EXPRESSION OF LEPTIN AND ITS RECEPTOR IN CORPUS LUTEUM DURING ESTROUS CYCLE IN BUFFALO (*BUBALUS BUBALIS)*

Thesis

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

in

VETERINARY PHYSIOLOGY

By

Dr. Lalit Kumar

Roll No. 4717

To

DEEMED UNIVERSITY

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Certified that the research work embodied in this thesis entitled **"Expression of Leptin and its Receptor in Corpus Luteum during Estrous Cycle in Buffalo (*Bubalus bubalis*)"** *submitted by* **Dr. Lalit Kumar, Roll No. 4717,** *for the award of* **Master of Veterinary Science** *degree in* **Veterinary Physiology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

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Date: **Lalit Kumar**

Abbreviations

%	Percentage
°C	Degree Celsius
A	Absorbance
Aa	Amino acid
AgRP-	Agouti-related peptide
Ang	Angiopoietin
ANOVA	Analysis of variance
BP	Base pairs
CART	Cocaine and amphetamine–regulated transcript peptide
CBB	Coomassie brilliant blue
cDNA	Complementary DNA
CL	Corpus luteum
CNS	Central nervous system
CP	Crossing point
CRH	Cytokine receptor homology module
Ct value	Threshold values
DAB	Diaminobenzidene tetrahydrochloride
DDW	Double distilled water
DEPC	Diethyl pyrocarbonate
DNA	Deoxy ribonucleic acid
dNTP's	Deoxynucleoside triphosphate
DW	Distilled Water
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
Etbr	Ethidium bromide
FSH	Follicle stimulating hormone
GC	Granulosa cell
GH	Growth hormone
GnRH	Gonadotropin releasing hormone
H_2O_2	Hydrogen peroxide
HRP	Horse radish peroxidase
IG	Immunoglobin
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IL-4	Interleukin 4

IRF	Interferon regulatory factor
JAK	Jenus Kinase
Kb	Kilo base
KDa	Kilo dalton
Kpa	Kilo Pascal
LH	Luteinizing hormone
M	Molar
MAPK	Mitogen-activated protein kinase
$MgCl_2$	Magnesium Chloride
ml	Mili litre
MM	Master mix
MMP	Matrix metalloproteases
Mol.Wt.	Molecular weight
mRNA	Messenger ribonucleic acid
ng	Nano gram
NO	Nitric oxide
NPY	Neuropeptide Y
NTC	No template control
Ob	Leptin
Ob	Obese gene
Ob-R	Leptin receptor
OD	Optical density
PA	Plasminogen activator
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PGF2a	Prostaglandin F 2 α
pH	Log Hydrogen ion concentration
PHG	Hypothalamus-pituitary-gonadal
pmol.	Pico mole
POMC	Pro-opiomelanocortin
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
SH2	Src homology 2
SREBP	Sterol regulatory element-binding proteins
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducer and activator of transcription
TAE	Tris Acetate EDTA buffer

Taq. Pol.	Thermus aquaticus Polymerase
TBE	Tris borate EDTA
TC	Theca cell
TIMP	Tissue inhibitors of metalloproteases
UV	Ultra violet
VEGF	Vascular endothelial growth factor
w/v	Weight/volume
µg	Microgram
µl	Micro liter

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Chapter I

Introduction

The domestic water buffalo, although derived from the wild water buffalo (*Bubalis arnee*), is the product of thousands of years of selective breeding in either South Asia or Southeast Asia. The water buffalo or domestic Asian water buffalo (*Bubalus bubalis*) is a large bovine animal. The classification of the water buffalo is uncertain. Some authorities list a single species, *Bubalus bubalis* with three subspecies, the river buffalo (*B. bubalis bubalis*) of South Asia, the carabao or swamp buffalo (*B. bubalis carabanesis*) of the Philippines and Southeast Asia, and the arni, or wild water buffalo (*B. bubalis arnee*).

Asia is the native home of the water buffalo, with 95% of the world population of water buffalo, with about half of the total in India. India has the largest livestock populations in the world. India has 57 percent of the world's buffalo population. In India the population of buffalo is one third as compared to cattle population, but contributes 55% of the total milk production.

The buffalo is a multipurpose animal. Not only is it a better source of milk than the cow, it also provides meat and works as a draught animal. Of all the domestic animals, the Asian buffalo holds the greatest promise and potential for production. It is well known that the buffalo is remarkable for its feed conversion ability. The production of buffalo milk in the Asian-Pacific region exceeds 45 million tonnes annually, of which over 30 million tonnes are produced in India alone. Buffaloes are laboured intensive and cost-effective. The domesticated water buffalo is often referred to as "the living tractor of the East", as it is relied upon for ploughing and transportation in many parts of Asia. They are the most versatile of all work animals in the variety of tasks, which they can be taught to undertake. All buffalo breeds have

a strong milk/meat entity. When a buffalo is fed well and managed for early slaughter (at a live weight of 350 to 450 kg), a yield of palatable, high-grade meat can be obtained at a competitive cost. The fat content of buffalo milk is the highest amongst farm animals and the butterfat is a major source of ghee in some Asian countries. The water buffalo has promise as a major source of meat, even the milking ones.

The water buffalo rumen has been found to contain a larger population of bacteria, particularly the cellulolytic bacteria, lower protozoa and higher fungi zoospores. In addition, higher rumen ammonia nitrogen (NH_3-N) and higher pH have been found as compared to those in cattle. Buffaloes are very efficient in the utilization of poor quality native roughages.

Good reproductive performance is critical for the efficient production of any livestock. Riverine buffaloes however, are sluggish breeders, beset with reproductive problems. A high percentage of buffalo cows (30–40%) experience a prolonged period of anestrous with the Indian farmers incurring an estimated loss of 19–20 million tonnes of milk each year due to this problem. Subestrous or silent estrous that has hormonal aetiology is perhaps the single largest factor responsible for poor reproductive efficiency in buffalo (Kanai and Shimizu, 1983; Prakash, 2002; Madan and Prakash, 2007). In buffalo reproduction, this is an important issue which has been the focus of many studies. Subestrous is more frequent in the early postpartum period, during the humid and low breeding seasons and also in underfed buffaloes, suckled buffaloes and hot season calvers. Persistent corpus luteum is associated in buffaloes with anoestrous and in as high as 45% of cases with endometritis. Inactive or non-functional ovaries are one of the most important causes of anoestrous and poor reproduction performance in buffaloes. Understanding of the endocrine factors that control estrus, are fundamental for the construction of strategies aimed at improving the detection of estrus and fertility improvement. With endocrine interventions, it might be possible to improve the fertility of these animals.

The ovarian cycle in mammals is central to reproduction and it is characterized by repeated patterns of cellular proliferation, differentiation and transformation that accompany follicular development and the formation and regression of the corpus luteum (CL). Pituitary derived gonadotropins and growth hormone are the primary regulator of final follicular maturation and CL function. However, it is also evident from several reports that locally produced factors such as steroid hormones, peptides and growth factors have essential modulatory role in follicular

development (recruitment, selection and dominance), ovulation and CL formation, function, development and regression (Fortune, 1988 and Berisha and Schams, 2005). These locally produced compounds constitute a complex intra-ovarian autocrine, paracrine and juxtacrine regulatory systems.

Leptin, a 16.4 kDa peptide hormone, product of the obese gene, is secreted primarily in adipocytes and is known to play a critical role in the regulation of body weight by inhibiting food intake and stimulating energy expenditure (Zhang *et al*., 1994). Apart from their role in the regulation of body weight and energy expenditure, evidence suggests that leptin also plays an important role in reproduction. Its role in reproduction includes important actions on the hypothalamus to bring about release of LH-releasing hormone, thereby triggering gonadotropin release and leading to development of the reproductive tract and induction of puberty (Caro *et al*., 1996). Administration of leptin to obese leptin-deficient mutant mice caused decreased food intake, body weight loss, increased ovarian weight, increase in ovarian follicles and restoration of fertility (Barash *et al*., 1996; Chehab *et al*., 1996 and Kikuchi *et al*., 1999).

Leptin signalling is accomplished via receptors which have six isoforms with sequence homology placing it in the Class I cytokine family (Tartaglia *et al*., 1995). Signalling of leptin receptors is brought about through JAK-STAT pathway. Leptin dose dependently modulate SREBP 1 & StAR transcription and in turn steroidogenesis in cells. Leptin receptors have been found in the hypothalamic center responsible for satiety (Tartaglia *et al*., 1995). In addition, leptin receptors exhibit widespread distribution in mammalian tissue, including liver, heart, kidney, lung, small intestine, testes, ovaries, spleen, pancreas and adipose tissues (Lee *et al*., 1996).

Although the general view has been that the principal effects of leptin are on the neuroendocrine component of reproduction (Chehab *et al*., 1996), evidence has emerged to indicate direct involvement of leptin in ovarian function. The expression of leptin receptors has been demonstrated in human, mouse, rat, pig and bovine ovaries (Karlsson *et al*., 1997; Kikuchi *et al*., 1999; Duggal *et al*., 2000; Ruiz-Corte's *et al*., 2000; Nicklin *et al*., 2007 and Sarkar *et al.*, 2009). Additionally, there are reports which indicate that reproductive dysfunction in aging obese mice is related to modified intra-ovarian leptin gene expression that is related to acquired obesity and declining fertility in this species and is related to progressive hyperleptinemia

and leptin resistance (Brannian *et al*., 2005, 2009). Given its positive effects on gonadotropin secretion and fertility, leptin is expected to have either a positive local effect or no effect at all. The majority of researchers have suggested that the direct effects of leptin on ovarian cells are inhibitory and can be attributed to attenuation of gonadotropin, insulin, insulin-like growth factor 1 (IGF-I) and/or glucocorticoid-mediated steroidogenesis (Spicer and Francisco, 1997, 1998; Zachow and Magoffin, 1997; Zachow *et al*., 1999; Duggal *et al*., 2000; Spicer *et al*., 2000; Ghizzoni *et al*., 2001 and Guo *et al*., 2001). Contrasting studies have revealed direct stimulatory effects of leptin in rat and human ovaries in the form of induction of angiogenesis and proliferation of ovarian cells (Spicer and Francisco, 1997 and Bouloumie *et al*., 1998) and more specifically progesterone production from the bovine corpus luteum (CL) (Nicklin *et al*., 2007). Moreover, the presence and turnover of mRNA and protein for leptin have been described in human granulosa and cumulus cells and the presence of leptin in mature human oocytes (Cioffi *et al*., 1997) and bovine ovaries (Sarkar *et al*., 2009).

Infertility in animals may be caused by a defective corpus luteum (CL), which can be attributed, in part, to incomplete vascularisation (angiogenesis) of the corpus luteum, causing a decrease in progesterone production. The angiogenic process is regulated by proangiogenic factors including vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1) and fibroblast growth factor 2 (FGF-2) that are expressed at different times during the luteal phase. Nevertheless, it is known that leptin induces angiogenesis (Bouloumie *et al*., 1998) and this may be reflected in the correlation between the luteinisation and leptin and leptin receptor in the bovine ovary. Leptin, a potent satiety hormone that influences the gene expression of some of these angiogenic hormones in non-ovarian tissues, has also been identified in the porcine and caprine corpus luteum. Therefore, leptin regulates the production of VEGF, Ang-1, and FGF-2 in developing luteal tissue and ultimately corpus luteum formation and progesterone production (Robin *et al*., 2009 and Jessica *et al*., 2008).

The expression of the leptin and its receptor in ovarian cells of many species suggest that leptin plays a role in an autocrine/paracrine fashion and take part in important processes concerning reproduction.

Given their role in controlling ovarian function, leptin and their receptors are hypothesized to be involved in corpus luteum formation, function and development during estrous cycle in an

autocrine / paracrine manner in buffalo. Hence, keeping in view of these facts, the present project has been formulated with the following objective.

- **To demonstrate the mRNA expression of leptin and its receptor (OB-Rb) in corpus luteum during different days of estrous cycle in buffalo.**

Chapter II

Review of Literature

2.1. Regulation of the oestrous cycle: Role of gonadotropins and locally produced factors in the ovary

Control of the oestrous cycle in dairy buffalo plays a central role in herd management of dairy farms. A high gravidity rate and short calving intervals assure the profitability of these farms. The exact knowledge of the physiological processes during oestrous cycle and gravidity is required to understand and treat pathological events correctly.

Each buffalo oestrous cycle (21 days) shows mostly three follicular waves (Evans and Fortune, 1997). Each wave contains the recruitment of a follicle cohort and the selection of one dominant follicle which continues growing whereas all others go through atresia (Stock and Fortune, 1993). Dominant follicles control the development of other follicles through hormones (like oestradiol, inhibin), which act locally as well as systemic (Savio *et al.* 1993). Follicle growth is promoted by two types of gonadotropins, which are secreted from the anterior pituitary as a reaction to pulsatile GnRH release from the hypothalamus (Roche 1996). These two types are the follicle stimulating hormone (FSH) and the luteinizing hormone (LH), both of them sequestered within the whole oestrous cycle. Naturally only the dominant follicle of the last wave comes to ovulation (Ginther *et al.* 1989). Ovulation of the Graafian follicle is the release of the oocyte. Compulsory for this impact is the LH peak, which arises through an increase of frequency and amplitude of the LH pulses (Piquette *et al.* 1991) triggered by the pulsatile release of oestradiol-17ß.

The above mentioned LH peak is also responsible for the formation of a temporary endocrine gland, the CL, which is built from the remaining granulosa and theca interna cells

after ovulation. This formation starts with the folding of the granulosa cell layer, its infiltration by theca cells and its vascularisation. During this process, also a variety of other cell types, such as pericytes, fibroblasts and macrophages enter the granulosa. The rapid angiogenesis in the early CL development provides a tense capillary network. Parallel occurs in the luteinisation of the theca and granulosa cells, which is marked through the inclusion of lipochroms and an increase in volume and synthesis rate. Cells of the follicle granulosa tissue develop into large luteal cells, while former theca cells become small luteal cells. These cubic cells synthesize steroid hormones, mostly progesterone. The preparation of the uterus mucosa for the implantation of the embryo as well as the maintenance of gravidity is the main functions of progesterone. Progesterone has a gonadotropin inhibiting effect; more precisely it decreases the pulse frequency. Thus, its inhibiting function aims at the LH peak which means that ovulation only can take place in the absence of a CL and progesterone secretion. Small luteal cells produce less progesterone, but are present in greater numbers than large luteal cells. If no fertilisation takes place, it is essential for the introduction of a new oestrous cycle, the CL regresses. Thus, the uterus releases prostaglandin PGF2a around day 16. PGF2a acts as physiological luteolysin and causes cellular events that lead to luteolysis and decreased steroidogenesis.

The life cycle of the CL involves several strictly regulated proteolytic processes that take place within the same CL during a relatively short period of time, including angiogenesis, tissue remodelling (Smith *et al.,* 1994) and tissue degradation (Kiya *et al.,* 1999). The intracellular signalling events that lead to structural regression of luteal tissue are poorly characterised. However, proteases (Curry and Osteen, 2003; Liu *et al.,* 1997) and apoptosis seem to play central roles in development and regression of the CL in several species (Bacci *et al.,* 1996; Yadav *et al.,* 2002). Locally produced vascular endothelial growth factors (VEGFs) are glycoproteins which induce angiogenesis. They are potent mitogens for endothelial cells (Ferrara and Henzel, 1989) and enhance the vascular permeability (Senger *et al.* 1986).

Matrix metalloproteases (MMPs), their tissue inhibitors (TIMPs) and the plasminogen activator system seem to play a critical role in development and regression of the CL. Degradation of specific extracellular matrix (ECM) components occurs by the action of MMPs (Woessner, 2002). All these processes occur during development of the CL, while during

luteolysis degradation of the different collagen components is necessary to enable the rapid regression of the CL. Activity of MMPs is strictly regulated by tissue inhibitors of metalloproteases (TIMPs). Also an important role in degradation of the ovarian ECM plays the plasminogen activator (PA) system (Smith *et al.,* 1999). It consists of plasminogen, the inactive form of plasmin. Plasmin is not only able to degenerate specific ECM components like types III and IV collagen, fibronectin, laminin and proteoglycans (Roldan *et al.*, 1990; Barnathan *et al.,* 1990), but also activates proenzyme forms of MMP- 1, MMP-2 (Monea *et al.*, 2002) MMP-3 and MMP-9 (DeClerck and Laug, 1996; Lijnen *et al.*, 1998; Makowski and Ramsby, 1998; Murphy *et al.*, 1999). Working hand in hand together the MMPs and the plasminogen activator system are able to degrade all ECM components in the ovary (Dow *et al.*, 2002).

However, in the ovary, the primary mediators of angiogenesis are regulated by gonadotrophin hormones, LH and FSH. Angiogenic factors include hypoxia-inducible factor (HIF), VEGF, and angiopoietins (Ang-1 and Ang-2). Other factors that are of lesser importance include endocrine gland vascular endothelial growth factor (VEGF), nitric oxide, and leptin.

Among all above mentioned locally produced factors in the ovary, leptin is notable one, as it has diverse role not only in ovary in the form of formation, function (steroidogenesis) and regression of CL but also act centrally on PHG axis and other tissues in the body which can affect reproduction and production potential of animal. Leptin is wonder molecule that answers the possible link between reproduction and nutrition. Future interventional studies involving leptin administration are expected to further clarify the role of leptin and may provide new therapeutic options for the reproductive dysfunction.

2.2. Leptin

The leptin story began in 1950, when researchers at Jackson Laboratory in Bar harbour, Maine, USA, noticed that they had a strain of mice that were obese, lethargic, insulin resistant and constantly hungry. This is called ob/ob mouse. The scientific explanation finally emerged in 1994, when Friedman and his colleagues published a landmark paper in the Nature, in which they identified a gene in mice and humans called obese (*ob*) that codes for a hormone 'he' later named leptin, after the Greek word leptos, for *thin*. It achieved hormonal status by virtue of its secretion into the bloodstream (Caro *et al*., 1996).

Friedman and colleagues showed that leptin is a hormonal signal made by the body's fat cells that regulates food intake and energy expenditure. When they gave the obese mice leptin supplements, the animal lost weight, became more active and began responding to insulin. Leptin has powerful effects on reproduction, metabolism, other endocrine systems and even immune function.

2.2.1. SITES OF PRODUCTION

White adipose tissue is the major source of leptin. It is also produced by brown adipose tissue, placenta (syncytiotrophoblasts), ovaries, skeletal muscle, stomach (lower part of fundic glands), mammary epithelial cells, bone marrow, pituitary and liver.

2.2.2. STRUCTURE OF LEPTIN

Leptin is a secreted protein that is encoded by the *ob* gene. It is a 16.4 KDa non-glycosylated polypeptide of 146 amino acids discovered in 1994 by Zhang *et al.* The precursor form of leptin contains 167 amino acids and is activated by the cleavage of a 21 amino acid residue (Zhang *et al*., 1994 and Ogawa *et al*., 1995). Leptin has similar structure with the family of helical cytokines that also includes interleukin (IL)-2 and growth hormone (Madej *et al*., 1995).

Further investigations with nuclear magnetic resonance analysis have revealed that it is a four-helix bundle (Fig. 1). Helix length and disulphide patterns suggest that leptin is a member of short helix cytokine family (Kline *et al.,* 1997). As described by Zhang *et al.,* the alpha-helices of leptin are very similar. The four helices can be superimposed upon each other with a high degree of sequence and structural similarity.

It is also important to note that the sequence of amino acids is highly conserved among species. There is a 67% amino acid sequence identity among such diverse species as human, gorilla, chimpanzee, orangutan, rhesus monkey, dog, cow, pig, rat and mouse (Zhang *et al.,* 1997). This sequence similarity suggests that leptin is an important protein required for regulation of fat.

2.3. LEPTIN RECEPTORS

Leptin acts on target cells after binding to the specific receptors. It is the product of *db* gene. The first leptin receptor (OB-R) has been cloned in the mouse (Tartaglia *et al*., 1995) and until now at least 6 isoforms have been identified as multiple alternative splice variants with distinct signalling functions (Tartaglia *et al*., 1997).

2.3.1. Structure and isoforms

The OB-R as a single-pass membrane receptor exhibits a structural similarity with the class I cytokine receptor family, which includes receptors for interleukin 6, leukemia inhibitory factor or granulocyte colony-stimulating factor. It has three domains *viz*. extracellular, transmembrane and intracellular (Fig. 2). Generally three major classes of OB-R can be distinguished: long (OB-Rb), short (OB-Ra, -Rc, -Rd, -Rf) and soluble (OB-Re). All isoforms characterize identical extracellular domain consisting of 816 amino acids but with differing intracellular domain. The different lengths of intracellular part, approximately 300 amino acids for long isoforms and more than 30 amino acids for short isoforms, generally distinguish leptin receptor forms. The short isoforms have a truncated intracellular domain and are generally considered to lack signalling capability (Tartaglia *et al*., 1995). Recent studies suggest that short forms may modulate the activity of the long isoforms and act as transport proteins for leptin (Banks *et al*., 1996) and/or may have some signalling capacity through mitogen-activated protein kinase (MAPK) activation, independent of the JAK system (Murakami *et al*., 1997 and Uotani *et al*., 1999). OB-Ra is considered as a predominant transporter of leptin by the blood-brain barrier to the CNS (Peiser *et al.,* 2000 and Kastin *et al.,* 2000). Ob-Re is the secreted form of the receptor and is capable of binding leptin, thereby serving a clearance function for leptin in the body (Li *et al*., 1998). The short 34 amino acid transcellular domain is typical for a long and short isoforms, but is absent in soluble OB-Re (Gavrilova *et al*., 1997 and Tartaglia *et al*., 1997) and serves as leptin binding protein (Yang *et al*., 2004). It is thought that only the long form of the receptor (OB-Rb), which has a complete intracellular domain containing the semi-conserved regions box 1 and box 2, is capable of activating the JAK-STAT signalling pathway and is responsible for most of the biological effects of leptin known to date.

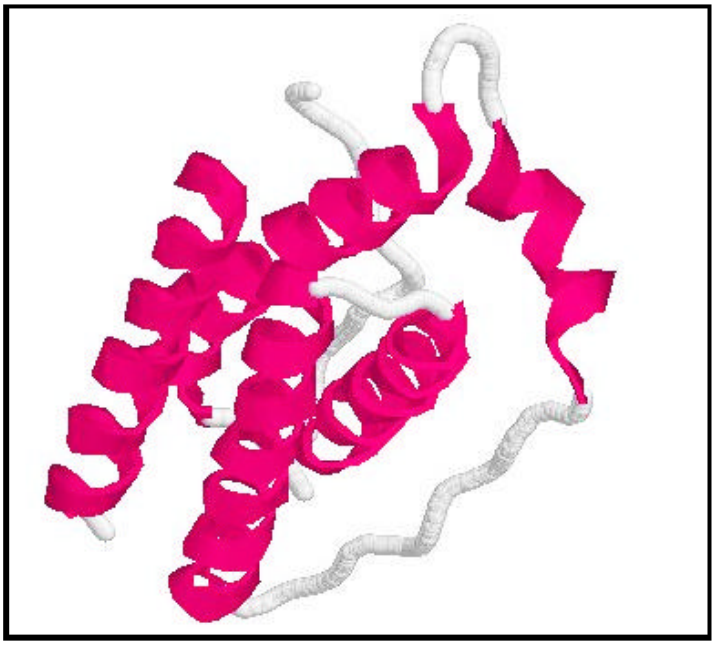


Fig. 1 : Four alpha helices of leptin

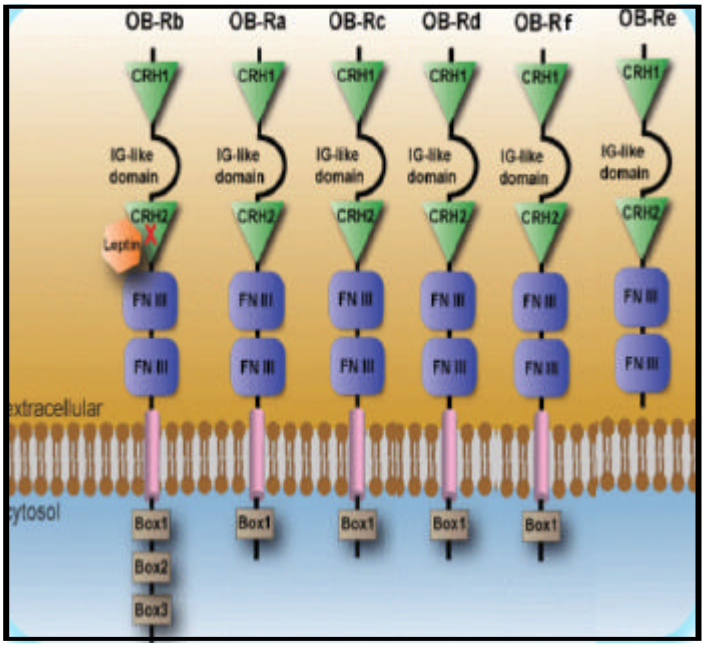


Fig. 2 : Structure of leptin receptor isoforms

OB-Rb contains the longest intracellular domain, which is crucial for leptin signalling. OB-Rb, OB-Rc and OB-Rd have varying short cytoplasmic domains. All isoforms except OB-Re contains the Box-1 motif known to bind the JAK kinase. Cytokine receptor homology module (CRH)-2 is main binding site for leptin. Immunoglobin (IG) like domain is for activation of receptors.

2.3.2. Distribution

Leptin receptors exhibit widespread distribution in mammalian tissue, including liver, heart, Kidney, lung, small intestine, testes, ovaries, spleen pancreas, and adipose tissue (Lee *et al*., 1996) and their expression fluctuated depending on physiological status (Table 1). It has been identified in the hypothalamus, gonadotroph cells of the anterior pituitary (Jin *et al*., 2000), granulosa, theca, and interstitial cells of the ovary (Karlsson *et al*., 1997), endometrium (Kitawaki *et al.,* 1999), and Leydig cells (Caprio *et al.,* 1999). The expression of leptin receptors has also been demonstrated in human, mouse, rat, pig and bovine ovaries (Karlsson *et al*., 1997; Kikuchi *et al*., 1999; Duggal *et al*., 2000; Ruiz-Corte's *et al*., 2000 and Nicklin *et al*., 2007).

Table 1: Location of leptin receptor in various tissues

Organ	References
Hypothalamus gonadotroph cells, Anterior pituitary	Jin *et al*., 2000
Mouse hypothalamus	Vaisse *et al*., 1996
Rat hypothalamus	Elmquist *et al.,* 1998
Rat pituitary gland	Sone *et al.,* 2001
Endometrium	Kitawaki *et al.,* 1999
Leydig cells	Caprio *et al.,* 1999
Human Ovary	Karlsson *et al.,* 1997
Mouse Ovary	Kikuchi *et al*., 1999
Rat Ovary	Duggal *et al*., 2000
Granulosa, Theca cells	Karlsson *et al*., 1997
Pig Corpus luteum	Ruiz-Corte's *et al*., 2003
Bovine Corpus luteum	Nicklin *et al*., 2007; Sarkar *et al*., 2009
Endometrium	Kitawaki *et al*., 1999

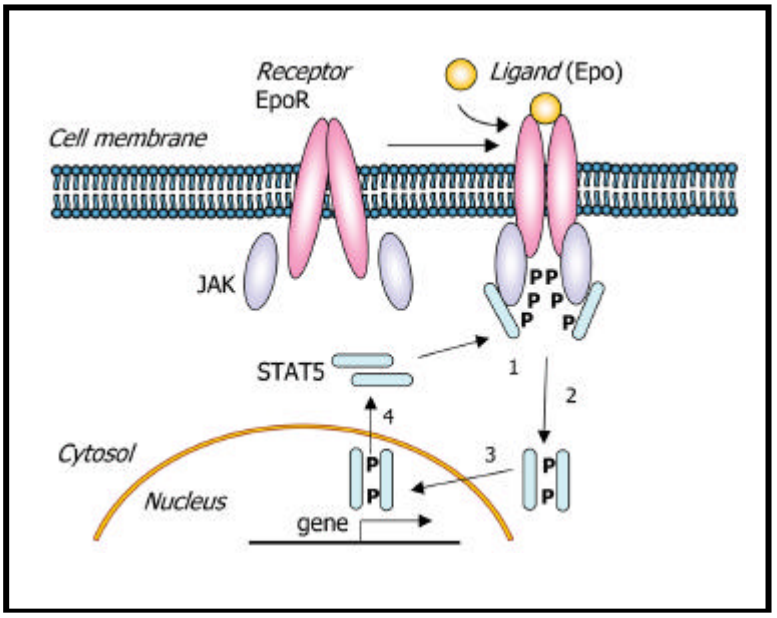


Fig. 3 : JAK/STAT pathway

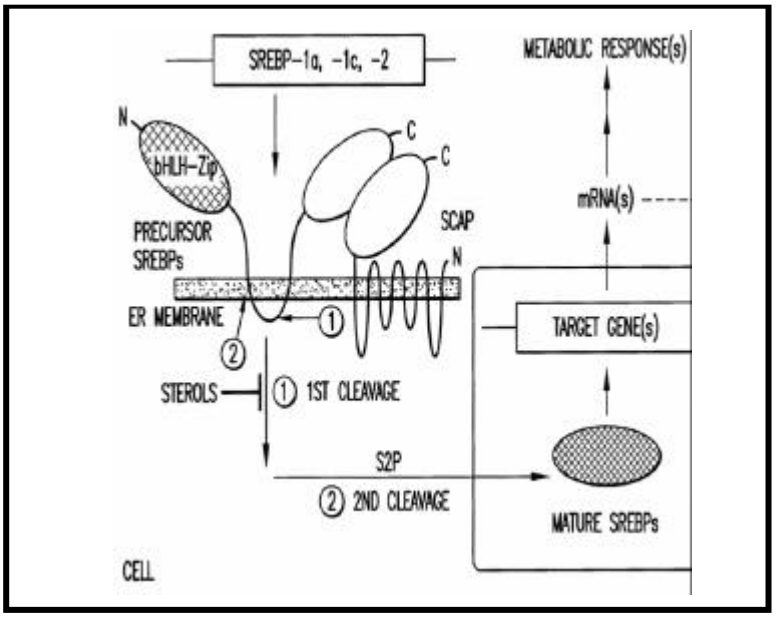


Fig. 4 : SREBP Pathway

2.3.3. Signalling of leptin receptors

OB-Rb, abundantly expressed in the central nervous system (CNS), participates in intracellular signal transduction by activation of Janus Kinase/signal transducer and activator of transcription (JAK/STAT) proteins, whereas short form is unable to stimulate this pathway (Bjorbaek *et al.*, 1997 and Zhang *et al.*, 2005).

2.3.3.1 JAK/STAT pathway

Upon ligand binding, the leptin receptor undergoes homodimerization, which is required for signalling activity, and activates receptor-associated Janus kinases (JAK) (White *et al*., 1997). Phospho-tyrosine residues of JAK interact with the SH2 (Src homology 2) domain of signal transducers and activators of transcription (STAT) (Bjorbaek *et al*., 1997), a family of transcription factors that are targets of JAK proteins (Fig.3). Upon interaction with JAK, STAT becomes phosporylated, dimerizes, translocates to the nucleus, and modulates transcription of target genes (White *et al*., 1997).

2.3.3.2 SREBP pathway

Steroidogenesis depends on the supply of its precursor, cholesterol, derived from intracellular and extracellular sources. Intracellular levels are tightly controlled by regulation of the uptake, storage, and synthesis by a unique family of transcription factors known as the sterol regulatory element-binding proteins (SREBP) (Hua *et al.*, 1993). These transcription factors are localized to the endoplasmic reticulum in an approximately 125-kDa precursor form under conditions of replete intracellular sterol/cholesterol stores. Upon depletion of cholesterol, the membrane-bound proteins are cleaved by proteases, releasing a 68-kDa transcription regulator. The mature SREBPs enter the nucleus, where they bind sterol regulatory sites located in the promoter regions of genes involved in cholesterol homeostasis and transport (Brown *et al.*, 1997) (Fig. 4). Leptin modulates SREBP1 activity by decreasing the amount of mRNA and of cleaved (transcriptionally active) SREBP1 protein (Soukas *et al.*, 2000) and regulates StAR (Shea-Eaton *et al.*, 2001). Leptin, acting through STAT-3, modulates steroidogenesis in a biphasic, dose-dependent manner and that SREBP1 induction of StAR expression may be in the cascade of regulatory events.

This multifocal expression of leptin, as well as the dense presence of Ob-Rs at all levels of the hypothalamus-pituitary-gonadal (HPG) axis, implies that the nutritional/leptin regulation of reproduction involves a complex network of interactions at multiple levels to regulate the HPG axis in a autocrine, paracrine and/or endocrine fashion.

2.4. ROLE OF LEPTIN

4.1. REGULATION OF FOOD INTAKE, ENERGY EXPENDITURE AND BODY WEIGHT

There is strong positive correlation of serum leptin concentrations with percentage of body fat. Leptin provides the body with an index of nutritional status. Leptin's effects on body weight are mediated through effects on hypothalamic centers that control hunger, body temperature and energy expenditure. Weight loss resulting from administration of leptin appears to result from a combination of at least two fundamental effects:

- **Decreased hunger and food consumption** mediated at least in part by inhibition of Neuropeptide Y synthesis. Neuropeptide Y is a very potent stimulator of feeding behaviour (Fig. 5).
- **Increased energy expenditure** , measured as increased oxygen consumption, higher body temperature and loss of adipose tissue mass.

Two opposing sets of neuronal activity that control appetite is present in the ARC of the hypothalamus the appetite-stimulating circuit and the appetite-inhibiting circuit. The two circuits send signals mainly to the PVN to modulate feeding behavior and energy expenditure. Peripheral hormones such as insulin, leptin, ghrelin, and PYY3-36 cross the blood-brain barrier and exert various effects on this system. NPY-neuropeptide Y; AgRP- agouti-related peptide; CART- cocaine and amphetamine–regulated transcript peptide; POMC- Pro-opiomelanocortin.

2.4.2 REPRODUCTIVE FUNCTION

It is well established that reproduction is very sensitive to nutritional status. Under nutrition delays the onset of sexual maturation negatively affects sexual behaviour. It has been suggested that food availability is the most important factor that influences mammalian

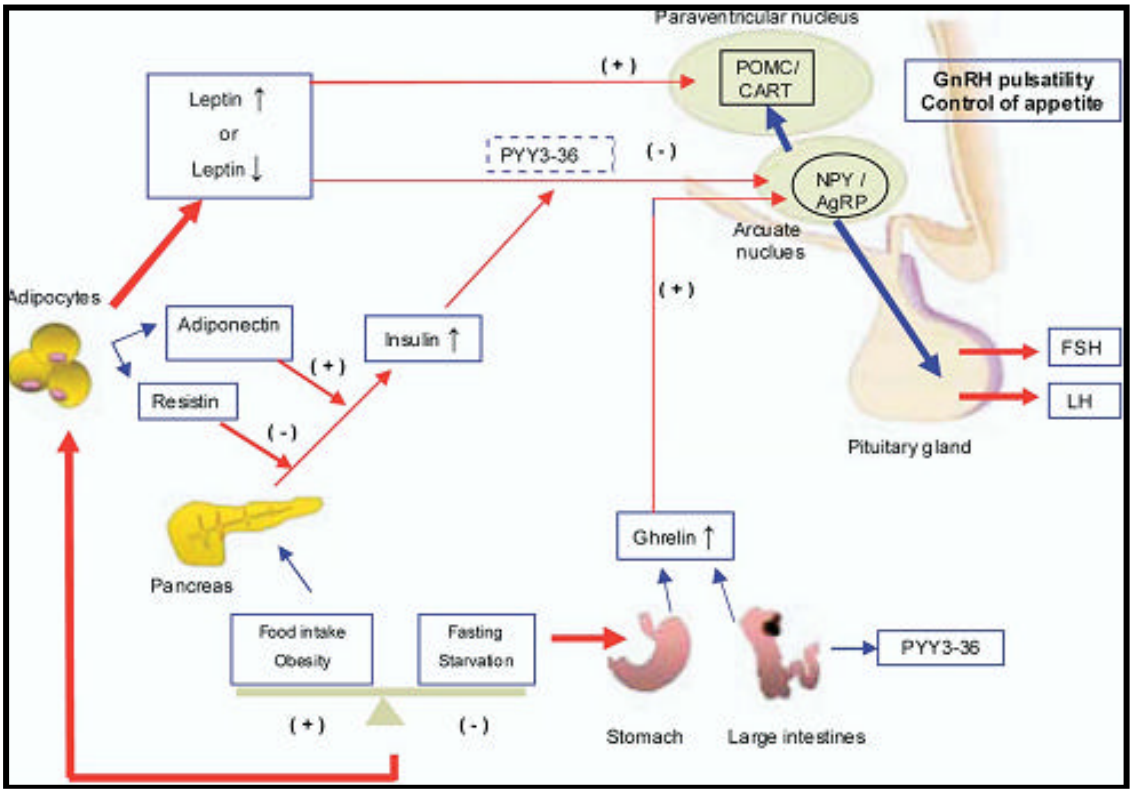


Fig. 5 : Regulation of food intake by leptin

reproduction. Links between nutrition and the reproductive axis is mediated by leptin. It has long been known that starvation adversely affect reproductive function. Also, the onset of puberty is known to correlate with body condition as well as age. Furthermore, different central and peripheral effects of this molecule have been described in reproductive organs, providing new insights into the physiology of reproduction.

2.4.2.1 ONSET OF PUBERTY

One of the first effects of leptin on reproduction is onset of puberty in animals. Its role in this regard includes important actions on the hypothalamus to bring about release of LH-releasing hormone, thereby triggering gonadotropin release and leading to development of the reproductive tract and induction of puberty (Caro *et al*., 1996). Prepubertal mice treated with leptin became thin, as one would expect, but also reached reproductive maturity and began cycling significantly earlier than control mice. Additionally, some humans with inactivating mutations in the leptin receptor gene not only are obese, but fail to achieve puberty (Montague *et al*., 1997).

2.4.2.2 FERTILITY

Leptin has been directly related to reproductive performance. Because of its role in fertility in animals, it is suggested that it is missing link between fat and fertility (Conway and Jacobs, 1997). The relations of leptin, fat stores and the reproductive axis have been studied in mice with a homozygous mutation for the *ob* gene, which developed obesity and sterility (Zhang *et al*., 1994). Obese (*ob/ob*) mice with a mutation in the leptin gene resulting in a pre mature stop codon are infertile, have subnormal gonadotrophin concentration and hypogonadism (Zhang *et al*., 1994). Administration of leptin to these obese leptin-deficient mutant mice caused decreased food intake, body weight loss, increased ovarian weight, increase in ovarian follicles and restoration of fertility (Barash *et al*., 1996; Chehab *et al*., 1996 and Kikuchi *et al*., 1999). Additionally, there are reports which indicate that reproductive dysfunction in aging obese lethal yellow mice is related to modified intra-ovarian leptin gene expression that is related to acquired obesity (Brannian *et al*., 2009) and injections of leptin to ob/ob mice, which have congenital deficiencies in leptin and are infertile, increased the number of graffian follicles (Barash *et al*., 1996) and correct their infertility (Chehab *et al*., 1996).

2.4.2.3 ACTION ON HYPOTHALAMIC-PITUITARY-GONADAL (HPG) AXIS

As the Ob-Rb isoform is highly expressed in the hypothalamus and considerable evidence has accumulated regarding the role of leptin in regulating the HPG axis, a number of studies have attempted to elucidate this mechanism at the level of the hypothalamus. It has been found that leptin accelerates gonadotropin-releasing hormone (GnRH) pulsatility, but not pulse amplitude, in arcuate hypothalamic neurons in a dose-dependent manner. In addition, although few studies have demonstrated that GnRH-secreting neurons express leptin receptors, leptin treatment of a GnRH-secreting neuronal cell line in vitro stimulates GnRH release. Ob-Rb isoforms are mainly present in arcuate and ventromedial hypothalamic nuclei controlling both sexual behavior and food intake. It is thus believed that leptin may facilitate GnRH secretion predominantly via indirect mechanisms, acting through interneurons secreting neuropeptides such as cocaine and amphetamine regulated transcript peptide (CART), galanin-like peptide, and/or melanocortin-concentrating hormone (MCH) in the hypothalamic zona incerta. In addition, leptin may increase release of nitric oxide (NO) from adrenergic interneurons, which then induces GnRH release from GnRH neurons by activating both guanylate cyclase and cyclooxygenase 1.

Leptin directly and indirectly stimulates GnRH release from the basal hypothalamus, affects pituitary gonadotropin release and regulates ovarian secretary functions. The main function of leptin is to act at various levels of the hypothalamic-pituitary-gonadal (HPG) axis via endocrine, paracrine, and/or autocrine pathways (Caprio *et al*., 2001).

The long form of the leptin receptor (Ob-R) has been localized in mouse (Vaisse *et al*., 1996) and rat (Elmquist *et al*., 1998) hypothalamus and in rat pituitary gland (Sone *et al*., 2001). It has a facilitatory effect on the central networks that regulate pituitary gonadotropin secretion (Ahima *et al*., 1996; Finn *et al*., 1998 and Pinilla *et al*., 1999) and involved in the regulation of growth and differentiation of pituitary cells (Jin *et al*., 2000).

2.4.2.4 OVARIAN ACTION OF LEPTIN

Although the general view has been that the principal effects of leptin are on the neuroendocrine component of reproduction (Chehab *et al*., 1996), evidence has emerged to indicate direct involvement of leptin in ovarian function. The expression of leptin receptors has

been demonstrated in human, mouse, rat, pig and bovine ovaries (Karlsson *et al*., 1997; Kikuchi *et al*., 1999; Duggal *et al*., 2000; Ruiz-Corte's *et al*., 2000 and Nicklin *et al*., 2007) and involved in regulation of reproductive processes.

Leptin is found in human follicular fluid (Cioffi *et al*., 1997), providing potential for direct effects of leptin on ovary. Additionally, there are reports which indicate that reproductive dysfunction in aging obese mice is related to modified intra-ovarian leptin gene expression that is related to acquired obesity and declining fertility and is related to progressive hyperleptinemia and leptin resistance (Brannian *et al*., 2005, 2009).

Leptin receptor mRNA and protein have been detected in bovine oocytes (Ryan *et al*., 2002 and Cervero *et al*., 2004) and it enhances meiotic maturation of bovine oocytes which is cumulus cell mediated.

The expression of Ob-R in ovary has been mainly related to a role of leptin in steroidogenesis (Cioffi *et al*., 1997; Karlsson *et al*., 1997 and Spicer and Francisco 1997). Moreover, the presence and turnover of mRNA and protein for leptin have been described in human granulosa and cumulus cells and the presence of leptin in mature human oocytes (Cioffi *et al*., 1997). Indeed, the influence of leptin on steroidogenesis remains the focus of many studies. Both positive and negative effects have been demonstrated in several species. More recently, Duggal *et al*., 2000 have reported variations in ovarian leptin mRNA at different times of the rat oestrous cycle.

Several authors have reported that leptin suppresses ovarian steroid synthesis in different species (Karlsson *et al*., 1997; Spicer and Francisco 1997; Zachow and Magoffin 1997; Agarwal *et al*., 1999; Barkan *et al*., 1999 and Caprio *et al*., 2001). In the human ovary, Ob-R mRNA which is found in the theca and granulosa cells (Karlsson *et al*., 1997) have an inhibitory effect on estradiol production through attenuation of gonadotropin, insulin, insulin-like growth factor 1 (IGF-I) and/or glucocorticoid-mediated steroidogenesis (Spicer and Francisco, 1997, 1998; Zachow and Magoffin, 1997; Zachow *et al*., 1999; Duggal *et al*., 2000; Spicer *et al*., 2000; Ghizzoni *et al*., 2001 and Guo *et al*., 2001). The inhibitory effects on theca cells comprise direct interference with ligand-induced steroidogenesis (Spicer and Francisco, 1998). Leptin is also known to interfere with adrenodoxin activity in ovarian cells (Barkan *et al*., 1999). Transcriptional inhibition by leptin has been evoked by reduction in

transcripts for steroidogenic enzymes in adrenal cortical cells (Zamorano *et al*., 1997), and abolition of glucocorticoid induced transcription in granulosa cells (Barkan *et al*., 1999). Interference with oestradiol synthesis was demonstrated in both rats (Zachow *et al*., 1999) and cow (Spicer and Francisco, 1997 and Spicer *et al*., 2000) granulosa cells *in vitro*.

Studies have also revealed direct stimulatory effects of leptin in rat and human luteinized granulosa cells in the form of induction of angiogenesis and proliferation of ovarian cells (Spicer and Francisco, 1997 and Bouloumie *et al*., 1998) and more specifically progesterone production from the bovine corpus luteum (Nicklin *et al*., 2007). The presence of leptin mRNA in porcine corpus luteum, theca, and granulosa cells has been reported (Ruiz-Corte's *et al*., 2000). The expression of the leptin receptor in ovarian cells of many species and leptin in bovine reported by Sarkar *et al*., 2009 suggest that it acts in the ovary in an autocrine / paracrine fashion and take part in important processes concerning reproduction by development of small follicles and formation, function and development of corpus luteum.

RT-PCR study in the porcine corpus luteum (Ruiz-Corte's *et al*., 2000) suggests that leptin receptor expression increases in association with luteinisation and declines coincidental with luteal regression. *In vitro*, leptin mRNA and protein expression increases at the time of morphological differentiation and during logarithmic progesterone accumulation over 96 hr of culture of porcine granulosa cell (Murphy and Dobias, 1999; Pescador *et al*., 1999 and Murphy, 2000). Thus, leptin receptor expression correlates with maximal progesterone production in both models, suggesting leptin has a positive effect on luteal function.

Leptin is directly involved in the angiogenic process in developing porcine corpus luteum (Robin *et al*., 2009). Associative relationship among the angiogenic factors Ang-1 and FGF-2 and the leptin receptor were also established during the luteal phase in the caprine species (Jessica *et al*., 2008).

Gregoraszczuk *et al.,* 2004 suggested that leptin decreased estrogen secretion in preovulatory follicles with concomitantly increased progesterone secretion. This provides evidence of leptin's action in the process of luteinisation, which starts just before ovulation and luteal functions thereafter.

Insulin like growth factors (IGF-1) has direct stimulatory effects on key components of the steriodogenic pathway to increase progesterone secretion in bovine luteal cells (Sauerwein

et al., 1992). In particular, IGF-1 is able to induce the expression of StAR mRNA in bovine granulosa-derived luteal cells (Mamluk *et al*., 1999). The stimulatory effects of leptin on porcine granulosa cell steriodogenic function have also been attributed to the induction of StAR transcription, as the result of sterol regulatory element binding protein 1 (SREBP-1) modulation (Ruiz-Corte's *et al*., 2003). It is therefore, feasible that it is the synergistic effects of leptin and IGF-1 on StAR transcription, which leads to a response great enough to increase progesterone synthesis. Results from the dispersed bovine luteal cell culture (Nicklin *et al*., 2007) and bovine luteinised GC cultures (Glister, 2001) showed that leptin alone had no significant effect on basal progesterone production, but, in the presence of IGF-1, leptin caused a significant increase in progesterone production.

Additionally, there is report which shows that negative effects of leptin on bovine theca and granulosa cell function are not mediated by an alteration in intraovarian IGFBP gene expression (Voge *et al*., 2004). Cell culture study demonstrated that leptin caused a dose-dependent inhibition of the IGF-1 augmentation of FSH stimulated oestradiol production in rat (Zachow and Magoffin, 1997), bovine (spicer and Francisco, 1997, 1998) and human follicle cells (Agarwal *et al*., 1999).

In recent papers a biphasic effect was shown for leptin. The authors measured progesterone production by cultured porcine granulosa cells and showed that leptin at 10 ng/ml increased progesterone production, while a dose of 1000 ng/ml decreased it (Ruiz-Corte's *et al*., 2003). In vivo serum porcine concentration rise during follicular phase and reach their peak during the luteal phase of spontaneous cycle (Ruiz-Corte's *et al*., 2003).

Incubation of rat Leydig cells with increasing concentrations of leptin (2–500 ng/ml) led to a significant and dose dependent inhibition of hCG stimulated testosterone production (Caprio *et al*., 1999). This was accompanied by a significant reduction of androstenedione and a concomitant rise of the precursor metabolites pregnolone, progesterone and 17-OH-progesterone.

Nicklin *et al*., in 2007 reported that progesterone production by luteal cells was increased by treatment with LH but treatment with leptin alone had no effect. However, in the presence of IGF-1, leptin caused a significant increase in progesterone production. They have shown that the leptin receptor is expressed in the bovine corpus luteum and have demonstrated a modulatory effect of leptin on luteal progesterone production in vitro.

Leptin alone has no influence on basal progesterone production. However, in bovine (Glister, 2001; luteinised) and porcine (Ruiz-Corte´s *et al*., 2003; non-luteinised) granulosa cells, leptin at low doses has been shown to stimulate progesterone production. Collectively, these results again highlight the contradictory effects of leptin often reported and indicate that the ability of leptin to regulate steroidogenesis is probably sensitive to tissue type, species and dose employed. In addition, it is feasible that leptin influences granulosa and luteal cell function in different ways, as the result of differentiation.

Gap in knowledge

mRNA expression of leptin & receptors in ovary found & documented in rat, pig, human, bovine (Caprio *et al*., 2001; Ruiz-Cortes *et al*., 2000; Nikline *et al.,* 1997; Sarkar *et al*., 2009). No study regarding expression of locally produced leptin and their receptor traced in buffalo ovary till date.

Hypothesis

Given their role in controlling ovarian function leptin and their receptor were hypothesized to be involved in CL formation, function and development in an autocrine / paracrine manner during estrous cycle in buffalo.

Chapter III

Materials and Methods

The present study was accomplished by using a number of materials and techniques which are described in this section. The first part deals with the chemicals used, followed by the methodologies, applied to achieve the target.

3.1 Materials

3.1.1 CHEMICALS / REAGENTS

Chemicals for molecular biology were obtained from different companies like Promega, Sigma, MBI Fermentas, Invitrogen, as per requirement. RNA*later*® Tissue Collection (Ambion applied biosystem), 100 bp and 50 bp plus DNA ladder, Loading dye was from (MBI, Fermentas) were used. The chemicals like Tris, Ethanol, Agarose, Ethidium Bromide were used.

3.1.2 COMMERCIAL KITS

SV Total RNA Isolation System (Promega Corporation, USA), RevertAid™ cDNA synthesis Kit (Fermentas, USA), DyNAmo™ HS SYBR^{R} Green qPCR kit (Finnzymes, Finland).

3.1.3 PRIMER SEQUENCES

To amplify the genes, a set of gene specific primers was designed from the published sequence. These primers were synthesized by the IDT Integrated DNA Technologies.

Table 2: Primer sequences

Genes	Sequences (5'-3')	Product Length
Leptin (Ob)	Forward: AGACCATAACAGCAGACAG; Reverse: TCCAGGCAATTCACTTCC	192
Leptin Receptor (Ob-R)	Forward: CCTCCTGGAATCTCAAAGAACAC; Reverse: ATCCAGCACTGTATGTTCC	254
Histone	Forward ACTGCTACAAAAGCCGCTC; Reverse: ACTGCCTCCTGCAAAGCAC	233

3.1.4 GLASSWARES AND PLASTIC WARES

For RNA work, RNase-free plastic wares and glassware were used, and they were thoroughly treated with 0.1% DEPC overnight at 37°C. It was further autoclaved to make it DNase and RNase free before use. For PCR and other DNA related work plastic wares were autoclaved (121^{o}C for 15 minutes at 101.3 kpa or 1 atmospheric pressure) and then used.

3.1.5 EQUIPMENTS

Major equipments used were as follows:

1. Agarose gel electrophoresis apparatus (Biorad)
2. Air displacement pipettes viz. P10, P100, P1000 (Finnpipette, Finland)
3. Gel documentation analysis system (AlphaImagerTM1220, Alpha Innotech Corporation, USA)
4. Hot air oven (Yorco instrument, Bombay)
5. Homogenizer (Aggen Hausser, Japan)
6. Ice flaking machine (Harrison Scientific Instrument Co. Delhi)
7. Refrigerated Microcentrifuge (Hettich, Germany)
8. Non refrigerated Centrifuge (Remi, India)
9. Refrigerator, BPL India Ltd.
10. -20^{o}C Deep freezer (Vestfrost)

11. -80^{0}C Sanyo Biomedical freezer
12. Spinix vortex machine
13. Nanodrop spectrophotometer
14. Thermal cycler, Eppendorf, Germany
16. Sanyo, microwave oven
17. Scientronic Double distillation apparatus
18. Weighing balance, Sartorius, Germany
19. Dry bath, Bangalore Genei
20. Laminar Flow, ESCO

3.1.6 COMPUTER AIDED MOLECULAR BIOLOGY SOFTWARE

The sequence analysis, phylogenetic tree based on evolutionary distances was constructed from nucleotide and amino acid sequences using Lasergene software (DNAstar Inc., USA).

3.2 METHODS

Standard protocols have been followed everywhere with slight modifications. The schematic representation of research work is given in Fig. 6.

3.2.1 COLLECTION OF SAMPLES

Ovaries containing corpus luteum were collected from buffaloes at a local slaughterhouse earliest after slaughter and were transported on ice to the laboratory. Precautions were taken to minimize the effect of ribonuclease activity while processing.

3.2.2 GRADING OF OVARIES

Sterile gloves were worn during the processing of samples. The stages of the oestrous cycle were defined by macroscopic observation of the ovaries (colour, consistency, number and size of follicles) as described previously (Berisha *et al*., 2000). CLs were assigned to the following stages; days 1–4, 5–10, 11–16 and >17 of oestrous cycle.

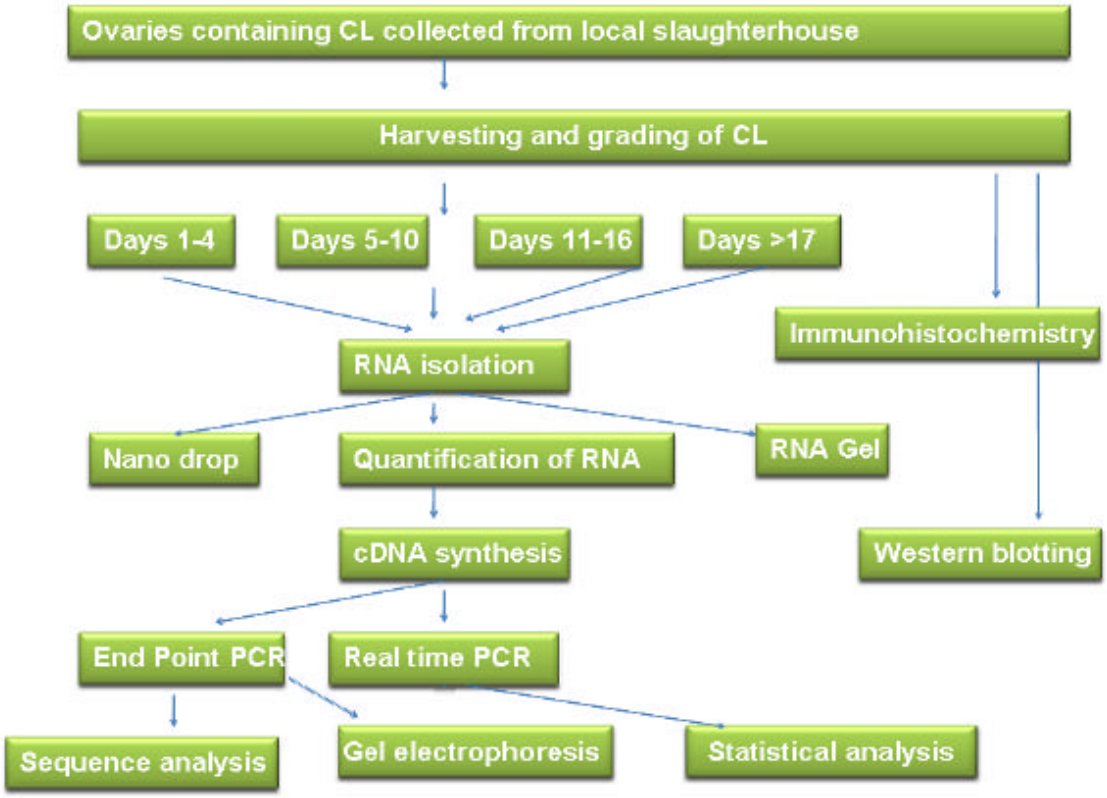
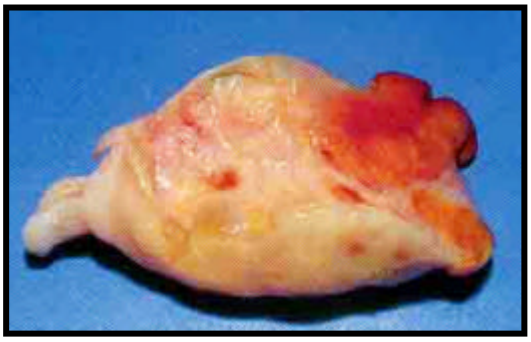
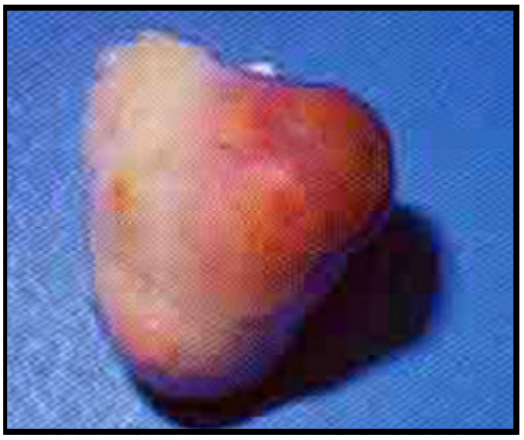


Fig. 6 : Schematic diagram

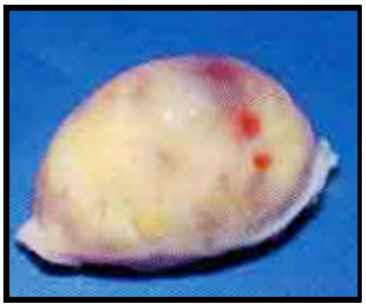


Day 1-4

Day 5-10



Day 11-16



Day >17

Fig. 7 : CL of different stages

Table 3: Grading of CL

Stages	External appearance on ovary	Appearance on CL
1-4 days	Ovulation depression, Recently ovulated, point of rupture not covered by epithelium	• Vasculature not visible • Red and hemorrhagic • Doughy • <1 cm in diameter.
5–10 days	Soft developing CL	• Vasculature, limited to periphery • Red in colour. • Soft • >1cm in diameter.
11–16 days	Fully developed	• Vasculature, limited to periphery • Reddish brown in colour. • Hard • >1cm in diameter.
>17 days	Firm CL	• Vasculature covers apex or not visible • Pale in colour • Very tough • <1cm in diameter

3.2.3 STORING OF SAMPLES IN RNA*later*® SOLUTION

Luteal tissues were immersed in RNAlater® Solution without freezing. Before immersion in RNA*later*® Solution, luteal tissue samples were cut to <0.5 cm in any single dimension. Fresh luteal tissues were place in 5-10 volumes of RNA*later*® Solution. Samples in RNA*later*® Solution were stored at -80^{o}C indefinitely. Samples in RNAlater® Solution was not frozen immediately instead, stored at 4^{o}C overnight (to allow the solution to thoroughly penetrate the tissue).

Luteal tissues were retrieved from RNA*later*® Solution with sterile forceps, quickly blotted away excess RNA*later*® Solution with an absorbent lab wipe or paper towel, and then submerged in RNA isolation lysis solution. Luteal tissues were homogenized promptly after placing it in lysis solution.

3.2.4 RNA ISOLATION

RNA isolation was done by using SV Total RNA Isolation System (Promega Corporation, USA). Before starting the isolation DNase I, RNA Lysis Buffer, RNA Wash Solution and DNase Stop Solution were prepared using the materials supplied in kit. All glassware & water used of molecular biology grade. Following steps were followed:

- Luteal tissue were retrieved from RNAlater® Solution with sterile forceps and quickly blotted away excess RNAlater® Solution with an absorbent lab wipe or paper towel.
- 170 mg weight of samples were cut by scissors and placed in 1 ml RNA Lysis Buffer (+ BME) placed in micro centrifuge tube.
- Sample were homogenised by homogeniser.
- 175 µl of homogenised mass were taken out in centrifuge tubes and 350 µl RNA Dilution Buffer were added and mixed by inverting 3-4 times and then heated at 70^0C for 3 minutes in water bath and centrifuged at 12,000–14,000 × g (at RT) for 10 minutes.
- Cleared lysate (approx 500 µl) were transfer into fresh micro centrifuge tube and 200 µl 95% ethanol was added and mixed well by pipet.
- Mixture was transferred to Spin Basket Assembly and centrifuged 12,000-14,000 × g (at RT) for 1 minute.
- Eluate was discarded. 600µl of RNA Wash Solution (+ ethanol) was added to the spin basket and centrifuged at 12,000–14,000 × g (at RT) for 1 minute.
- Again eluate was discarded.
- DNase incubation mix was prepared by adding 40 ìl of Yellow Core Buffer, 5 µl of $MnCl_2$ and 5 µl of DNase 1.
- 50 µl of DNase mix were added to membrane and incubated at RT for 15 minutes.
- 200 µl DNase Stop Solution **(**DSA**)** (+ ethanol) were added and centrifuge for 1 minute.
- Again 600 µl RNA Wash Solution (RWA) was added and centrifuge for 1 minute and elutes was discarded.
- Finally 250 µl RNA Wash Solution (RWA) were added and centrifuged for 2 minutes.
- Then cap was removed from spin basket and spin Basket were transferred to Elution Tube.

- 100 µl Nuclease-Free Water were added to membrane and centrifuged for one minute to elute the RNA.
- RNAs were stored at -80°C.

3.2.5 QUANTIFICATION OF RNA

The purity and concentration of total RNA was checked using the spectrophotometer using nanodrop reading. Quantification of RNA was done spectrophotometrically. 1 µl of total RNA was used and absorbance at 260 nm and 280 nm wavelengths were recorded against nuclease free water as blank. RNA samples showing the OD 260: OD 280 value more than 1.8 would be expected to contain no protein and taken for further use.

3.2.6 CONFIRMATION OF RNA BY GEL ELECTROPHORESIS

The integrity of the total RNA was checked using denaturing agarose gel (1%) electrophoresis and visualization under UV light. Intact bands of 28s and 18s with smearing indicated good quality and intactness of RNA.

3.2.7 SYNTHESIS OF cDNA

The first strand cDNA was synthesized from the isolated total RNA. RT-PCR was done using reverse transcription system (FERMENTAS, USA) following manufacturers instruction. Reverse transcription was carried out in 20 µl reaction mixtures. Calculation was done by using the concentration of total RNA from nanodrop reading (ng/µl) to take two microgram of total RNA for each reaction and dissolved in nuclease free water to make final volume 11 µl. One microgram of random hexamer primer was added and then incubated at 70^0C for 5 minutes. Then snap cooled in ice and following mixture was added:

Components of reaction mixture	**Quantity**
5X RT Buffer	4 µl
dNTP mix. (10mM)	2 µl
Ribonuclease Inhibitor (20 Units/µl)	1 µl
Reverse transcriptase enzyme (200 Units/µl)	1 µl

Reaction mixture was mixed to RNA-primer complex and spinned, followed by incubation at 25^oC for 5 minutes and 42^oC for 60 minutes. Reaction was stopped by incubating at 70^oC for 5 min and finally at 4^oC forever. The cDNA was stored at -20^oC for long term use.

3.2.8 CONFIRMATION OF cDNA WITH HISTONE PRIMERS

The integrity of the cDNA was checked by PCR with histone primers. The amplification of 233 bp histone gene fragment from the cDNA indicated that the cDNA was made from the RNA extracted from luteal tissue.

3.2.9 END POINT PCR OPTIMIZATION

PCR was standardized to amplify buffalo Ob and Ob-R gene sequence. Factor specific primers were used for the amplification genes. The annealing temperature was standardized using cDNA prepared from mRNA of buffalo CL by PCR. The reaction was carried out at different annealing temperatures. The optimum temperatures of 62^{o}C for leptin and 63^{o}C for leptin receptor were found to be most suitable for annealing for primers and were used in subsequent polymerase chain reaction. The reaction mixture was put according to standardized PCR reaction mixture composition. cDNA was diluted to ten times with nuclease free water. Following reactant were added to a nuclease free thin walled 0.2 ml micro centrifuge tube pre-chilled on ice in same order.

Components	**Volume**
cDNA template	2.50 µl
Nuclease free water	16.80 µl
10x PCR buffer	2.50 µl
25 mM $MgCl_2$	1.50 µl
2.5 mM dNTP mix	0.50 µl
Forward primer10 pmol/µl	0.50 µl
Reverse primer10 pmol/µl	0.50 µl
Roche Taq Polymerase	0.20 µl
Total	**25.00 µl**

The contents were gently vortexed and then spun down to collect at the bottom of tube by brief centrifugation. The reaction was carried out in a thermal cycler using the following cycling parameters that have been found optimum for amplification of gene fragments.

S.N.	Cycling steps	Ob		Ob-R	
		Temp.	Time	Temp.	Time
1	Initial denaturation	95°C	4 min	95°C	4 min
2	35 cycles of				
	Denaturation	95°C	30 sec	95°C	30 sec
	Annealing	62°C	45 sec	63°C	50 sec
	Extension	72°C	1 min	72°C	1 min
3	Final extension	72°C	10 min	72°C	10 min
4	Hold	4°C	α	4°C	α

3.2.10 AGAROSE GEL ELECTROPHORESIS

The confirmation of amplification of specific RT-PCR amplicon was done by agarose gel electrophoresis (appendix). 2% agarose was mixed with 1X TAE buffer and melted in a microwave oven. When the molten gel had cooled to about 42^{o}C, ethidium bromide was added to make final concentration 0.5 µg/ml. The gel was mixed thoroughly by gentle swirling and then poured into the gel casting tray fitted with the comb. The gel was allowed to solidify and the comb was removed. The PCR product were mixed with 1X gel loading dye (Final concentration) and loaded into the wells. For the comparison, a 50 bp molecular weight marker was gel electrophoresed in parallel to the RT-PCR amplicons. The gel was run at a voltage of 10 V/cm till the running dye crossed at least two third of the gel. The bands were visualized under UV light and recorded on a gel documentation system (GELDOC, USA).

3.2.11 REAL TIME PCR

Quantitative Real-time PCR was performed with DyNAmo™ HS SYBR^{R} Green qPCR kit and Mx3000P spectroflouremetric thermal cylcer operated by MxPro™ QPCR software. Reaction setup was performed in area separate from nucleic acid preparation or PCR product analysis. Pipetting was done with sterile filter tips. Exposure of light to the qPCR mastermix was minimised. Careful pipetting was done without creating bubbles to avoid interference in reading of fluorescence by the instrument. No template control (NTC) was put for either gene quantification for checking the contamination in the reaction components other than the cDNA. To ensure the cDNA samples were not contaminated with genomic DNA, reactions were set up using 10 ng of non-reverse transcribed RNA in place of cDNA in NTC. Failure to generate

a detectable signal signified the samples as DNA free. For reaction set up optically clear caps were used. cDNA was diluted two times and 2.5 µl of cDNA was taken. Following master mix was prepared:

Components	**Volume**
Nuclease free water	6.5 µl
Forward primer (10 pm/µl)	0.5 µl
Reverse primer (10 pm/µl)	0.5 µl
Syber green MM	10 µl
Total volume	**17.5 µl**

Touching of the optical surface of the caps without gloves was avoided. Strips were centrifuged before starting the cycling programme to force the solution to the bottom of the tubes and to remove any possible bubbles.

Thermal profile setting and cycling parameters used in real time quantitative PCR

Segment	**Thermal profile**	**Time**	**Thermal profile**	**Time**	**No. of cycle**	**Comments**
	Ob		**Ob-R**			
Segment 1	95^0C	15 min	95^0C	15 min	1 cycle	Hot start PCR
Segment 2	95^0C	30 sec	95^0C	30 sec	40 cycles	Denaturation
	62^0C	40 sec	**63^0C**	40 sec		Annealing
	72^0C	40 sec	72^0C	40 sec		Extension
Segment 3	95^0C	1 min	95^0C	1 min	1 cycle	Dissociation
	63^0C	30 sec	63^0C	30 sec		curve
	63-95^0C	2 degree per min	63-95^0C	2 degree per min		analysis
	95^0C	30 sec	95^0C	30 sec		

The amplification and denaturation data was acquired for further calculations.

3.2.12 DETERMINATION OF COPY NUMBER

A standard curve was obtained by serial dilution of the cDNA containing the template and a regression line equation in relation to the threshold values ("Ct") was formulated. The initial copy number was estimated directly from that regression line equation of the standard curve and the expression level was quantified by normalizing each gene copy number against the reference gene in each tissue samples.

3.2.13 STATISTICAL ANALYSES

The statistical significance of differences in mRNA expressions of the examined factors was assessed by one-way ANOVA followed by the Holm-Sidak as a multiple comparison test. Differences were considered significant if $P<0.05$. All experimental data are shown as 40 minus the mean of CP±SEM.

3.2.14 SEQUENCING OF LEPTIN AND LEPTIN RECEPTOR GENE

Sequencing was done by automated sequencer using Sanger's dideoxy chain termination method. The PCR products and primers were submitted along with picture of gel. Sequencing was done from 5'-3' as well as 3'-5' directions.

3.2.15 ANALYSIS OF LEPTIN AND LEPTIN RECEPTOR GENE SEQUENCES

The sequences obtained were first checked manually and blasted (www.ncbi.nlm.nih.gov/BLAST) to ascertain that sequences were of leptin and leptin receptor gene. The related sequences identified from blast results were retrieved from Genbank (www.ncbi.nlm.nih.gov). The sequences were edited by using EditSeq option of DNASTAR to get comparable sequences. The sequence homology comparisons were made between the leptin and leptin receptor gene nucleotide sequence and related nucleotide sequences of other species of farm animals using the clustalIV method of MegAlign Programme of Lasergene Software (DNA STAR). Phylogenetic analysis was also performed to determine the evolutionary relatedness between buffalo and other species of farm animals.

3.2.16 SDS-PAGE AND WESTERN BLOTTING

SDS-PAGE analysis was carried out in a vertical maxigel electrophoresis apparatus (ATTO, Japan). Glass plates were cleaned and set in a gel moulding tray of electrophoresis apparatus and bottom and sides were sealed with agarose. Gradient separating gel (7.4-15%)

solution was poured between the two SDS-PAGE glass plates and 1 ml DDW was poured over the gel. After polymerization, DDW was removed by tilting the plates and 7 ml of 3% stacking gel was poured over the separating gel and later on suitable comb was inserted. The polymerization gel was mounted into the electrophoresis chamber and buffer reservoir was filled with 1X Tris glycine buffer. Before loading, 30 µl sample buffer was added to each sample. The samples were boiled for 10minutes and kept on ice. The samples were briefly centrifuged before loading. A standard protein marker (MBI- Fermentas) was included along with the samples. Electrophoresis was carried out at a constant current of 150 V and 150 mA, until the tracking dye reached the bottom of the gel. The gel was removed from the plates and stained with Coomassie brilliant blue for 1 hour and then destained with de-staining solution.

Proteins were characterized by Western blot analysis in order to confirm specificity. The protein was run in 7.5-14% gradient SDS-PAGE gel along with pre-stained protein marker. After electrophoresis the gel was taken out from the plates and kept in Western Blot buffer. Four Whatman filter papers, PVDF membrane (Sigma, USA), SDS-PAGE gel, and four Whatman filter papers were stacked in respective order one by one on the anode plate of blotting apparatus (ATTO, Japan) after soaking with the Western blot buffer. Care was taken for avoiding air bubbles. The complete stack was saturated with ice cold transfer buffer before the cathode plate was placed in position over the stack and a current of 0.8mA/cm^2 was applied for 1hr. After the transfer, the gel was stained to check the efficiency of transfer of protein from gel to the membrane and the membrane was subjected to immunological detection. The membrane, after transfer, was incubated over night at 4°C in 1% bovine serum albumin diluted with PBS. After blocking, the membrane was washed thrice with PBS-T (PBS+0.01% Tween 20) for 5 minutes each and incubated with primary antibody 1:100 dilution which was raised in rabbit for 2 hr. After incubation, membrane was washed thrice with PBS-T (PBS+0.01% Tween 20) for 5 minutes each then added antirabbit goat IgG HRP (Horse Radish Peroxidase) conjugate for 1hr at 37°C. After washing, the antigen antibody reaction was detected by incubating the membrane with substrate diaminobenzidene tetrahydrochloride (DAB). The colour reaction was terminated by washing the membrane with distilled water to prevent background coloration.

3.2.17 IMMUNOHISTOCHEMISTRY

Serial sections of 5 µm thickness were cut on a microtome. Sections were kept overnight at 4°C in acetone for fixation. It is then rehydrated with graded (absolute, 90%, 80%, 70%) ethanol. A moist chamber was made and slides were placed into it. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 minutes in dark. Slides were washed with chilled PBS for 5 min, placed in 0.01 M citrate buffer (pH 6.0), and incubated for 15 min for antigen retrieval. Slides were washed with chilled PBS. Background blocking was performed with blocking solution containing 5% BSA in PBS and non-immune serum (1:5 dilutions) taken from the animal (Goat) that produced the secondary antibody and kept for 30 minutes. The tissue sections were incubated for 2.5 hours at 4°C with a rabbit polyclonal antibody specific for leptin (Y-30 sc-843; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 for both Ob and Ob-R in 5% BSA. The detection system used was the consisting of a goat anti-rabbit Ig secondary antibody coupled to a peroxidase labelled dextran polymer. The sections were incubated with this reagent diluted 1:250 for both Ob and Ob-R for 1.5 hours at 37°C and washed in chilled PBS for 5 minutes. The peroxidase activity was revealed using 3, 3 diaminobenzidine (DAB) solution consisting H_2O_2 in PBS for 15 minutes. The reaction was stopped by washing in DDW. Then slides were dried in air and visualised under microscope and photographs were taken.

Chapter IV

Results

This chapter has documented the outcome of present study, which covers the collection of corpus luteum, grading of corpus luteum, isolation, characterization and quantification of RNA, cDNA synthesis, cDNA confirmation by end point PCR, real time PCR, sequencing, immunohistochemistry, Western Blotting and sequence analysis.

4.1 SAMPLE COLLECTION AND GRADING

CLs were harvested from ovaries collected from slaughter house and were graded in laboratory on ice as early as possible into four grades. The changes in the dimension of the CL are shown in Fig. 7. The CLs were cut into pieces and kept in RNA *later* solution until the total RNA isolation.

4.2 RNA ISOLATION AND QUANTIFICATION

Tissues were then retrieved from RNA *later* solution and the total RNA was isolated using Promega, kit (Promega, Madison, WI, USA) according to the manufacturer's instructions with slight modification. The integrity of total RNA was checked on 1.0% agarose gel using 1x TBE as electrophoresis buffer. Total RNA was in good yield in all the samples. The bands of 28sRNA and 18sRNA reflected the high quality of extracted total RNA (Fig. 8).

The purity and concentration of total RNA was checked using nanodrop. Isolated RNA samples were free from the protein contamination as the OD 260: OD 280 values were more than 1.8. The concentrations of the RNA samples were in the range of 200-1000 ng/µl.

4.3 cDNA SYNTHESIS AND ITS CONFIRMATION

2 µg of total RNA was directly used for cDNA synthesis in thermo cycler using random hexamer primer. cDNA integrity was checked by histone gene amplification with already published primer whose reaction conditions were already known. After running on 2% agarose gel, single band of 233 bp was visualized (Fig. 9). In order to ensure the amplification of specific fragment with higher yield, the PCR protocol was optimized with respect to reaction conditions as well as cycle parameters.

4.4 OPTIMIZATION OF END POINT PCR PROTOCOL

Primers were synthesized using HUSAR software (DKFZ, Heidelberg). Gradient PCR conducted in special cyclers that allow different temperature profiles to be programmed for each cavity in the cycler. Gradient PCR used to optimize PCR conditions with respect to the primer annealing temperature. All samples were treated equally, but different annealing temperatures were used. It was observed that annealing temperature of 62°C gave the best result for leptin and 63°C for leptin receptor.

In an agarose gel the PCR efficiency analyzed investigating intensity and integrity of the product bands. The reaction conditions were optimized using different combinations of the primers, $MgCl_2$ and dNTPs for both the genes. The optimized concentrations that gave the best results were 1.5 mM of $MgCl_2$, 200 mM of dNTPs and 10 pM of each primer. Finally PCR was carried out in 25 µl volume of reaction mixture containing optimized concentrations of $MgCl_2$, dNTPs and primers, and 0.5 µl cDNA as template, 1X PCR assay buffer and 1.5 unit of *Taq* DNA polymerase.

4.4.1 AMPLIFICATION OF LEPTIN GENE

Following PCR, the amplicon length was checked using agarose gel electrophoresis. As expected a single and specific band of 192 bp was amplified from the cDNA (Fig. 10).

4.4.2 AMPLIFICATION OF LEPTIN RECEPTOR GENE

Following PCR, the amplicon length was checked using agarose gel electrophoresis. As expected a single and specific band of 254 bp was amplified from the cDNA (Fig. 11).

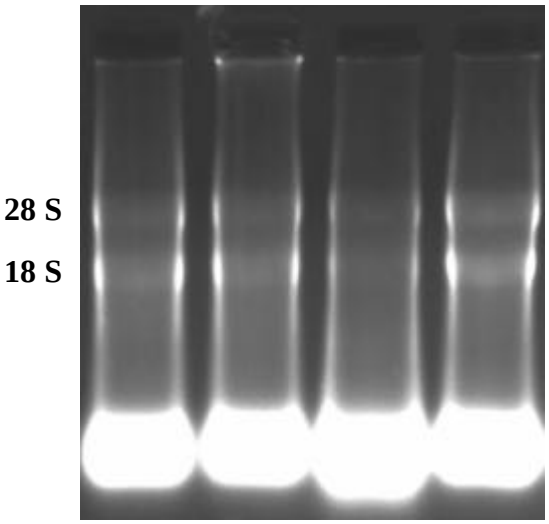


Fig. 8 : Gel showing integrity of total RNA

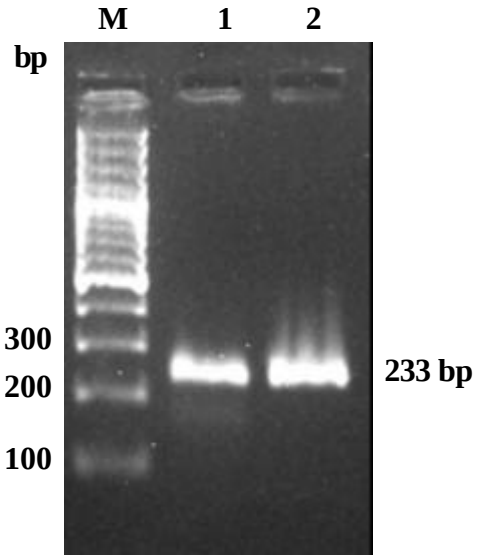


Fig. 9 : RT-PCR amplification of Histone gene

Lane M : Molecular weight Marker (100 bp)

Lane 1&2 : 233 bp PCR product

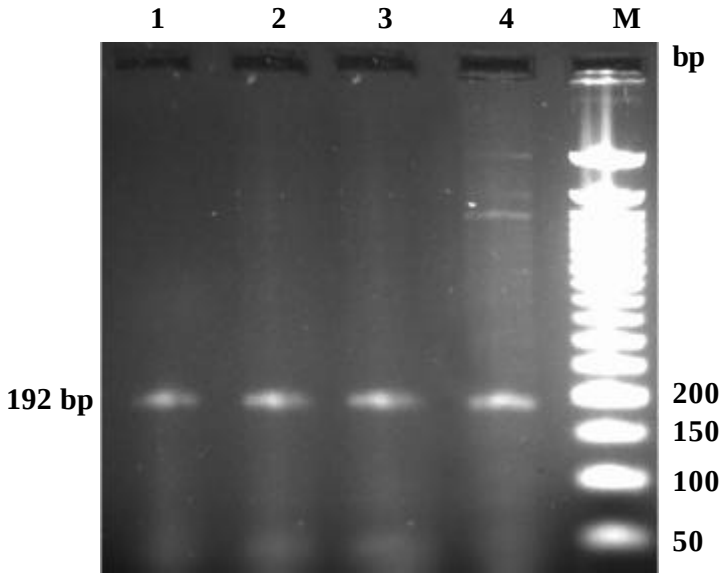


Fig. 10 : RT-PCR amplification of Leptin gene

Lane M **:** Molecular weight Marker (50 bp)

Lane 1,2,3&4 **:** 192 bp PCR product

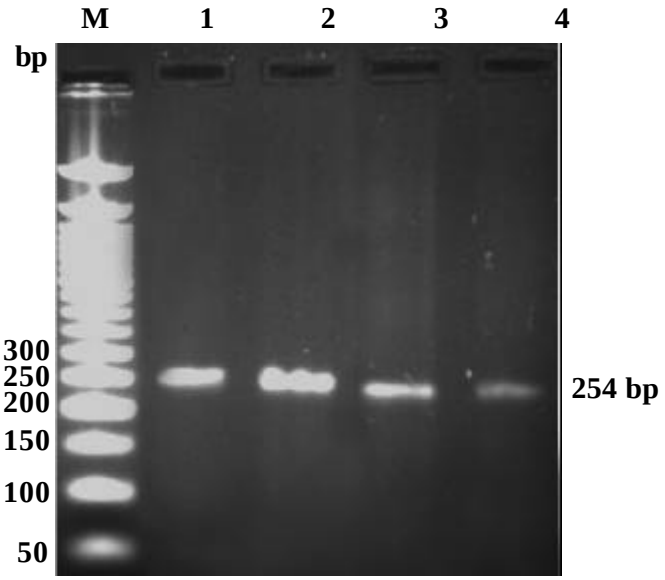


Fig. 11 : RT-PCR amplification of Leptin receptor gene

Lane M **:** Molecular weight Marker (50 bp)

Lane 1,2,3&4 **:** 254 bp PCR product

4.5 SEQUENCE ANALYSIS

Sequences obtained were aligned to remove the vector sequence. The sequence was submitted to NCBI Genbank and the accession numbers HM775400 for leptin and HM775401 for leptin receptor have been obtained.

Following are the nucleotide sequences of leptin:

Origin

1 gtcctctgac ctccagagtg cctcttgaac caggtggtag tctctggaga tgtgaaaaaa

61 gtagggcagg gagggcagga gtgtttgctg gaagagagga gttccgaggc ctattttgca

121 ggcggtgagg gaagtgaatt gcctgga

Following are the nucleotide sequences of leptin receptor:

Origin

1 agagatttac tgttgtaaca aatgccttcc cttcaatgtc gtctgtatat acagagaagt

61 ttttatcttc ctcgctccaa aagcaacagt ggaaagttgt tctggatgat aagttagata

121 agtaggtacc acttgaatta agcttagttt caacaactgc ctcataacgt ccattcaaat

181 ttgaagtgtt ctttgagatt ccaggagga

These nucleotide sequences were aligned and compared with available respective sequences of other domestic species. All leptin and leptin receptor gene cDNA sequences of different domestic species were aligned using ClustalV method of MegAlign program (DNA star, USA) which reveals the nucleotide substitutions. The homology (percentage similarity and divergence) and the relative significance of the obtained sequence of buffalo leptin and leptin receptor gene with that of domestic species were studied.

The entire nucleotide sequence of buffalo leptin gene showed 96.6%, 63.4% and 53.7% homology with that of Bos taurus, Canis lupus familaries and Felis catus, respectively (Fig. 12). The entire nucleotide sequence of buffalo leptin receptor gene showed 99.0%, 95.6%, 88.2%, 87.6%, and 87.0% homology with that of Bos taurus, Ovis aries, Sus scrofa, Equus caballus and Canis lupus familaries respectively (Fig. 13).

The phylogenetic tree was drawn by MegAlign programme of Lasergene software (DNASTAR Inc, USA) at nucleotide level. Buffalo and cattle leptin gene showed identical lineage. However, dog and cat are having similarity with buffalo which have different lineage

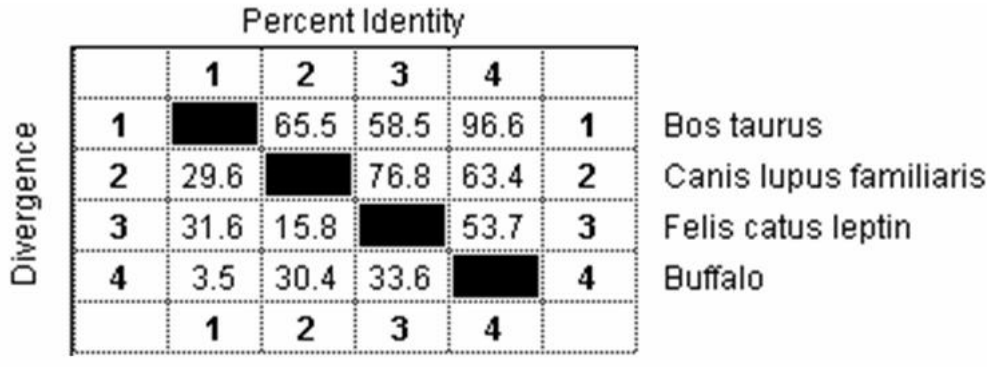


Fig. 12 : Homology for leptin

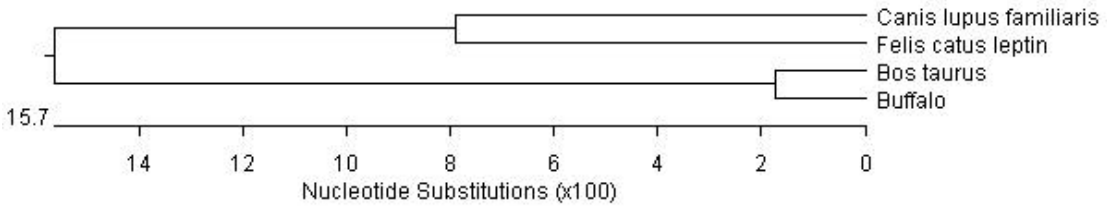


Fig. 14 : Phylogenetic tree for leptin

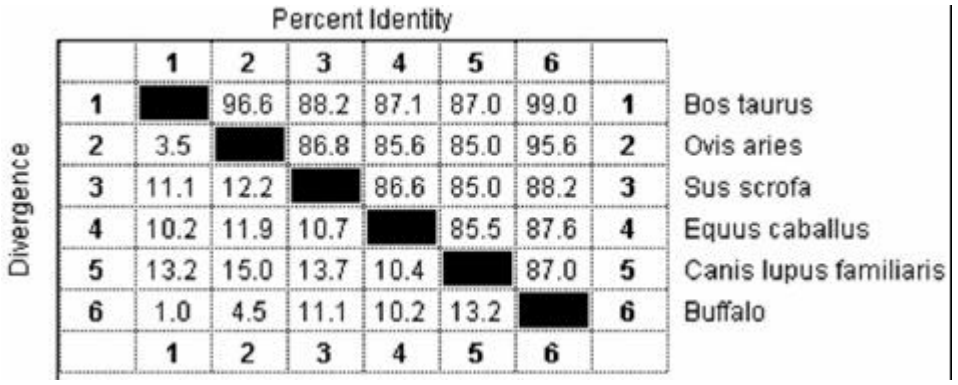


Fig. 13 : Homology for leptin receptor

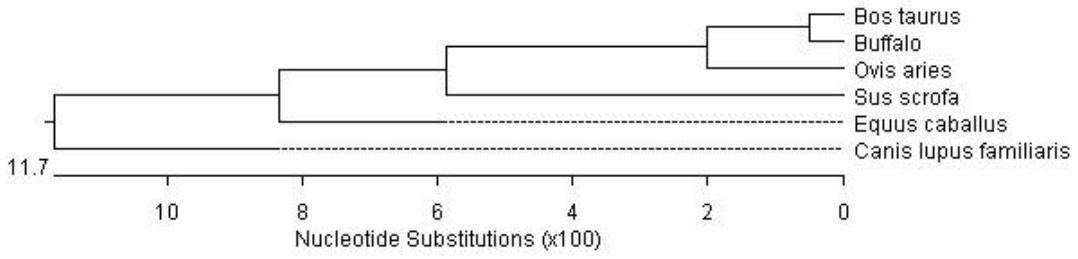


Fig. 15 : Phylogenetic tree for leptin receptor

(Fig. 14). Phylogenetic tree of leptin receptor gene also showed identical lineage of gene in buffalo and cattle. Sheep was closely related to cattle and buffalo while pig, horse and dog were distantly related (Fig. 15).

4.6 REAL TIME PCR

Real time PCR was optimized using different dilutions of templates of cDNA. 1.25 µl of the template gave good results for both genes. The mRNA expression of the housekeeping gene histone was not statistically regulated. Thus, it is assumed that equal amounts of mRNA were used in each sample.

After standardization real time PCR was performed for both the genes taking all the samples. After the run has ended, crossing point (CP) values and amplification plot for Ob (Fig. 16) and Ob-R (Fig. 17) were acquired by using the "comparative quantitation" method of the Rotor-Gene Analysis Software V5.0. The PCR-product was identified by the characteristic melting curve of Ob and Ob-R (Fig. 18 & 19). The melting curve showed only one peak.

4.6.1 EXPRESSION OF mRNA FOR LEPTIN

The expressions of mRNA for leptin in CL tissue during oestrous cycle are presented in (Fig. 20). The abundance of transcripts of both leptin and its receptor varied through the luteal phase. The mRNA expression during early-luteal stage (days 1–4) maintained low but detectable, as was the case in the regressed CL and after that expression level started to increase and reached maximum ($P<0.05$) in the mid cycle CL (days 11–16) followed by gradual decline during late-luteal stage (days >17).

4.6.2 EXPRESSION OF mRNA FOR OB-R

Expression of Ob-R mRNA during oestrous cycle closely followed to that of leptin mRNA (Fig. 21). Significantly higher ($P<0.05$) expression during mid cycle and low but detectable level during early-luteal stage and regressed CL was observed for Ob-R mRNA.

4.7 IMMUNOLOCALIZATION OF Ob AND Ob-R

Positive staining for Ob (Fig. 22) and Ob-R (Fig. 23) were detected in the CL of buffalo by using monoclonal antibody. Within the CL, a strong positive reaction for Ob and

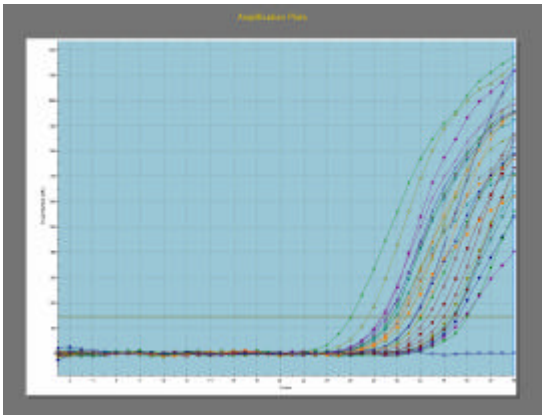


Fig. 16 : Amplification plot of Ob

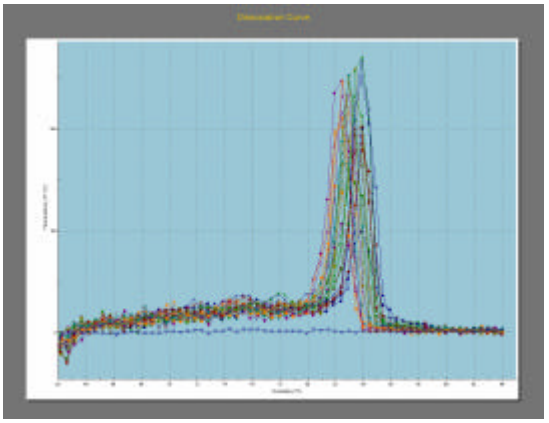


Fig. 18 : Dissociation curve of Ob

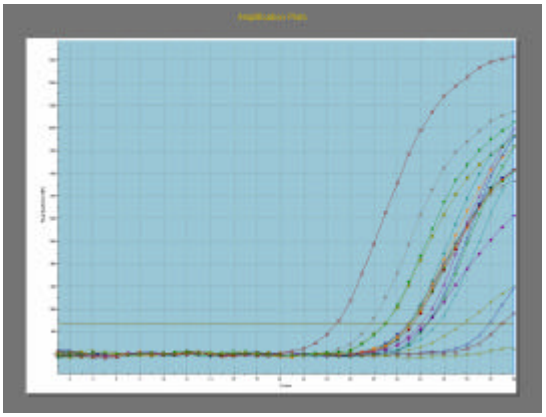


Fig. 17 : Amplification plot of Ob-R

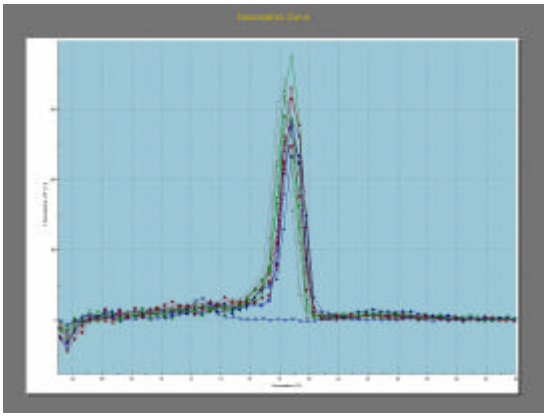


Fig. 19 : Dissociation curve of Ob-R

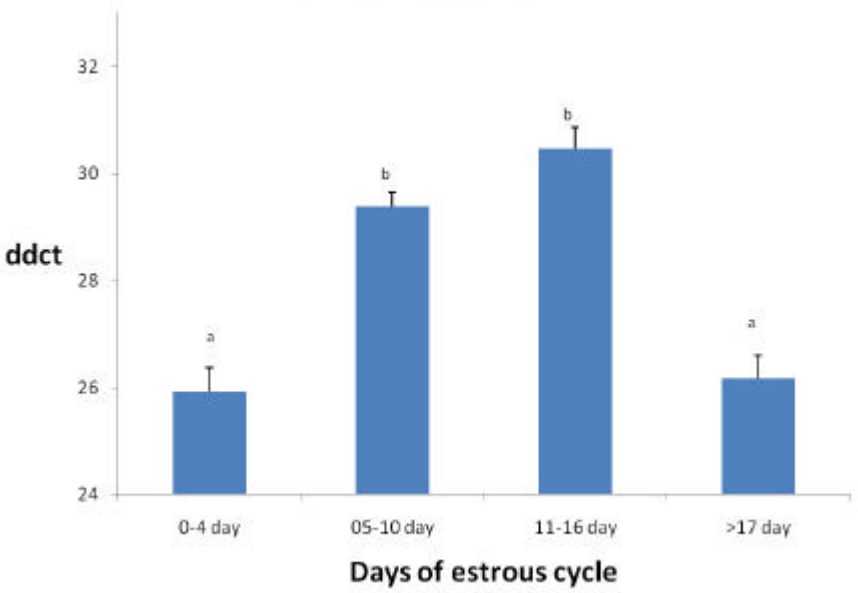


Fig. 20 : Leptin mRNA CL

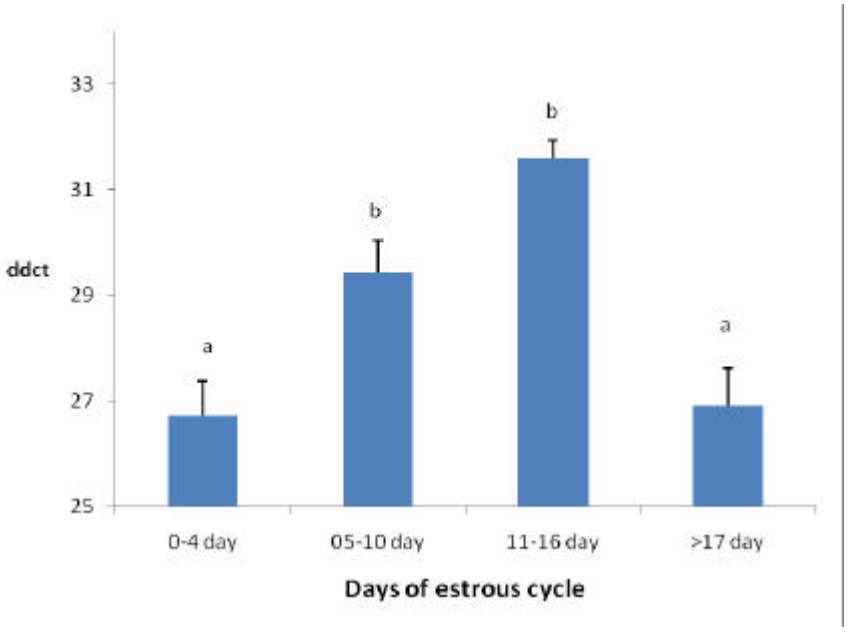


Fig. 21 : Leptin receptor mRNA CL

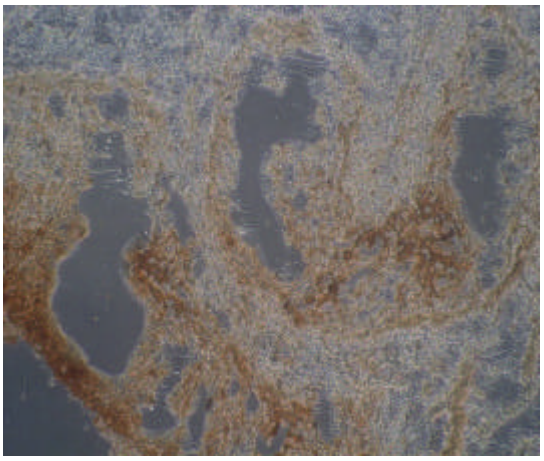


Fig. 22 : Immunohistochemistry for leptin

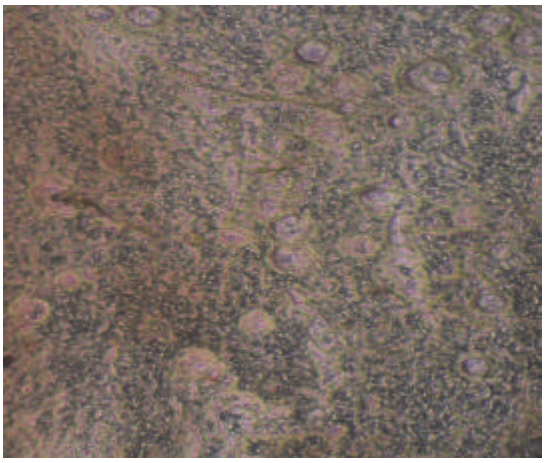


Fig. 23 : Immunohistochemistry for leptin receptor

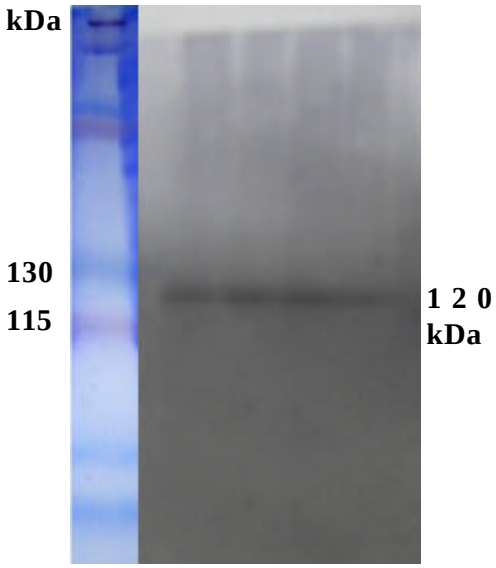


Fig. 24 : Western blotting for leptin receptor

Ob-R was localised primarily in the large luteal cells as indicated by the staining in their cytoplasm and also in the small luteal cells, although less evident.

4.8 WESTERN BLOT ANALYSIS

Western blot analysis of Ob-R protein expression revealed a strong band of approximately 120 kDa size in the immune precipitated derived fraction from whole luteal protein extracts of buffaloes (Fig. 24).

Chapter V

Discussion

The ovarian cycle is central to reproductive function in mammals. It is characterised by repeating patterns of cellular proliferation, differentiation and transformation that accompany follicular development, ovulation and the formation, function and regression of CL and gives females opportunity to become pregnant throughout their productive life time. The cycle is well regulated by the hypothalamic–pituitary–gonadal axis, which produces hormones that dictate reproductive events. However, it is also evident from many reports that locally produced factors such as steroid hormones, peptides and growth factors have essential modulatory role in follicular development (recruitment, selection and dominance), ovulation and CL formation, function, development and regression (Fortune, 1988; Berisha *et al*., 2000; Berisha and Schams, 2005).

These locally produced compounds constitute a complex intra-ovarian autocrine, paracrine and juxtracrine regulatory system. A direct involvement of leptin in ovarian function has been demonstrated in human, mouse, rat, pig and buffalo ovaries (Karlsson *et al*., 1997; Kikuchi *et al*., 1999; Duggal *et al*., 2000; Ruiz-Corte's *et al*., 2000; Nicklin *et al*., 2007).

The objective of the present study was to determine the expression of leptin (Ob) and its receptor (Ob-R) in buffalo CL obtained from different stages of the oestrous cycle.

The results obtained using qPCR indicate that leptin and its receptor is expressed in the buffalo CL throughout the oestrous cycle and may influence the function and/or development of this gland. Present study, demonstrated leptin and its receptor transcripts consistent with *in vivo* luteinisation of buffalo CL and decline coincidental to luteal regression.

Results of present study are consistent with RT-PCR studies in the porcine CL (Ruiz-Corte's *et al*., 2000) which showed that in the pig leptin receptor expression increases in association with luteinisation and declines coincidental with luteal regression. Results are also consistent with studies in the bovine CL (Sarkar *et al.,* 2009) which showed that both leptin and its receptor expression increases in association with luteinisation and declines coincidental with luteal regression.

In vitro, leptin mRNA expression increases at the time of morphological differentiation and during logarithmic progesterone accumulation over 96 h of culture of porcine granulosa cell (GC) (Murphy and Dobias, 1999; Pescador *et al*., 1999; Murphy, 2000). Hence, in the present study, expression pattern of leptin and its receptor which corresponds to the pattern of progesterone secretion suggest a possible positive effect of leptin on luteal steriodogenic function in buffalo.

Indeed, the influence of leptin on steroidogenesis remains the focus of many studies. Both positive and negative effects have been demonstrated in several species. On the positive side, treatment of the ob/ob mice with recombinant leptin was found to markedly upregulate cytochromo P450 side chain cleavage and P450-17a hydroxylase mRNA levels in the ovary (Zamorano *et al*., 1997). Similarly, studies in human luteinised granulosa cells indicate that leptin stimulates oestrogen production by increasing the P450 aromatase mRNA and protein expression and, consequently, aromatase activity (Kitawaki *et al*., 1999). Nonetheless, most reports suggest that leptin has inhibitory effects on ovarian steroidogenesis. Interference with oestradiol synthesis was demonstrated in both rat (Zachow *et al*., 1999) and cow (Spicer and Francisco, 1997; Spicer *et al*., 2000) granulosa cells in vitro. The effects on granulosa cells appear to result from interference with growth factors (Spicer and Francisco, 1997; Agarwal *et al*., 1999; Zachow *et al*., 1999), glucocorticoid (Barkan *et al*., 1999) and/or insulin (Agarwal *et al*., 1999) interactions with stimulatory ligands. The inhibitory effects of leptin on theca cells comprise direct interference with ligand-induced steroidogenesis (Spicer and Francisco, 1998). Additionally there is report which shows that negative effects of leptin on bovine theca and GC function are not mediated by an alteration in intraovarian IGFBP gene expression (Voge *et al*. 2004). The mechanisms of leptin inhibition of steroid synthesis are not well known, although there is evidence for interference with adrenodoxin activity in ovarian cells (Barkan *et al*.,

1999). Transcriptional inhibition by leptin has been evoked by demonstration of reduction in transcripts for steroidogenic enzymes in adrenal cortical cells (Zamorano *et al*., 1997), and abolition of glucocorticoid-induced transcription in granulosa cells (Barkan *et al*., 1999). Similarly, incubation of rat Leydig cells with increasing concentrations of leptin (2–500 ng/ml) led to a significant and dose dependent inhibition of hCG stimulated testosterone production (Caprio *et al*., 1999). This was accompanied by a significant reduction of androstenedione and a concomitant rise of the precursor metabolites pregnolone, progesterone and 17-OH-progesterone, consistent with a leptin-induced lesion in 17, 20 lyase activity. This model of role of leptin in selective ovarian steroidogenesis may hold good for buffalo too.

Leptin, acting through the nuclear transcription factor signal transducer and activator of transcription 3 (STAT-3), modulates sterol regulatory element-binding protein 1 (SREBP1) thereby increasing steroidogenesis. Steriodogenic acute regulatory protein (StAR) is an interesting candidate for leptin regulation, especially since SREBP1 regulates StAR (Shea-Eaton *et al.*, 2001).

Progesterone is important steroid hormone produced by the CL that not only maintains the pregnancy but also responsible for various events of estrous cycle. The production of progesterone concentration varies with the days of estrous cycle. The initial rise in plasma progesterone in individual buffaloes was observed on day 4 post estrus and reached the peak levels of 2.0 to 4.0 ng/ml in luteal phase by day 10 to 14 post estrus (Fig. 25) (Palta *et al*., 1996).

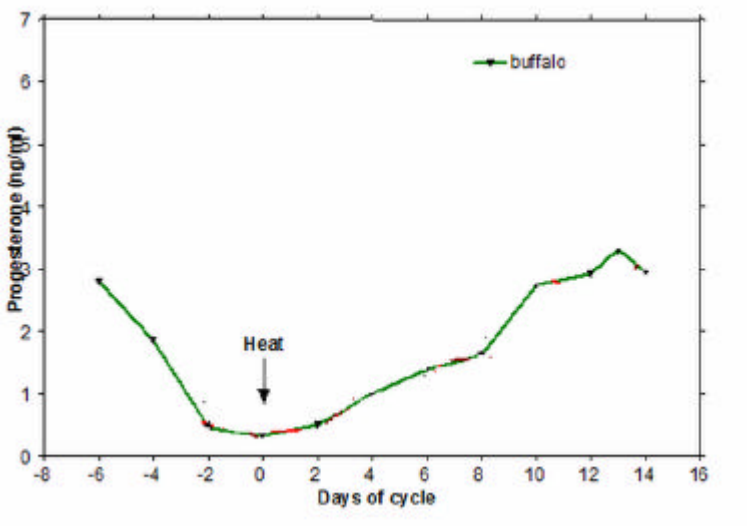


Fig. 25 : Plasma progesterone profiles during different days of estrous cycle in buffalo (Palta *et al*., 1996).

However, results from the dispersed bovine luteal cell culture (Nicklin *et al*., 2007) and bovine luteinised granulosa cell cultures (Glister, 2001) showed that leptin alone had no significant effect on basal progesterone production, but, in the presence of IGF-1, leptin caused a significant increase in progesterone production. Interestingly, in one earlier study, tendency of an increase IGF-1 mRNA expression during mid- and late-luteal phase has been observed in bovine (Schams *et al*., 2002). Hence, in vivo, leptin in conjunction with IGF-1 play an important role on luteal steriodogenic function in buffalo.

A crossover in the cellular responses elicited by each factor may go some way to explaining the interaction between leptin and IGF-1. IGF-1 has direct stimulatory effects on key components of the steriodogenic pathway to increase progesterone secretion in bovine luteal cells (Sauerwein *et al*., 1992). In particular, IGF-1 is able to induce the expression of StAR mRNA in bovine granulosa-derived luteal cells (Mamluk *et al*., 1999).

The stimulatory effects of leptin on porcine GC steriodogenic function have also been attributed to the induction of StAR transcription, as the result of sterol regulatory element binding protein 1 (SREBP-1) modulation (Ruiz-Corte's *et al*., 2003). It is therefore feasible to suggest that it is the synergistic effects of leptin and IGF-1 on StAR transcription, which leads to a response great enough to increase progesterone synthesis.

The interaction between leptin and IGF-1 on luteal function demonstrates that the corpus luteum may be under endocrine influences that can be manipulated by diet. Nutrition is an important modulator of IGF-1 and experiments in vitro provide evidence that nutrition acting via the IGF system can influence luteal efficiency and function (Sauerwein *et al*., 1992). The role of leptin in relating nutritional status to the reproductive system is also well documented (Caprio *et al*., 2001).

Angiogenesis is the differentiation and growth of new blood vessels from pre-existing microvasculature. Ovarian function is critically dependent on angiogenesis for follicle development, ovulation and corpus luteal function. Corpus luteum formation involves recurrent, regulated, and self-limited angiogenesis. The initial steps of endothelial cell invasion and migration are mediated by proteolytic enzymes (urokinase, tissue-type plasminogen activators and matrix metalloproteinases) and cell adhesion molecules (integrins and cadherins) (Mignatti *et al.*,

1996; Bischoff *et al.,* 1997). "Angiogenic factors" including vascular endothelial growth factor (Dvorak *et al.,* 1995), basic fibroblast growth factor (Presta *et al.,* 1986) and angiopoietins (Suri *et al.,* 1996; Holash *et al.,* 1999) that promote endothelial cell survival, proliferation, and differentiation are attached to the extracellular matrix (ECM).

Angiogenesis in the ovary enables the hormone-producing cells to obtain the oxygen, nutrients and also precursors necessary to synthesize and release different hormones essential for the maintenance of the ovarian functions. Luteolysis is characterized by degeneration of vasculature and steroidogenic cells with consequent decline in progesterone secretion. Leptin has both angiogenic and mitogenic effects. Leptin stimulats the expression of vascular endothelial growth factor (VEGF).

Leptin that influences the gene expression of some of these angiogenic hormones in non-ovarian tissue has also been identified in the porcine and caprine corpus luteum. Leptin regulates the production of VEGF, Ang-1, and FGF-2 in developing luteal tissue and ultimately corpus luteum formation and progesterone production (Robin *et al*., 2009 and Jessica *et al*., 2008). Vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1) and fibroblast growth factor 2 (FGF-2) are proangiogenic factors that regulate angiogenic process.

The production of leptin in the postovulatory corpus luteum by IHC and western blotting was detected. The results of the present study coincide with previous reports that have detected leptin mRNA by RT-PCR (Löffler *et al*., 2001) and protein by IHC (Löffler *et al*., 2001; Ryan *et al*. 2002). The number of luteal cells observed in the study containing leptin protein increases during maturation and decreases during regression of the corpora lutea. Immunoreactivity for leptin appears throughout the cytoplasm of these steroid-secreting cells. Löffler *et al*. (2001) also reported variations in leptin mRNA and protein in the corpus luteum progression. However, contrary to present findings, these authors reported that the greatest amounts of leptin was found in the developing corpus luteum, whereas lesser amounts were found in the secretory phase. This contradiction may be explained by the fact that they obtained the stain not in the steroid-producing cells but in fibroblast-like cells derived from the thecal layer.

Results from immunohistochemical staining indicate that the leptin produced in the corpus luteum may be acting through functional receptors in all parts of the corpus luteum,

specifically at blood vessels. Leptin may play a role in angiogenesis, as leptin receptors are expressed on vascular endothelial cells, possibly by playing a role in matrix remodelling by regulating the expression of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases

The study suggest that leptin may also act to control the production of its own receptors via actions through existing receptors, the results showed an increase in receptor mRNA production immediately after an increase in leptin mRNA production.

There is currently speculation about the role of leptin within the CL, without any clear evidence to indicate its precise function in this organ. Study suggest that leptin has actions on the CL via its specific receptors at ovulation and/or immediately after ovulation and may act on ovarian macrophages or infiltrating monocytes, to promote cytokine production and phagocytosis that occurs during tissue remodelling throughout ovulation and corpus luteum production; and blood vessels within the ovary, to promote angiogenesis during development of the corpus luteum. Present study provides evidence that the production of leptin and its receptors is regulated within the CL, and study further suggest that the leptin produced in the CL may regulate its own receptor.

On the basis of the results of the present study it can be concluded a possible involvement of locally produced leptin and leptin receptor system in the buffalo ovary where they influence the function and/or development of CL in an autocrine/paracrine fashion.

Chapter VI

Summary and Conclusions

Leptin, a 16.4 kDa peptide hormone, product of the obese gene, is secreted primarily in adipocytes and is known to play a critical role in the regulation of body weight (Zhang *et al*., 1994) and reproduction.

Leptin signalling is accomplished via receptors which have six isoforms (Tartaglia *et al*., 1995) and brought about through JAK-STAT pathway. Leptin modulates SREBP 1 & StAR transcription and in turn steroidogenesis in cells. Leptin receptors exhibit widespread distribution in mammalian tissue (Lee *et al*., 1996). The expression of leptin receptors has been demonstrated in human, mouse, rat, pig and bovine ovaries (Karlsson *et al*., 1997; Kikuchi *et al*., 1999; Duggal *et al*., 2000; Ruiz-Corte's *et al*., 2000; Nicklin *et al*., 2007 and Sarkar *et al.,* 2009). Leptin induces angiogenesis and proliferation of ovarian cells (Spicer and Francisco, 1997 and Bouloumie *et al*., 1998) and more specifically produce progesterone from CL (Sarkar *et al.,* 2009).

Ovaries containing corpus luteum were collected from slaughterhouse and were assigned to the following stages; days 1–4, 5–10, 11–16 and >17 of oestrous cycle. Luteal tissues were stored in RNAlater® Solution till RNA isolation. RNA isolation was done by using SV Total RNA Isolation System (Promega Corporation, USA). The purity and concentration of total RNA was checked using nanodrop reading. RNA samples showing the OD 260: OD 280 values more than 1.8 were used further. The integrity of the total RNA was checked using agarose gel (1%) electrophoresis and bands of 28s and 18s with smearing indicated good quality and intactness of RNA.

RT-PCR was done using reverse transcription system (FERMENTAS, USA) by taking 2 µg of total RNA. The cDNA was stored at -20°C for further use. The integrity of the cDNA was checked by PCR with histone primers and desired band of 233 bp is obtained. Block PCR was standardized using factor specific primers to amplify buffalo ob and ob-R gene sequence and product of desired length were obtained. qPCR was performed with DyNAmo™ HS SYBR® Green qPCR kit following standard procedure with slight modification. Specificity of the desired products were documented using analysis of the melting temperature, which is product specific and a high resolution gel electrophoresis to verify that the transcripts were of the exact molecular size predicted and further confirmed by sequence analysis. This was done at DNA sequencing facility at ILS by automated sequencer.

The mRNA expression during early-luteal stage (day 1-4) maintained low but detectable, as was the case in the regressed CL and after that expression level started to increase and reached maximum ($p<0.05$) in the mid cycle CL (day 11-16) followed by gradual decline during late-luteal stage (days >17).

Harvested CLs were immediately processed for immunohistochemistry and sections of 5 µm thickness were cut on a microtome and fixed in acetone. Then rehydrated with graded ethanol. Endogenous peroxidase activity was blocked with 3% H_2O_2. Slides were washed for 5 min, placed in 0.01 M citrate buffer (pH 6.0), and incubated for 15 min for antigen retrieval.

Blocking was performed 5% BSA in PBS and non-immune serum (1:5 dilutions) taken from the Goat. The tissue sections were incubated for 2.5 hours at 4^0C with a rabbit polyclonal antibody specific for leptin diluted 1:200 for both ob and ob-R in 5% BSA. The sections were incubated with secondary antibody diluted 1:250 for both ob and ob-R for 1.5 hours at 37°C and washed in chilled PBS for 5 minutes. The peroxidase activity was revealed using DAB solution and visualised under microscope.

SDS-PAGE analysis was carried out using gradient separating gel (7.4-15%). Samples were boiled before loading. Electrophoresis was carried out and stained with Coomassie brilliant blue for 1 hour and then destained. Proteins were characterized by Western blot analysis in order to confirm specificity. Four Whatman filter papers, PVDF membrane, SDS-

PAGE gel, and four whatman filter papers were stacked in respective order one by one on the anode plate of blotting apparatus after soaking with the western blot buffer. The cathode plate was placed in position over the stack and a current was applied for transfer of protein. The membrane was subjected to immunological detection. The membrane, after transfer, was incubated overnight at 4°C in diluted with PBS. After blocking with 1% BSA, the membranes were washed with PBS-T and incubated with primary antibody in 1:100 dilutions for 2 hr. Again washed with PBS-T then Goat antirabbit IgG HRP conjugate for 1hr at 37°C were added. After washing, the antigen antibody reaction was detected by incubating the membrane with substrate. The colour reaction was terminated by washing the membrane with distilled water to prevent background coloration.

CONCLUSIONS

- The abundance of transcripts of both leptin and its receptor varied through the luteal phase.
- The mRNA expression of Ob and Ob-R during early-luteal stage maintained low, as was the case in the regressed CL and after that expression level started to increase and reached maximum in the mid cycle CL.
- Results from IHC and western blotting indicate that the leptin produced in the corpus luteum may be acting through functional receptors in all parts of the corpus luteum, specifically at blood vessels.
- Leptin plays a role in angiogenesis, CL formation, remodelling and regression.
- Expression pattern of leptin and its receptor which corresponds to the pattern of progesterone secretion thus may play an important role in ovarian steroidogenesis.
- Leptin and leptin receptor may act in autocrine and paracrine fashion in regulating the ovarian function.

Final thoughts

- Expression of leptin and its receptor mRNA was done by quantitative RT-PCR and protein for the same by the immunohistochemistry and western blotting in corpus luteum during different days of estrous cycle in buffalo.
- The mRNA expression of Ob and Ob-R during early-luteal stage maintained low, as was the case in the regressed corpus luteum and after that expression level started to increase and reached maximum in the mid cycle corpus luteum as is also evident from findings of immunohistochemistry and Western Blotting.
- Leptin and its receptor may act in autocrine and paracrine manner in corpus luteum and may play an important role in angiogenesis, formation, function as a regulator of steroidogenesis.

Chapter VII

Mini Abstract

Leptin, a 16.4 kDa peptide hormonal product of the obese (Ob) gene, has role in the regulation of body weight, energy expenditure and reproduction. Its role in reproduction includes important actions on the hypothalamus to bring about release of LH-releasing hormone, thereby triggering gonadotropin release and leading to development of the reproductive tract and induction of puberty. Leptin signalling is accomplished via receptors which have six isoforms and exhibit widespread distribution in mammalian tissue is brought about through JAK-STAT pathway. Leptin modulates SREBP 1 & StAR transcription and in turn steroidogenesis in cells. Large population of buffalo remain unproductive due to sub-oestrous, anoestrous, infertility which has hormonal aetiology. With endocrine interventions, it might be possible to improve the fertility of these animals. The present study has demonstrated the expression of leptin and its receptor (Ob-R) in buffalo CL obtained from different stages of the oestrous cycle. Real-time RT-PCR was applied to investigate mRNA expression of examined factors. Specificity of the desired products were documented using analysis of the melting temperature and high resolution gel electrophoresis to verify that the transcripts are of the exact molecular size predicted and further confirmed by sequence analysis. Proteins of leptin and its receptor were also localised with the immunohistochemistry and Western Blotting. The mRNA expression of Ob and Ob-R during early-luteal stage maintained low, as was the case in the regressed CL and after that expression level started to increase and reached maximum in the mid cycle CL as is also evident from findings of immunohistochemistry and Western Blotting. Leptin plays a role in angiogenesis, CL formation, remodelling and regression. Leptin and leptin receptor regulate steroidogenesis in autocrine and paracrine manner in CL.

Chapter VIII

लघु सारांश

लेप्टिन जो 16.4 किलो डालटन का पेप्टाइड है, ओ बी जीन की हार्मोनल उत्पाद है। इसका शरीर के वजन, ऊर्जा व्यय और प्रजनन के विनियमन में प्रमुख भूमिका है। प्रजनन के महत्वपूर्ण कार्यो में लेप्टिन की भूमिका अधश्रेतक से एल एच निस्तारक हार्मोन स्रावित करना है, जिससे गोनैडोट्रोपीन की रिहाई होती है, और प्रजनन पथ का विकास तथा यौवन का आगमन होता है। लेप्टिन संकेतन, जो प्रापक के माध्यम से पूरा होता है। जिसके छह समरूप हैं तथा जिसका स्तनधारी ऊतक में व्यापक वितरण है, जैक ऐसटैट के रास्ते होता है। लेप्टिन ऐस आर ई बी पी$_1$ और स्टार प्रतिलेखन को प्रभावित करता है। जिससे कोशिकाओं के स्टेरॉयड सृजन में उतार–चढ़ाव आता है। भैंस की बड़ी आबादी छोटा–कामोंमाद, अमदकाल, बांझपन, जिसका कारण हार्मोन है, के कारण अनुत्पादक रह जाती है, अंत स्त्रावी हस्तक्षेप से इन जानवरों की प्रजनन क्षमता में सुधार संभव हो सकता है। वर्तमान अध्ययन ने लेप्टिन और इसके प्रापक की अभिव्यक्ति का प्रदर्शन भैंस के मद चक्र के विभिन्न चरणों से प्राप्त पीत–पिण्ड में किया है। वास्तविक समय आर्टी–पीसीआर का उपयोग कारकों के ऐम आर एन ए की अभिव्यक्ति की जांच करने के लिए किया गया वांछित उत्पादों की विशष्टिता, पिघल–वक्र के विश्लेषण तथा उच्च संकल्प जेल वैद्युतकणसंचलन से प्रलेखित किया गया तथा यह सत्यापित किया गया कि प्रतिलेख का ऐक सटीक भविष्यवित आणविक आकार हैं, और इसकी आगे अनुक्रमन विश्लेषण द्वारा पुष्टि की गई। लेप्टिन और उसके प्रापक के प्रोटीन को प्रतिरक्षाऊतिकीरसायन और वेस्ट्र्न बलौटींग से भी स्थानीयकृत किया प्रारंभिक चरण के पीत–पिण्ड में ओ बी तथा ओ बी–आर का ऐम आर एन ए अभिव्यक्ति कम था। प्रत्यावर्ती सीएल का भी यही मामला था पर उसके बाद अभिव्यक्ति का स्तर बढ़ाना प्रारंभ होकर मध्य चक्र के पीत–पीण्ड में अधिकतम तक पहुंच गया, और इसकी पुस्टी प्रतिरक्षाऊतिकीरसायन और वेस्ट्र्न बलौटीग के निष्कर्षो से भी होती है। लेप्टिन रक्तनलिकासृजन, पीत–पिण्ड रचना, पुनृढालन और प्रत्यावर्तन में महत्वपूर्ण भूमिका निभाता है। लेप्टिन और लेप्टिन प्रापक पीत–पिण्ड में औटोक्रायन और पराक्रायन तरीके से स्टेरॉयड सृजन को नियंत्रण करता है।

Chapter IX

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Appendix

1. SOLUTIONS USED FOR IMMUNOHISTOCHEMISTRY

1. PBS 0.01M, 7.4 pH

$Na_2HPO_4:2H_2O$	1.86 gram/litre
KH_2PO_4	0.43 gram/litre
NaCl	7.20 gram/litre

2. 5% BSA in PBS.
3. 3% H_2O_2 (Freshly prepare, protect from light).
   Dilute 10 times of 30%
4. 0.01M Citrate buffer. (MW 294.1)
   For 10 ml take 29.41 mg
5. Substrate solution

PBS	10 ml
DAB	50 µl
30% H_2O_2	10 µl

2. SOLUTIONS USED FOR SDS-PAGE AND WESTERN BLOTTING

1. Separation gel buffer (pH 8.8)

Tris	18.17 gm
SDS	0.40 gm

   Distilled water was added to make the final volume to 100 ml.
2. Stacking gel buffer (pH 6.8)

Tris	3.025 gm

   Distilled water was added to make the final volume to 100 ml.
3. Gradient separating gel (7.5-15%)

	7.5%	15%
Separating buffer	2.0 ml	2.0 ml
APS (20%)	0.08 ml	0.08 ml
30% acryl amide	4.00 ml	8.00 ml
Autoclaved distilled water	9.92 ml	5.92 ml
Total	16 ml	16 ml

4. Stacking gel 5%

Stacking buffer	1.0 ml
30% acryl amide	1.066 ml
APS 20%	0.040 ml
Autoclaved distilled water	5.894 ml
Total	**8.00 ml**

5. Tris Glycine electrophoresis buffer (5X)

Tris base	15.1 gm
Glycine (pH 8.3)	93.8 gm

Dissolve in 900 ml deionized water. 50 ml of 10% SDS solution was added and the volume was adjusted to 1 litre with deinozed water.

6. Sample buffer (2X)

Stacking gel buffer	1.7 ml
10% SDS	4.5 ml
Glycerol	1.0 ml
Bromophenol	2.0 ml
Distilled water	2.8 ml

7. 10% Ammonium persulphate

APS	1.0 gm
Distilled water	10 ml

Stored at 4°C.

8. 30% acrylamide

Acrylamide	29.2 gm
N-N-methylene bis acrylamide	0.80 gm

Dissolve in 60 ml distilled water by heating at 37°C and adjust the volume to 100 ml. Filtered and stored in amber colored bottle at 4°C.

9. Gel staining solution

Coomassie brilliant blue	0.25 gm
Methanol : deionized water (1:1)	90.0 ml
Glacial acetic acid	10 ml

The solution was filtered through Whatman filter paper no. 1.

10. Gel destaining solution

Methanol : deinoized water (1:1)	90 ml
Glacial acetic acid	10 ml

11. Phosphate buffered saline (PBS)

NaCl	8.00 gm
KCl	0.20 gm
Na_2HPO_4 (anhydrous)	1.44 gm
KH_2PO_4	0.24 gm

Distilled water was added to make the final volume upto 1litre. The pH was adjusted to 7.4 with HCl. The resulting solution was autoclaved and stored.

12. Transfer buffer

Tris base	2.90 gm
Glycine	11.95 gm
SDS-PAGE	0.185 gm
Methanol	100 ml

Distilled water was added to make the final volume upto 50 ml.

13. Blocking buffer

	PBS	1000 ml
	Tween-20	0.05%
	BSA	1%
14.	Dilution buffer	
	PBS	1000 ml
	Tween-20	0.05%

3. REAGENTS USED IN AGAROSE GEL ELECTROPHORESIS

1. Tris-acetate-EDTA (TAE) buffer 50 X

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 ml EDTA (pH 8.0)	100 ml

Distilled water was added to make up to a final volume of 100 ml. A working solution of I X was used.

2. Ethidium bromide stock solution (10 mg/ml)

Ethidium bromide	100 mg
Distilled water	10 ml

The solution was mixed and stored at 4°C. A concentration of 0.5 µg/ml was used in preparing agarose gel.

3. Loading dye (6X)

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)

Sucrose 40% (w/v)

Store at 4°C.

Vitae

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