reproductive defects associated with lead poisoning have been reported since classical times (Bell and Thomas, 1980). A study among the cohort battery workers having a mean lead level of 61 μg/dl demonstrated lower sperm counts as compared to the sperm count of the control having mean blood lead level of 18 μg/dl (Assennato et al., 1987), without any change in serum testosterone and pituitary hormones. Ng et al. (1991) reported an overall significant increase in serum levels of LH and FSH in males exposed to lead (32 Pb μg/dl) as compared to unexposed males (8.3 μg/dl). Reduction in sperm counts due to lead exposure was more evident in individuals with specific genotypes of the enzyme ALAD (Alexander et al., 1996).

Table 12: Summary of lowest observed adverse effect levels (LOAELs) for lead induced reproductive effects in males.

Lead level (μg/dl)	Effects	Reference
30 to 40	Severe depression of sperm count	Alexander et al., 1996
40 to 50	Decreased function of prostrate	Telisman et al., 2000
60	Male infertility	Telisman et al., 2000

Cadmium is known to cause oligospermia and azoospermia in human. A population study in Singapore reports that Cd in seminal plasma is related to low semen volume (Xu et al., 1993). Blood Cd level was positively correlated to an increase in abnormal sperm morphology and serum testosterone level and decrease in sperm motility in exposed healthy males of Croatia region (Teliesman et al., 2000).

Animal Studies

Studies were carried out in animal models to understand the reproductive toxicity of both these toxicants. It was proposed that lead can act both at the level of the hypothalamic-pituitary unit and directly at the gonadal sites. In rat models, different effects of toxicity have been reported including altered circulating levels of testosterone, variable effects on pituitary and serum concentrations of LH, decreased LH binding sites in the testis, and decreased sperm counts (Sokol et al., 1985; Sokol and Berman, 1991). Lead exposure is also reported to cause testicular damage and inhibit spermatogenesis (Hilderberg et al., 1973, Eyden et al., 1981). Contradictory results were reported with adult male rats exposed to lead under various experimental conditions where plasma or

serum testosterone levels remained, either unchanged (Nathan et al. 1992; Pinon-Lattaillade et al., 1993) or decreased (Sokol and Berman, 1991). At the level of pituitary, dose dependent elevation in pituitary LH level following lead exposure was observed (Klein et al., 1994). However, in contrast to the above study, no consistent elevation was observed in pituitary LH β mRNA (Ronis et al. 1996).

In rodents, severe testicular necrosis and sterility has been reported at the dose of 1mg of Cd / kg. b. Wt (CopiusPeereboom, 1981). Absence of spermiation (6 mg Cd / kg. b.wt) and depression in both sperm and steroid production (0.18 to 0.34 mg Cd / kg. b.wt) without any morphological changes (Hew et al., 1993; Laskey et al., 1991) occurs at lower doses when administered to rats. Zenick et al. (1982) reported that male rats receiving 10 mg of Cd/Kg.b.wt orally for 6 weeks daily had significant decrease on sperm production as well as on the alkaline phosphatase and lactate dehydrogenase activity, FSH and LH levels showed no difference while testosterone level showed a decrease in treated rats compared to control.

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). He had 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. Dose dependent decrease in testosterone production was reported in lead exposed leydig cells (Thoreux-Manlay et

al., 1995a) . Liu et al. (2001) demonstrated that lead directly inhibited steroidogenesis by decreasing StAR protein expression and the activities of P450scc and 3 beta-HSD enzymes with dose response trend in MA-10 mouse leydig cells. Ng and Liu (1990) 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.

Female reproductive system

Epidemiological studies

Lead

Several workers have reported that lead exposed industrial workers have higher blood lead level (Al-Neamy et al., 2001; Hwang et al, 2000; 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 impairment (Hu, 1991).

Cadmium

There is 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).

Animal studies

Lead

Experimental studies in animals indicate that lead is able to produce irregular estrous cycle (Lach and Srebro, 1972) in mice and decreased the frequency of pregnancy in mice and rats (Schroeder and Mitchner, 1971). Female monkeys exposed to lead for a longer period showed more variable menstrual cycles, shorter menstrual flows and suppressed luteal flow (Franks et al., 1989). Female physiology is under the control of Hypothalamic-Hypophyseal –Gonadal axis. The pituitary hormones regulates the ovarian cycle in adult females. *In vitro* lead exposure causes a change in morphology of the granulosa cells and decreased progesterone content (Paksy et al., 2001). It has been reported that lead exposure through drinking water, either prior to pregnancy or during gestation and lactation, caused an alteration in steroidogenesis, with significant change in ovarian gonadotropins (FSH, LH) binding (Wiebe et al., 1988).

Table 13: Summary of lowest observed adverse effect levels (LOAELs) for lead induced reproductive effects in females.

Lead level (μg/dl)	Effects
300	Irregularity of the estrus cycle
500	Persistant vaginal estrus
>500	Development of ovarian follicular cysts with the reduction in number of corpus lutea

Cadmium

There are numerous animal studies where the biochemical effects of cadmium are studied using various animal models. There are reports which demonstrate that cadmium accumulation occurs in the thecal layer of the ovary in golden hamsters as well as in non-luteal ovarian tissue of pseudopreganant rats (Donker, 1975; Paksy et al., 1990). The parenteral administration of Cd salts (1mg/kg.b.wt) to adult female rats does not result in sterility (Copius Peereboom et al., 1981). Baranski and Sitarek (1987) reported that female rats when exposed to cadmium (0.04 to 40 mg Cd/Kg/ day) for 14 weeks (5 days per week), had an increase in mean duration of estrus cycle, by lengthening diestrus stage after 6 weeks of treatment and was more significant in those females receiving 40 mg Cd/Kg. A single s.c. injection of 1 to 6 mg Cd/Kg. b.wt caused hemorrhage and necrosis in the ovaries of hypophysectomised, immature or nonovulating rats in persistent estrus (Rehm and Waakles, 1988; Kar et al., 1959; Copius Peereboom-Stageman and Jongstra-Spaapen, 1979). In mature cycling animals ovarian hemorrhagic necrosis was seen within 24 hour after administration of 1 to 3 mg Cd/ kg.b.wt in hamsters, mice and in some strains of rats (Rehm and Waakles, 1988; Saksena and Salmonsen, 1983. A single dose of Cd administered in diestrus or proestrous stage (3.0-7.5 mg/kg. b.wt S.C.)

caused a reduction in serum progesterone (P4) concentrations within 20-48 hours after exposure (Paksy et al., 1992; Paksy et al., 1990). He (1989) also reported that Cd given to rats at diestrus stage as a single s.c. injection inhibited ovulation in 50% of animals with lowered proestrous LH levels. Perturbations in serum concentrations of steroid hormones due to cadmium exposure has been reported in hamsters (Saksena and Salmonsen, 1983). Several findings have shown that cadmium affects hypothalamic-pituitary axis. Acute Cd exposure (5,7.5 mg Cd / kg.b.wt S.C) in rats given in diestrus caused temporary anovulation, which was preventable by simultaneous luteinizing hormone releasing hormone (LHRH) stimulation (Paksy et al., 1989). He also demonstrated that serum LH and serum FSH levels were decreased in proestrous rats after 24 hours of cadmium treatment. Piasek et al. (1996) reported a reduction in concentration of serum estradiol and serum progesterone in proestrous rats which received 3 or 5 mg of Cd / kg. body.wt.

There are many studies with " *in vitro*" cadmium exposure on ovarian cells, to understand the toxic effects. I*n 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). He later (1997) demonstrated that 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.

EFFECTS OF LEAD AND CADMIUM DURING PREGNANCY AND DEVELOPMENT

Epidemiological Studies

Lead

Several studies indicates that lead affects the physiological state of pregnancy. Several studies in Europe reported that greater fetal lead level than maternal lead level in occupationally exposed pregnant females (Romero et al., 1990; Truska et al., 1989; Wibbereley et al., 2000). There are reports which suggests a positive correlation of lead content between maternal and umbilical cord blood and lesser degree of correlation between lead content of maternal blood and breast milk in non-occupational exposed females (Ong, 1985; Mc Michael et al., 1986). Andrews et al. (1994) reviewed the available epidemiological studies on prenatal lead exposure in relation to premature rupture of membranes (PROM), preterm delivery, gestational age, low birth weight and mean birth weight adjusted to gestational age

Cadmium

Cadmium is known to accumulate in placenta and exert the toxic effects on the growing fetus. Epidemiological studies from regions around the world have indicated that Cd is accumulated in placenta with occupationally exposed women and smokers (Milnerowicz et al., 2000; Boadi et al., 1992; Peereboom-Stageman et al., 1983). Cadmium exposure during gestation causes morphological changes in the placenta (Cho, 1989; Hazelhoff Roelfzema et al., 1988). Salpietro et al. (2002) found that birth weight is inversely correlated with maternal and cord blood cadmium concentrations. Epidemiological data have revealed that women who smoke cigarettes are estrogen deficient (Bernstein et al., 1989; Baron et al., 1990) and have significantly decreased

concentration of serum estradiol, human chorionic gonadotropins and sex hormone binding globulin in early pregnancy.

Animal Studies

Lead

The effects implicated in lead toxicity have been studied using animal models. Toxic effect caused by lead is due to its ready passage across the placental-fetal barrier (Goyer, 1990; Pinon-Lattaillade et al., 1995), causing a direct relationship between lead exposed mother and the possibility for irreversible effects to her offspring (Rom, 1976). It is reported that lead is excreted into the milk during lactation and as a result nursing offspring is exposed to lead (Hallen et al., 1995), which leads to several defects during development. Several studies had shown developmental effects such as delayed vaginal opening, delayed onset of puberty in experimental animals when they were exposed to lead during gestation and lactation (Dearth et al., 2002; Ronis et al., 1996, Mc Giveren et al., 1991). Reduced birth weights, stillbirths associated with in *utero* lead exposure were also reported (Ronis et al., 1996). Lead is easily permeable through placental membrane and adversely affect the fetal viability and fetal development (ASTDR, 1999). Nayak et al. (1989) demonstrated that Swiss Webster mice exposed to lead from gestation day 9 had increase in rate of resorption of the fetus and reduced placental weight.

Hammond et al. (1993, 1990,1989) demonstrated lead induced depression of growth in weanling female Sprague-Dawley female rats, following exposure to 250 ppm of lead in the drinking water. A significant drop in circulating LH levels and 50% drop in estradiol concentrations along with complete disruption of estrus cyclicity, without any change in LH β mRNA levels has been reported (*in utero)* lead exposed females (Ronis

et al., 1996). It is known that IGF-1 acts as a metabolite signal for LHRH release during puberty (Hiney et al., 1996). It is proposed that lead exposure during gestation, lactation or at both times can decrease IGF-1 levels that could mediate the delayed puberty (Dearth et al., 2002).

Lead is known to get accumulated in the uterus and cause several deleterious effects (Hangfelt et al., 1977). A study by Standevan et al. (1994) demonstrated that chronic exposure of female B6C3F1 mice to high concentration of unleaded gasoline induces uterine changes. Mice treated with lead chloride intravenously had lesser number of uterine estradiol binding affinities compared to the control (Wiebe et al., 1988). Administration of lead to pre-implantation mice resulted in increased metabolic activity of their blastocysts (Nilsson et al., 1991). It was demonstrated that i.v. injection (75 ppm) of lead chloride on day 4 of pregnancy in mice, caused a failure in implantation, by causing an interference in activity of ovarian steroid hormones on the endometrium (Wide and Nilsson, 1979). Along with these, he also observed an increase in sister chromatid exchange in bone marrow cells, decrease in nuclear organizing regions and deletion in fetal and maternal bone marrow aberrations (Wide and Nilsson, 1979).

It has been demonstrated that rats when exposed to lead (0 to 500 mg/L) through water throughout pregnancy, caused a decrease in brain weight, cell growth, cell division, total protein with no change in placental weights, kidney weight (Dilts and Ahokas, 1980). Lead acetate (300 mg/L) administered through drinking water throughout gestation caused a decrease in DNA and RNA contents of male pups with an increase in proliferative activity, though gross testicular structure did not show any change (Corpas et al., 1995). Generation study done in lead exposed rats revealed that lead levels in milk

were 2 times higher than blood lead level and 6 times higher brain lead content (Hallen et al., 1995). Female rats receiving i.v. injection of radiolabelled Pb alone or in combination with lead nitrate (5 / 25 mg/kg) causes disruption of egg cylinder at 6 hour post injection of 25 mg/kg.b.wt at gestation day 9 and 100% mortality at gestation day 15 (Hackett et al., 1982). Lead is known to cross the blood brain barrier. Couple of studies have demonstrated that lead affects the neurotransmitter levels like Brain Norepinephrine, GABA and Acetylcholine levels (Singh and Ashraf, 1989; Gietzenet and Wooley, 1984).

Cadmium

Biological effects by cadmium exposure have been experimentally studied in various animal models. Cd is a known teratogen in mammals, when it is administered in early post implantation period (Denker, 1975; Gale and Horner, 1987), in mid pregnancy (Samawickrama, 1979) and late gestation (Parizek, 1983). Several reports suggest that that Cd accumulates after a single oral or parenteral administration with minimal concurrent fetal uptake (0.01% of the injected dose late in pregnancy (Levin et al., 1983; Webb and Samarawickrama, 1981; Ahokas and Diits, 1979). Paksy et al. (1992) reported that Cd given on day 1 of pregnancy does not cause any deleterious effect on oviductal transport, cleavage number and location of ova in rats during the preimplantation time. Preimplanted embryos incubated with cadmium chloride (1 µg/ml) caused inhibition in development of both 8-cell stage and morulae with shrunken and pyknotic cells (Abraham et al. 1986). Cadmium when given to rabbits for 1 to 7 days before mating; pregnancy was interrupted in 60-75% of the animals (Saksena et al., 1983).

Parenteral Cd administration (0.3 to 3.0 mg Cd/kg.b.wt) between 7 to 16 days of gestation in rodents induces various teratogenic effects, depending on the dose and

species strain specific (Samawarickrama and Webb, 1981; Gale and Layton, 1980; Chernoff, 1973). It has been observed that rats when exposed to Cd (0-100 ppm) through water from gestation day 6 to 20 demonstrated dose dependent accumulation of Cd and higher doses (50, 100 ppm) caused a decrease in fetal and maternal weights (Sorell and Graziano, 1990). It has been reported that Cd treatment through day 8 to 10 during gestation caused an increase in fetal deaths, increased resorption and decreased placental weight (Nayak et al., 1989). It has been observed that there are high incidences of placental necrosis and fetal death 96 hours after single SC injection of 5 mg Cd/ kg b wt on gestation day 15 (Piasek and Laskey, 1994). A study by Di'Sant Agnese et al. (1983) demonstrated that Cd injection(40μMmol/kg.) on day 18 of gestation caused fetal death, placental necrosis.

Paksy et al. (1992) found a delay in the rise of serum progesterone content in pregnant CFY rats (3 to 4 days into pregnancy) following Cd treatment (0.9, 1.8, 3.6 mg Cd/Kg. b.wt) with no effects on other reproductive parameters. Reductions in concentration serum estradiol and serum progesterone of pregnant rats on gestation day 8, 24 hours after treatment with 3 or 5 mg / kg.b.wt. (Piasek et al., 1996). She also (2002) reported that Cd administration (3 or 5 mg/ kg.b.wt s.c. as a single dose) interfered with steroidogenesis in early pregnancy, ovarian estradiol being affected the most.

An "*in vitro* "study by Jolibios Jr. (1999a) revealed that 72 hour exposure of Cd (5-20μM) to human trophoblastic cells, causes a decrease in release of progesterone and exposure to highest dose of cadmium (20μM) did not inhibit syncial formation. *In vitro* exposure of Cd (0.04 μM, 16μM) to JEG-3 cells caused an alteration in cell integrity and altered calcium transport at higher dose (Lin et al., 1997). Yu and Chan (1987) reported

Cd exposure "*in vitro*" caused a significant retardation of trophoblastic outgrowth. In studies on the "*in vitro*" isolated perfused human placental lobule, toxic effects of cadmium demonstrated dose related functional alterations in the production of hCG and zinc transfer followed by changes in placental morphology (Wier et al., 1990). Human placental explants with acute cadmium exposure at high concentrations caused imbalance between thromboxane A2 and prostacyclin, a disturbance seen in late gestation in preclampsia (Eisenmann and Miller, 1995).

Cadmium is known to accumulate in uterus (Bires et al., 1995, Massanyi et al., 1995). Sipowicz et al. (1995) demonstrated that cadmium (*in vitro)* inhibits spontaneous contractile activity of myometrium. Cadmium is known to cause difference in nuclear and nucleolar changes, cytoplasmic changes of uterus (Morselt et al., 1983; Peereboom-Stageman and Jongstra-Spapen, 1979). "*In vitro*" exposure to cadmium caused a inhibitory effect on 17β estradiol binding to its endometrial receptors (Young et al., 1977).

Cadmium is also known to affect the micronutrient status. A study by Sowa and Steibert (1985) revealed that cadmium (50 ppm) exposure to female dams caused a decrease in Zn, Cu and Fe status of the fetus as well as decreased Cu content in placenta, fetal intestine, brain and kidney. They also found a decrease in nuclear and cytoplasmic Zn content in subcellular fraction of fetal liver as well as a decrease in serum Zn, Fe and ceruloplasmin levels. Calcium supplementation during suckling period reduces oral Cd absorption in Cd (0.5 mg/kg.b.wt) treated rats (Saric et al., 2002). Studies have shown that exposure to Cd during gestation (gestation day 12) inhibits Zn transport system (Webb and Samarawickrama, 1981; Samarawickrama and Webb, 1979) and inhibition of

Zn dependent thymidine kinase (TK), thereby decreasing rate of DNA synthesis of the fetuses. A study by Chertok et al.(1984) reports that i.v. injection of Cd , increased the clearance of ^{67} Cu in pregnant guinea pigs, thus suggesting cadmium can compete with copper.

Exposure to cadmium is also known to affect the biochemical status. Studies have shown that Cd administration during gestation causes decrease in placental alkaline phosphatase activity (Itoh et al., 1996; Saillenfait et al., 1992) and a decrease in placental glycogen levels (Roelfzema et al., 1987) and gets concentrated around the embryo (Gutierrez correa et al., 1991). Cadmium (1 or 2 mg/ Kg. b.wt) given on 3, 10, 17 postnatal days, caused a decrease in non-esterified cholesterol (Gulati et al., 1986). Placental Metallothionein (MT) is a protein that is expressed in placenta during pregnancy in response to Cd injection (Breen et al., 1995). Basal levels of MT were increased already at gestation day 8 in rats with no treatment. Several studies have shown MT induction in response to Cd toxicity. The fetus is likely to be protected from cadmium exposure due to such an induction. Goyer and Cherian (1992) reported concentrations of metals and MT in placental tissue from 55 mothers of Canada, who stated that they were not smoking during pregnancy, but 16 were smokers in the past. He found a strong correlation between MT and Zn, Cu concentrations but a negative correlation with Cd and MT.

AIM AND OBJECTIVES OF THE PRESENT STUDY

Reproduction is a major aspect of human life. Environmental pollutants are known to cause deleterious effect on human health. Lead and cadmium are well known reproductive toxicants. Environmental and biological monitoring has clearly shown

increased concentration of these pollutants. National Institute of Occupational Health (NIOH) Ahmedabad had conducted a preliminary longitudal study in school children and have reported that low level lead could delay the sexual maturity in both boys and girls. In several urban cities high population of old vehicles along with congested traffic contributes to 90% of lead in the atmosphere (Samanta et al., 1995; Ramanthan 1999). A study by D'Souza et al. (2002) showed that school children (Age 4-12 yrs) in Bangalore city have strong correlation with lead content and zinc protophorphyrin content. Saxena et al. (1994) reported that maternal blood lead levels was higher in those who experienced abnormal deliveries in city of Lucknow. A recent study indicated that pregnant females in urban slums of Lucknow have elevated blood lead levels, associated with higher risk of low birth weight babies (Aswathi et al., 2002). Report from our lab has indicated that high level of lead in urban and suburban population of Vadodara compared to rural population (Gupta et al., 2001). Population study with cadmium exposure has shown increased level of cadmium in follicular fluid of smoker women (Piasek et al., 2001). Thus, population data suggests that lead and cadmium can cause reproductive dysfunctions.

Available data on animal studies in the literature suggests that metals demonstrate difference in sensitivity due to difference in species, sex, physiological status and dose of toxicants. Moreover, most of studies are dealt with single metal exposure. But, in reality, population is exposed simultaneously to more than one metal toxicant at low level. Net effect of such an exposure would contribute to additive, antagonistic or synergistic effect. There are few studies to understand the combined effect of lead and cadmium in various systems like immune system (Yucesoy et al., 1997) and male reproductive system (Der et

al., 1976). But, to best of our knowledge, there are no studies with simultaneous exposure to lead and cadmium on female reproductive system. A preliminary animal studies were carried out in our lab, where combined exposure to lead and cadmium even at level as low as 0.1 mg/kg.b.wt for 30 days in female rats, caused a significant inhibition of δ-Amino Levulinic Acid Dehydratase (δ-ALAD) activity, marker for lead and cadmium toxicity (Gupta et al., 1994). **In view of this and various deleterious effects caused by these metals on female reproductive function, it is worthwhile to study the effect of lead and cadmium either alone or in combination on ovarian function in rats. Attempts have also been made to understand the mechanism of interaction of these heavy metals at cellular level.**

Objectives

- To study the dose and time dependent effect of lead and cadmium in isolation and combination on ovarian function in both proestrous and estrous stages of estrus cycle.
- To study the effect of lead and cadmium in isolation and combination on reproductive performance and on biochemical parameters of placenta and ovary.
- To study the effect of lead and cadmium at cellular level using granulosa cell as the model.
- To understand the mechanism of lead and cadmium toxicity, in combination and in isolation.