EXPRESSION AND LOCALIZATION OF VISFATIN IN CORPUS LUTEUM OF BUFFALO AND ITS INFLUENCE ON PROGESTERONE SECRETION

THESIS

Submitted

in partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY PHYSIOLOGY

BY

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(INDIA)

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I hereby declare that the experimental research work and Interpretation of the thesis entitled **"EXPRESSION AND LOCALIZATION OF VISFATIN IN CORPUS LUTEUM OF BUFFALO AND ITS INFLUENCE ON PROGESTERONE SECRETION"** or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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"Dedicated To My Parents and Guide"

Date :

Place: **Nagpur** ***Ankita Thakre***

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ABBREVATIONS

%	:	Percentage
<	:	Lesser than
>	:	Greater than
°C	:	Degree Celsius
3β-HSD	:	3β-hydroxysteroid dehydrogenase
ANOVA	:	Analysis of variance
BAX	:	Bcl-2-associated X protein
β-Actin	:	Beta actin
BP	:	Base pairs
BSA	:	Bovine serum albumin
CBB	:	Coomassie brilliant blue
Cdna	:	Complementary DNA
CL	:	Corpus luteum
CO_2	:	Carbon dioxide
Ct value	:	Cycle threshold values
CYP11A1	:	Cytochrom p450 A1 (cholesterol side chain cleavage)
DAPI	:	4,6-diamidino-2-phenylindole
DEPC	:	Diethyl pyrocarbonate
DMEM/F12	:	Dulbecco Modified Eagle and F-12 Ham Medium
DF	:	Dominant follicle
DNA	:	Deoxy ribonucleic acid
DW	:	Distilled Water
EDTA	:	Ethylene diamine tetra acetic acid
FBS	:	Fetal Bovine Serum
FF	:	Follicular fluid
M	:	Molar
Mg	:	Milligram
Min	:	Minutes

$MgCl_2$	:	Magnesium Chloride
Ml	:	Millilitre
Mm	:	Millimeter
mM	:	Millimolar
MM	:	Master mix
Mol.Wt.	:	Molecular weight
mRNA	:	Messenger ribonucleic acid
Fig	:	Figure
GC	:	Granulosa cell
H	:	Hour/s
H_2O_2	:	Hydrogen peroxide
HRP	:	Horse radish peroxidase
KDa	:	Kilo Daltons
LN_2	:	Liquid nitrogen
NFW	:	Nuclease free water
Ng	:	Nanogram
NTC	:	No template control
OD	:	Optical density
P_4	:	Progesterone
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PCNA	:	Proliferating cellular antigen
Pmol	:	Pico mole
PVDF	:	Polyvinylidene difluoride
qPCR	:	Quantitative PCR
qRT-PCR	:	Quantitative real-time PCR
RNA	:	Ribonucleic acid
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SEM	:	Standard Error of Mean
SD	:	Standard deviation

Sec	:	Seconds
SDS	:	Sodium dodecyl sulphate
TAE	:	Tris Acetate EDTA buffer
TI	:	Theca interna
Mg	:	Microgram
Ml	:	Microliter
g	:	Gram

INTRODUCTION

Water buffalo, *Bubalus bubalis* is an economically important livestock species in Asian and Mediterranean countries. It is a triple purpose animal producing milk, meat and draught purposes. The world buffalo population is estimated at 185.29 million across 42 countries, of which 179.75 million (97%) are in Asia (Thiruvenkadan *et al*., 2013). During the last 10 years, the world buffalo population increased by approximately 1.49% annually, by 1.53% in India, 1.45% in Asia and 2.67% in the rest of the world.

The country has made a grand spectre on the World stage being top milk producer and leading meat exporter with crucial contribution of 71.3 Million tonnes milk (53 % of total) and 86 per cent of the exported meat from buffalo alone. Buffalo meat export earned foreign exchange to the tune of 26,000 crore rupees in 2013-14.

Despite of economic importance of buffaloes in agriculture, dairy and meat industries, very little information is available on their gene organization, evolution and transcriptional status, including the molecular regulation of ovarian functions.

The cycle of reproduction is characterized by alternating pattern of cellular proliferation and differentiation that leads to follicular development along with the development and regression of corpus luteum in cyclic manner (Evans and Fortune, 1997). In buffalo, the follicular growth is characterized by 2-3 follicular waves during estrous cycle and when the follicle in a wave attains around 8.5 mm size, this follicle continue growing to about 14-20 mm size then it normally ovulates or begins regressing (Ginther *et al.,* 2003). The Corpus Luteum (CL) plays a central role in the regulation of the estrous cycle and in the maintenance of pregnancy. This function is carried out largely by progesterone, which is the main steroid synthesized by this transient endocrine gland. If the oocyte is not fertilized, the CL regresses, allowing a new cycle to begin. These events are finely controlled by various signaling molecules secreted by various tissues in the ovary.

Adipose tissue comprises of adipocytes and secrete a variety of substance called adipokines, thus describes as body’s largest endocrine gland

(Ahima *et al.,* 2006). Chief adipokines secreted from adipose tissue are leptin, adiponectin, resistin, visfatin, chemerin and apelin etc. (Tsatsanis *et al*., 2015).

Out of these signaling molecules, Visfatin has indeed arisen as a multifaceted and ubiquitously expressed molecule in the last few years that exerts multiple biological actions beyond the adipose tissue. The term visfatin refers to visceral fat, since it was initially suggested that visfatin was mainly produced in visceral fat compared to subcutaneous fat in both mice and humans. It is constitutively synthesized by adipose tissue and many other tissues, including reproductive tissue (Reverchon *et al.,* 2014) and having pleiotropic biological effects (Samal *et al.,*1994). It exists in two molecular forms, the extracellular is cytokine type and intracellular is enzymatic type (Revollo *et al*., 2004).

Visfatin is a 52-kDa dimer protein, containing 491 amino acids in each monomer in humans. The dimerization is essential for it's catalytic activity. Each monomer contains 19 β-strands and 13 α-helices and is organized into two structural domains (Sonoli *et al.,* 2011)

Visfatin is also known as Nicotinamide phosphoribosyl transferase (NAMPT) or a pre-B cell colony – enhancing factor (PBEF) (Stephens and Vidal-Puig, 2006; Dahl *et al.,* 2012). NAMPT was originally identified 40 years ago (Dietrich *et al.,* 1966, Powanda *et al.,* 1969, Streffer & Benes 1971) and was found to act as an intracellular enzyme that catalyzes the rate-limiting conversion of nicotinamide to nicotinamide mononucleotide (NMN), a substrate in the biosynthesis of NAD. Several years later, the peptide was rediscovered as pre-B cell colony-enhancing factor, a presumptive cytokine that enhances maturation of B-lymphocyte precursors in the presence of interlukin-7 and stem cell factor (Samal *et al.,* 1994) and directly correlates with the fetal growth in gestational diabetes, PCOS and uterus (Shang *et al*., 2009; Ma *et al*., 2010; Dikmen *et al*., 2011; Guducu *et al*., 2012; Rafraf *et al*., 2012; Kaygusuz *et al*., 2013).

Visfatin promotes synthesis and storage of fat, in vivo regulates the glucose and lipid metabolism and is expressed widely in bone marrow stromal cells, macrophage, liver, uterus, muscle tissue, lymphocytes, pancreas, embryolemma and trophoblast cells besides visceral adipose tissue (Samal *et al*., 1994; Ognjanovic *et al*., 2005; Ons*et al*., 2010; Kover *et al*., 2013; Jung *et al*.,

2013), Kendal and Bryant-Greenwood, 2007 and Dahl *et al.,* 2012). The expression is up regulated during obesity and type II diabetes (Beltowski, 2006; Chen *et al.,* 2006). It mimics insulin and stimulates glucose uptake in skeletal myocytes and adipocytes (Fukuhara *et al.,* 2005; Bohler *et al.,* 2010; El-Mesallamy *et al*., 2013).

There are positive correlations between visfatin/NAMPT serum concentrations and age, blood pressure, creatinine, free fatty acid (FFA) and the inflammatory markers IL-6 and CRP. On the contrary, other groups suggest that decreased expression of visfatin, as a pro angiogenic factor, may be associated with the pathogenesis of pre-eclampsia (Fasshauer *et al.,* 2008)

NAMPT has been located in human ovarian follicles and suggested that it is directly involved in regulating reproductive functions in mammals (Reverchon *et al*., 2013).

A correlation between the concentration of NAMPT in the follicular fluids and the number of oocytes retrieved in women undergoing controlled ovarian stimulation was reported by Shen *et al. (*2010).

Gestational diabetes, polycystic ovary syndrome and fetal growth in uterus directly correlates with visfatin (Kaygusuz *et al.,* 2013). The relationship between the expression of visfatin in uterus and embryolemma and intrauterine fetal growth, gestational diabetes and polycystic ovary syndrome suggests that visfatin might functionally relate with the reproductive system. Visfatin expression in testies of avian species and sexual maturation in avians is associated with an increase in testicular NAMPT expression and circulating NAMPT levels in broiler breeder chickens (Ocon-Grove *et al.,* 2010). It is immunolocalized in diabetic and normal rat testes (Gurusubramanian and Roy, 2014).

Visfatin/NAMPT is induced *in vitro* by inflammatory stimuli in cells that are involved in immunity responses and also itself potently induce the release of inflammatory cytokines in PBMCs in a dose dependent manner from patients with unstable angina when compared with cells from healthy control (Dahl *et al*., 2007). Recombinant visfatin/NAMPT induce the release of IL-1, TNFα, and IL-6 in human monocytes and extracellularly promotes chemotaxis and upregulates adhesion molecule (Moschen *et al.,* 2010)

Visfatin/NAMPT has been recently proposed as a candidate in the pathogenesis of endothelial dysfunction in polycystic ovary syndrome (PCOS), the main androgen excess disorder in women. Visfatin was expressed more specifically in leydig cells, spermatocytes and sperm in the testes of rat while it has been shown to increase testosterone production in cultured leydig cell of Sprague Dawley rats (Hameed *et al.,* 2012) Further, visfatin in mouse ovary has regulatory effect on IFN-γ and progesterone secretary role in hen granulose cells. (Diot *et al.,* 2015b)

As per the literature available, Visfatin has been shown to regulate gonadal functions in rats, humans and chickens. However, no report is available regarding existence of visfatin in buffalo ovary. Therefore, the present study is proposed to investigate the expression and localization of visfatin in corpus luteum of buffalo ovary and its *in vitro* effect on progesterone secretion.

OBJECTIVES

1. To study the expression of Visfatin mRNA and protein during different developmental stages of corpus luteum in buffalo ovary.
2. To study the localization of Visfatin during different developmental stages of corpus luteum in buffalo ovary.
3. To study the effect of Visfatin on progesterone secretion in cultured luteal cells.

REVIEW OF LITERATURE

The literature available on relevant aspects of present study was reviewed extensively. The important and related findings of research work done by various scientists are presented below.

2.1 Expression of Visfatin mRNA and protein

Tan *et al*. (2006) detected visfatin mRNA expression in adipose tissue and in adipocytes with normal and polycystic ovary syndrome (PCOS) women. They showed the expression of visfatin in subcutaneous (sc) and omental (om) adipose tissue for both mRNA and protein levels and also reported the presence and significance of up regulated visfatin gene expression and protein in adipose tissue depots in women with PCOS.

Chang *et al.* (2010) suggested visfatin as a proinflammatory cytokine and a marker of adipose tissue associated with systemic insulin resistance and hyper lipidemia. The mRNA level of Visfatin/PBEF/NAMPT macrophages specific marker CD68 and proinflammatory gene were measured predominantly in paired visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) and found no difference in between the level of mRNA visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) and neither were associated with the measures of obesity but were positively correlated with steady state plasma glucose concentration.

Ma *et al. (*2010) observed a significantly higher expression of visfatin mRNA and protein in placenta of gestational diabetic mellitus (GDM) women than those with control groups and positively correlated the serum visfatin level with mRNA and protein expression in placenta whereas in SAT and VAT it did not associated with it's mRNA and protein expression.

Ocon-Grove *et al*. (2010) by using real time quantitative PCR found that NAMPT mRNA quantity was significantly greater in adult chickens as compared to prepubertal chicken. To detect NAMPT protein in chicken testis and whether the NAMPT protein quantity differs between prepubertal and adult chickens western blot was performed and concluded that the testicular NAMPT protein quantity were not significantly different between adult and prepubertal chickens.

Seow *et al.* (2011) investigated the visfatin expression in tissue from two different sources, i.e. omental adipose tissue and peripheral blood mononuclear cells (PBMCs) in women with polycystic ovary syndrome (PCOS) and in controls and expressed higher visfatin mRNA in both omental adipose tissue and PBMCs in women with PCOS, it indicated that mononuclear cells are a source of secreted visfatin in addition to adipose tissue in women with PCOS.

Choi *et al*. (2012) investigated expression of visfatin mRNA and protein from two groups of differently aged mice (6–11 and 26–31 weeks) and observed that visfatin was expressed in the ovary regardless of the age of female mice. Its expression was increased with advancing follicular development in both aged and young mice after PMSG administration. However, expression of both visfatin mRNA and protein was significantly decreased in aged mice compared with young mice.

Reverchon *et al*. (2013) identified visfatin in human ovarian follicle and human ovarian granulosa-like tumors cell line (KGN).They detected visfatin in granulosa cells (GC), cumulus cells ,oocytes and theca cells but visfatin was expressed more particularly in granulosa cells (GC).They next investigated the effect of two insulin sensitizers, metformin (MetF) and rosigilitazone on visfatin mRNA and protein expression and observed that MetF increases expression of visfatin mRNA in a dose dependent manner and the level of visfatin protein is increased by more than 2 fold whereas rosigilitazone increased visfatin mRNA expression only at a particular concentration and did not affect visfatin protein expression. They studied that visfatin expression is regulated by MetF through AMP activated protein kinase (AMPK) and Sirtinol, an inhibitor of sirtuin-1 (SIRT1) in hGCs and KGN.

Gurusubramanian and Roy (2014) showed expression of visfatin in the diabetic rat testis with a significant decrease in visfatin expression as compared with control testis by western blot analysis.

Diot *et al.* (2015a) showed the mRNA and protein expression of visfatin in ovarian cells (granulosa and theca cells) in turkeys (*Meleagris gallopavo).* mRNA expression of visfatin was higher in theca cells compared to granulosa cells in both F1 and F3/4 hierarchical follicles and the protein levels were confirmed by immunoblotting.

Diot *et al*. (2015b) concluded that the NAMPT is present in ovarian cortex and in granulose and theca cells from hierarchical follicles in hen. Significantly higher mRNA expression was observed in granulosa cell in F1 and F2 hierarchical follicles and the level of expression was similar in granulosa and theca in F3 hierarchical follicle. Also investigated NAMPT mRNA and protein expression in hen during follicle development in both granulosa and theca cells. As the follicle develops there was a clear decrease in expression of NAMPT mRNA in theca cell where as in granulosa cell it remained stable.

Novak *et al.* (2015) determined the physiological values of serum visfatin concentration and visfatin mRNA expression in prepubertal healthy boys and found visfatin mRNA higher in subcutaneous adipose tissue (SAT) when compared with visceral adipose tissue (VAT).

Reverchon *et al*. (2016) showed that NAMPT/ Visfatin is highly expressed in large follicle (LF) than the small follicle (SF) of granulosa cells (GC) and used adipose tissue and mammary gland as positive controls and observed that visfatin protein is present in bovine CL and different follicular cells. Level of protein was high in LF than SF of granulosa cells and furthermore, they found that the amount of visfatin decreases in oocytes by in vitro maturation (IVM) but it does not affect the level of visfatin protein in cumulus cells. They investigated the effect of FSH, IGF-I and Visfatin itself on NAMPT mRNA expression from small follicle (SF) in primary bovine granulosa cell and showed significant increase in NAMPT mRNA expression after 48 hour of stimulation by IGF-1 and Visfatin but not by FSH.

2.2 Localization of Visfatin by Immunohistochemistry

Ocon-Grove *et al*. (2010) localized NAMPT in prepubertal and adult chicken testis using immunohistochemistry. They observed that in prepubertal chicken testis NAMPT was localized in the nucleus of myoid cells, sertoli cells and in leydig cells. However in adult chicken NAMPT immunostaining was observed in the cytoplasm of leydig cells, sertoli cells, primary spermatocytes, secondary spermatocytes, round spermatids and elongated spermatids but not in spermatogonial cells.

Choi *et al*. (2012) localized visfatin expression in mice ovary by immunohistochemistry and showed decreased expression of visfatin in aged

mice compared with young during Immunostaining of ovary sections. The expression was localized in granulosa cells, cumulus cells, stromal cells, and endothelial cells and, the immunoreactivity of visfatin revealed more in granulosa and cumulus cells of young mice compared with aged mice

Reverchon *et al*. (2013) localized visfatin in human ovarian follicle by immunochemistry. By using antibodies against visfatin, DAB- immunoperoxidase staining was performed on paraffin embedded human ovary. The immunospecific staining was brown and then the section was counterstained with haematoxylin. Visfatin was detected in granulosa cells (GCs), cumulus cells (CC), theca cells (TC) and oocytes (OO) of human ovarian follicles.

Song *et al*. (2014) verified the correlation between visfatin and reproduction by observing the round and oval visfatin immunoreactive cells in different developmental stages (primary follicle and secondary follicle) of mouse ovarian follicles that also specially in the granular layer of growing ovarian follicle. It also indicated visfatin action on ovarian follicle for development and function due to its expression in granulose cells. The cells seen in the granular layer of growing follicle were numerous and compressed. Though the amount was fewer in granular layer of mature follicle they showed significantly positive reaction. In oocytes the visfatin cells were weakly positive and rarely expressed and lacked in germinal epithelium, theca folliculi, follicular matrix and atretic follicle.

Gurusubramanian and Roy (2014) localized visfatin in diabetic rat testis and observed that affected seminiferous tubules showed mild immunostaining in comparison with normal tubules of diabetic and control rat testis. While in normal seminiferous tubules of both diabetic and control rat showed a similar pattern of visfatin. Visfatin was observed in Leydig cells, spermatids and sperm while faint staining was observed in Sertoli cells and primary spermatocytes of normal seminiferous tubules of control and diabetic rat testes and visfatin was clearly seen in cell membrane of round spermatids. The diabetic rat with affected tubules showed giant cells with strong immunostaining.

Reverchon *et al.* (2016) performed immunohistochemistry with ovarian sections from bovine follicles and confirmed that the finding of immunoblot and RT-PCR revealed that visfatin is also present in the theca cells but less in granulosa cells.

2.3 Progesterone Secretion from CL cells

Ma *et al.* (2010) determined effects of TFN-α on visfatin expression in BeWo cells. Visfatin expression was measured in dose dependent way and interestingly at 48 hr incubation with TNF- α, visfatin secretion from BeWo cells increased at a concentration of 100 ng/ml when compared to non stimulated controls.

Reverchon *et al*. (2013) determined the effect of human recombinant visfatin on basal and IGF-I or FSH- induced progesterone production by KGN and hGCs cells. In basal state and in the presence of IGF-I visfatin treated with 10 ng/ml was increased by 2 fold secretion of progesterone and no effect was observed in the presence of FSH and confirmed the effect of visfatin on IGF-I induced steroid producing using FK866, a specific inhibitor of enzymatic activity of visfatin/ NAMPT.

Diot *et al*. (2015b) investigated the effect of recombinant human NAMPT (rhVisf) on progesterone secretion in granulosa cells from hierarchical follicles in hen. *In vitro* treatment with human recombinant NAMPT (100 ng/ml) for 48 hours halved basal and IGF1-induced progesterone secretion and this was associated with reduction in STAR and HSD3B protein levels and MAPK3/1 phosphorylation levels in granulosa cells. These effects were abolished by the addition of FK866, a specific inhibitor of NAMPT enzymatic activity. They concluded that NAMPT is present in hen ovarian cells and inhibits progesterone production in granulosa cells.

Reverchon *et al.* (2016) observed that IGF-I and visfatin (10 and 100 ng/ml) but not FSH increased mRNA expression levels of NAMPT after 48 hours of stimulations in the bovine granulosa cells and also observed that rhVisf increased the release of progesterone secretion and this was associated with an increase in the protein level of STAR, HSD3B activity, and the phosphorylation levels of IGF-1 R and MAPK ERK1/2 in the presence or absence of IGF1. Thus, in cultured bovine granulosa cells, visfatin improves basal and IGF1- induced steroidogenesis and IGF1 receptor signaling through SIRT1.

MATERIALS AND METHODS

The present study was conducted in the Department of Veterinary Physiology, Nagpur Veterinary College, Nagpur 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 Chemicals/ reagents/ kits

Chemicals for molecular biology were obtained from different companies like Promega, Sigma, Invitrogen, BioLab, Himedia, Roche as per requirement. 6X Loading dye, $MgCl_{2,}$ Reverse Transcriptase, oligo-dT15primer, PCR nucleotide Mix were from Promega, Madison WI USA. The chemicals like Tris, Chloroform, Isopropanol, Ethanol, Agarose, DEPC, RIPA buffer, Bradford reagent, nitrocellulose membrane (NC), bovine serum albumin Fraction v (BSA) from Himedia; 50 bp DNA ladder from BioLabs, New England; Trizol reagent from Invitrogen; DAB from Roche, Germany; GoTaq® Green Master Mix kit (Lot no- 0000291538) from Promega, Madison, WI, USA; AEC staning kit (Catlog No. AEC 101) from Sigma and Progesterone kit (Lot no.- #0289) from Neogen.

3.2 Antibodies

3.2.1 <u>Primary antibodies –</u>

- Rabbit anti PBEF 1(Lot – QC12802, Sigma)
- Rabbit Beta Actin (Lot-14, Cell signaling Technology)

3.2.2 <u>Secondary antibodies-</u>

- Goat Anti Rabbit IgG peroxidase (Lot- 026M4782V, Sigma)

3.3 Primer sequences

To amplify the genes, a set of gene specific primers were designed from the published sequence and for primer design the Fast PCR. (Version: 6.2.73) software was used. All the detail of primer sequence and amplicon length is shown in Table 1.

Table 1: Target gene, primer sequence, amplicon length and accession number for qRT-PCR used in the study

Target Gene	Primer sequence 5'-3'	Amplicon length (Bp)	Accession no./ Reference
Visfatin	For: GCGTGTGAGAAAACATGGGG Rev: TCATGC CTTCCA CAA TCT CTT G	250	MH675886
Beta actin	For: TCT CAC GGA GCG TGG CTA CAG Rev: CTGCTCGAA GTCCAG GGC CACGTA	100	NM_001290932.1
RPL15	For: TGGGCTACA AGGCCA AACAA Rev: GCT TCGAGCAAACTTGAGCTGG	140	MG969348
3β-HSD	For: GATCATCTGCCTGTTGGTGGA Rev: GTGGATGACCACTGAGGTGC	191	Kumar *et al.,* 2012
CYP11 A1	For: AGTTCGAGGGATCCTACCCAGA Rev: AGCCATCACCTCCGTGTTCAG	146	Gupta *et al.,* 2014
PCNA	For: ACCTGCAGAGCATGGACTCGTC Rev: CATGCTGGTGAGGTTCACGCCCA	160	NM_001034494.1
BAX	For: TCTGACGGCAACTTCAACTG Rev: AAGTAGGAGAGGAGGCCGTC	250	NM_173894.1

Target genes: Visfatin, beta actin and RPL15 are used for mRNA expression; 3β-HSD, CYP11A1, PCNA and BAX are used for *in vitro* mRNA expression

3.4 Glasswares and Plastic Wares

For RNA work, RNase-free plastic wares and glassware were used. They were thoroughly treated with 0.1% DEPC (Lot no- 0000296843, Himedia, India) 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°C for 15 minutes) and then used.

3.5 Equipments

Major equipments used were as follows:

1. -20^{0}C Deep freezer (Hoshizaki)
2. Agarose gel electrophoresis apparatus (Cat no- #MGU-202T, C.B.S. Scientific)
3. Real-Time qPCR System instrument (Insta Q 96, Himedia)
4. 96 well Thermal cycler (Thermofisher Scientific)
5. Nanodrop (Eppendorf, USA)
6. Blotting apparatus (Genaxy)
7. Cell counter (e.g., Countess® Automated Cell Counter or hemacytometer
8. Cell culture hood (i.e., laminar-flow hood or biosafety cabinet)
9. Gel documentation analysis system(BioRad)
10. Homogenizer (Cole Parmer LabGEN 7,USA)
11. Hot air oven
12. Incubator (humid CO_2 incubator recommended)
13. Inverted microscope
14. Laminar Flow
15. Liquid nitrogen (LN_2) freezer
16. Power Pac
17. Refrigerated centrifuge (BioEra)
18. Refrigerator (4 °C)
19. Double distillation apparatus
20. Spinner (BioEra)
21. Sterilizer (i.e., autoclave)
22. Water bath
23. Weighing balance (Contech)
24. Heating Mantle
25. Microwave Oven

3.6 Collection and classification of CL

Buffalo ovaries were collected from a local slaughter house after slaughter and were transported on ice to the work place. Total 40 ovaries, each with corpus luteum were used to extract 10 CL per group for RNA extraction, Western blotting and immunohistochemistry studies. The CL was classified into following stages:

early luteal phase, mid luteal phase, late luteal phase and regressed CL as described by Sarkar *et al.,* 2010. The criteria used for classification of CL is presented in Table 2. Luteal tissue was stored at -20°C until the RNA and protein isolation.

Table 2: Classification of Corpus Luteum

STAGE	External appearance on ovary	Appearance on CL
Early Luteal phase (day1-4)	Ovulation depression, recently ovulated and rupture not covered by epithelium	Vasculature not visible, red and hemorrhagic and doughy <1 cm in diameter
Mid Luteal phase (day5-10)	Soft developing CL	Vasculature limited to periphery, red in color and soft >1cm in diameter
Late Luteal phase (day 11-16)	Fully developed CL	Vasculature limited to periphery, reddish brown in color and hard >1 cm in diameter
Regressed CL(day>17)	Regressing CL	Surrounded by yellow lutein (white arrowhead)

3.7 Primers

For primer design the Fast PCR (Version: 6.2.73) software was used. The details of the primers used are shown in Table 1.

3.8 Quantitative RT-PCR analysis

Total RNA was isolated from different stages of CL (10 CL per group) by Trizol reagent (Invitrogen) according to manufacturer instructions. RNA quality, quantity and integrity were verified by agarose gel electrophoresis and spectrophotometric readings. Total RNA were transcribed using Go Script ™ 5X reaction buffer (Lot no.- 0000217608,Promega, Madison, WI, USA), $MgCl_2$ (Lot no.- 0000219154, Promega, Madison, WI, USA), PCR nucleotide mix (Lot no.- 0000226978, Promega, Madison, WI, USA), RNase inhibitor, Reverse Transcriptase (Lot no.- 0000230162, Promega, Madison, WI, USA) and oligo-

(dt15) primer (Lot no.- 0000222720,Promega, Madison, WI, USA). The resulting complimentary DNAs (cDNAs) were used in qRT-PCR reactions.

3.8.1 Total RNA extraction from CL

Total RNA extraction from CL was done using TRIZOL reagent (Invitrogen) described as below:

1. The CL was isolated from the ovary and stored at -20°C for further analysis.
2. Approximately 100mg of the luteal tissue was chopped.
3. The finely chopped luteal tissue were taken in eppendorf tube containing 1ml Trizol reagent, homogenized with the help of homogenizer (Cole Parmer LabGEN 7, USA) and kept at room temperature for 10 min.
4. The tube was centrifuged at 12000 x g at 4°C for 10 minutes and 200µL of chloroform per 1 ml of TRIZOL reagent was added to the supernatant and kept at room temperature for 10 minutes.
5. The upper aqueous phase was transferred without disturbing the interphase into the fresh tube.
6. Then 500 µL of isopropanol was added to the aqueous phase per 1 ml of TRIZOL reagent and kept at room temperature for 10-15 min after proper mixing.
7. It was centrifuged at 12000 x g at 4°C for 10 min to precipitate the RNA as pallet.
8. The supernatant was removed completely and the RNA pallet was washed with 75% ethanol and centrifuged again at 7500 x g at 4°C for 5 min.
9. The pellet was air dried and dissolve in 30 µL of nuclease free water (NFW) (Lot no.- 0000213084, Promega, Madison, USA)

3.8.2 RNA Integrity and Purity

The integrity of total RNA was checked on 1.5% agarose gel using 1X TAE as electrophoresis buffer. Total RNA was in good yield in all the samples. The purity and concentration of total RNA was checked using nanodrop (Eppendorf, USA). The isolated RNA samples were free from the protein contamination as the OD 260: OD 280 values were more than 1.8.

3.8.3 Synthesis of cDNA

The first strand cDNA was synthesized from the isolated RNA. RT-PCR was done using5X Reaction Buffer, $MgCl_2$, PCR Nucleotide Mix, RNase Inhibitor, Reverse Transcriptase, Oligo- (dt 15) Primer, RNA template, NFW. Reverse transcription was carried out in 20 μL reaction mixture depicted in Table 3.

Table 3: Reaction mixture for cDNA synthesis

Components of reaction mixture	**Quantity**
5X Reaction Buffer	4 μL
$MgCl_2$	3 μL
PCR Nucleotide Mix	1 μL
RNase Inhibitor	0 .5 μL
Reverse Transcriptase	1 μL
Oligo- (dt 15) Primer	1.5 μL
RNA template	1 μL
NFW	8 μL
Total	**20 μL**

Reaction mixture was mixed and spinned, followed by incubation for 15 minutes at 50°C and 2 minutes 30 seconds at 42°C and finally hold at 4°C.The cDNA was stored at -20°C for long term use.

3.8.4 Gradient PCR/ End point PCR optimization

PCR was standardized to amplify buffalo gene sequence using RT-PCR. Specific primers were used for the amplification of genes. The annealing temperature was standardized using cDNA prepared from mRNA of buffalo CL by PCR. The optimum temperatures were found to be suitable for primer annealing and were used in subsequent polymerase chain reaction. The components of reaction mixture used for optimization of primers is presented in Table 4.

Table 4: Reaction mixture for optimization of primers

Components of Reaction Mixture	Quantity
GoTaq® Green Master Mix	10 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
DNA template	1.0 µl
NFW	8.0 µl
Total	**20 µl**

The contents were vortexed and then spun down to collect at the bottom of tube by spinner (BioEra). The reaction was carried out in a Real-Time qPCR System instrument (Insta q96, Himedia) using the following cycling parameters that have been found optimum for amplification of gene fragments.

Table 5: Cycling conditions for optimization of primers

S.N	Cycling Steps	Temperature	Time
1.	Initial Denaturation	95°C	2 min
2.	40 cycles of Denaturation	95°C	20 sec
3.	Annealing	58°C/60°C	20 sec
4.	Extension	72°C	30 sec
5.	Final Extension	72°C	30 sec
6.	Hold	4°C	

58°C – annealing temperature for Beta Actin and 60°C for Visfatin and RPL15

3.8.5 Agarose gel electrophoresis

The confirmation of amplification of specific RT-PCR amplicon was done by agarose gel electrophoresis.1.5% agarose (Cat no.- MB229,Himedia) was mixed with 1X TAE buffer and melted in a heating mantle. When the molten gel had cooled, ethidium bromide was added. The gel was mixed 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 blue/orange loading dye 6X (Lot- 0000255933, Promega, USA) and loaded into the wells. For the comparison, a 50 bp molecular weight marker (New England, BioLab) were 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 (BioRad).

3.8.6 Real time PCR

Quantitative Real-time PCR was performed with Go Taq Green Master Mix (Lot no.- 0000225642, Promega, USA), Real-Time qPCR System instrument (INSTA Q 96, Himedia) 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 master mix 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. It was ensured that the cDNA samples were not contaminated with genomic DNA. For reaction set up optically clear caps were used 1µl of cDNA was taken. The master mix used for reaction of qRT-PCR is presented in Table 6.

Table 6: Reaction for mixture qRT-PCR

Components of Reaction Mixture	Quantity
GoTaq® Green Master Mix	7.5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
DNA template	1.0 µl
NFW	5.5 µl
Total	**15 µl**

The qRT-PCR for each cDNA and the housekeeping genes β-actin and RPL 15 was performed. 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. The contents were vortexed and then spun down to collect at the bottom of tube by spinner (BioEra). The reaction was carried out in a Real-Time qPCR System instrument (Insta q96, Himedia) using the following cycling parameters that have been found optimum for amplification of gene fragments.

Table 7: Cycling steps for qRT-PCR

S.N	Cycling Steps	Temperature	Time
1.	Initial Denaturation	95°C	2 min
2.	40 cycles of Denaturation	95°C	20 sec
3.	Annealing	58°C-60°C	25 sec
4.	Extension	72°C	30 sec
5.	Final Extension	72°C	15 sec
6.	Hold	4°C	

3.8.7 Calculation of Relative Expression

Optical data were collected at end of each extension step and relative expression of PCR product was determined by the $2^{-\text{Delta Delta Ct}}$ method. The tissue with lowest expression (highest Ct) was taken as calibrator. The geometric mean of Ct of beta actin and RPL 15 was taken as reference/ internal control.

3.9 SDS- PAGE and Western Blotting

The Detailed procedure SDS-PAGE and western blotting is given below:

3.9.1 Isolation and quantification of total proteins

1. Different stages of CL (luteal cells) were triturated in LN_2 and homogenized in RIPA lysis buffer (Lot no- 0000307900, Himedia, India) with the help of homogenizer.
2. The homogenate was centrifuged at 10000 x g for 10 minutes.
3. The supernatant containing mostly the total soluble proteins was taken in a separate eppendorf tube and kept in 4°C for future use.
4. The protein concentration was determined by Bradford method.

3.9.2 Vertical gel electrophoresis

SDS PAGE was performed as per the protocol of Laemmli (1970) in a vertical surecast handcast electrophoresis apparatus (Invitrogen). The glass and notch plates were cleaned and set in a gel caster. Running gel was prepared by accurate combination of given chemicals, poured between the two plates. 0.2-0.3 ml Isopropanol was poured over the gel to prevent oxidation. After polymerization, isopropanol was thoroughly removed. Stacking gel was layered

over the running gel with appropriate comb. The polymerized gel was set onto the electrophoresis unit along with running buffer. The sample stored in 4°C was thawed, diluted in lamellae buffer and boiled for 5 min and immediate cooling in ice for 5 min. 30 µl of each sample were loaded along with a standard prestained protein ladder (Lot no- 0000341265,Himedia, India). Electrophoresis was carried out at a constant voltage of 60 V for a period of 15 minutes or until sample crossing the stacking gel and then with constant voltage of 150 V till the tracking dye reaches the bottom of the gel or constant 90 V throughout the gel. After the run, gel was removed from the unit and stained with Coomassie brilliant blue staining solution, (Lot no. -0000258865, Himedia, India) for 1 hour and then destained with de-staining solution.

3.9.3 Transfer of proteins from gel to NC/PVDF membrane

Proteins were characterized by Western blot analysis in order to confirm specificity. The protein was run in 12% SDS-PAGE gel along with prestained protein marker (Lot no- 0000341265, Himedia, India). After electrophoresis the gel was taken out from the plates and kept in transfer buffer for atleast5 minute prior to transfer. Nitrocellulose (NC) blotting membrane roll (Lot no- 0000291538, Himedia, India) was soaked in methanol for 5 min followed by transfer buffer. Four Whatman filter papers (pre-soaked with transfer buffer), NC membrane, gel and another set of pre-soaked Whatman filter papers (Cat no. – 3030-6189, Whatman™, UK) were stacked one by one in respective order on the anode plate of blotting apparatus (Genaxy). Care was taken for avoiding air bubbles in between gel and nitrocellulose membrane. The complete stack was saturated with ice cold transfer buffer before the cathode plate was placed in position over the stack and a constant current of 0.8 mA/cm^2 was applied for 1 h. After 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 2 h at 37°C in 3% bovine serum albumin diluted with TBS in rotary shaker.

3.9.4 Incubation with antibody

After blocking, the membrane was washed thrice with TBS-T (TBS-0.01% Tween 20) for 5 minutes each and incubated with primary antibody PEBF-1at a

1:1000 dilution and anti-β actin in 1:1000 dilution for overnight at 4°C. After incubation, membrane was washed thrice with TBS-T (TBS+0.01% Tween 20) for 5 minutes each then respective secondary antibody conjugated with radish peroxidase was added and incubated at 37°C for 1h. After washing 3 to 4 times in TBS-Tween 20 solution, the positive signals were detected by incubating the membrane using 3,3 diaminobenzidine (DAB, Roche) in 1X TBS (pH 7.4) containing 0.06% H_2O_2 for 10-15 min. The bands were visualized under white light and recorded on a gel documentation system (BioEra).

3.10 Immunohistochemistry

CLs of different stages were separated from ovary and were fixed with 10% neutral buffer formalin (NBF), dehydrated through a series of graded alcohols, paraffin-embedded, serial sectioned (5μm), mounted on Mayer's albumin coated slides and dried at 37°C overnight. Five representative CLs each group were used for immunohistochemistry studies. Deparaffinization was carried out in xylene, followed by rehydration in a series of graded alcohols at room temperature, subjected to antigen retrieval in sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% Tween-20) for 30 min and then allowed to cool at room temperature. After washing twice in a phosphate buffered saline (PBS) for 5 min sections were immersed in peroxidase blocking reagent for 15 min at room temperature to quench endogenous peroxidase activity. After washing twice in PBS for 5 min, non specific background was eliminated by blocking with 5 % BSA (dissolved in 1xPBS) for 2 h at 37°C. Subsequently, sections were probed with rabbit anti PBEF1 1:100 dilutions. Sections were washed twice for 5 min in PBS and were incubated for 1-1.5 h at 37°C by secondary antibodies. The slides were washed twice in PBS and staining was revealed by using AEC staining kit according to manufacturer's instruction. The control slides were processed under similar conditions except for the omission of the primary antibody. Finally sections were washed with distilled water and observed under microscope (Axio Lab A1 from Zeiss). Images were captured using installed camera of Axio Cam ERC 5C.

3.11 Cell culture and estimation of progesterone concentration

3.11.1 Preparation of stock and working solutions

All the stock and working solutions required for luteal cell culture were prepared using autoclaved distilled water. Visfatin stock solution was prepared by reconstituting 100 µg of lyophilized desiccate of visfatin in 1% BSA-PBS to yield working solutions of 1 ng/µl and 10 ng/µl made in aliquots and stored at -20°C until used. The working solutions were added @ 1 µl/ml of DMEM/F12 media (containing serum -FBS at 1%) so as to yield final treatment concentrations respectively of 1 and 10 ng visfatin/ml media. The two above treatment concentrations were made fresh from the working solutions immediately before the treatment of luteal cells in wells.

3.11.2 Luteal Cell Culture

In order to evaluate the effect of visfatin on luteal function, a luteal cell culture model was developed with cells isolated from fresh CL. Luteal cells were cultured as described by Gupta *et al.,* 2014. Ovaries were collected from a local abattoir and transported back to the laboratory in 1XPBS at 37°C. In all experiments, only stage II CL (days 5-10) were used and these were selected based on criteria applied previously (Sarkar *et al.*, 2010). The CL was removed from the ovary with all connective tissue and then sliced up using BP blades. The minced luteal tissue was washed 3 times for 5 min at 12000 × g with dispersing medium. All cells (including luteal, endothelial, pericytes and fibroblasts) were dispersed by incubating the luteal tissue in DMEM/F12 medium (Lot no. -0000313737, Himedia, India) containing 2mg/mL collagenase I type 1A (Lot no. 0000324051, Himedia, India), 25 µg/ml DNase I and 0.5% BSA Fraction (Lot no.-0000268629, Himedia, India) for 2×45 min in a incubator at 37°C and shaking manually at 10 min interval. The dispersed cells from each incubation were pooled together and then filtered through 70 µm cell strainer (Lot no.-0000301022, Himedia, India) to remove nondissociated tissue fragments. The filtrate was washed twice by centrifugation for 5 min at 250×g with DMEM/F12 media (Lot no. -0000313737, Himedia, India). Supernatant was discarded. Later, erythrocyte lysis was accomplished by washing the pellet with RBC lysis buffer (Lot. no. -0000308804, Himedia, India) and further one washing step was performed. Cells were resuspended in DME/F12 medium (Lot no.-0000313737,

Himedia, India); containing 10% Fetal Bovine Serum (Lot no.- 42G5176K, Gibco life technologies, South America) and Antibiotic & Antimycotic solution (10,000 units Penicillin, 10 mg Streptomycin, 25 µg Amphotericin B per ml) (Lot no.- #067M4754V, Sigma, USA). Cell viability, determined by trypan blue exclusion dye (Lot no.- 0000320404, Himedia, India), and was higher than 90%. The cells were then plated out at 1.5×10^5 viable cells per well in a 24-well plate in a humidified CO_2 (5%) incubator at 38.5°C. The cells were allowed to attach and grow (75-80% confluent) for 48 h and there after the media was replaced with fresh media containing different concentrations (1 and 10 ng/ml) of visfatin and were maintained for 48 h. The doses of the visfatin were selected based on the earlier report in bovine granulosa cells (Reverchon *et al.,* 2016). Control cells were grown in media without visfatin. The cells were allowed to attach for 24 h and after a further 24 h period the medium was changed. After 48 h, the medium and cells were collected for mRNA isolation.

3.11.3 Identification of luteal cells

Luteal cells were identified based on their typical morphological characteristics such as shape, size, presence of cytoplasmic lipid droplets, location of nucleus and cytoplasm to nuclear ratio viewed under a phase contrast microscope.

3.11.4 Quantitative RT-PCR and gene expression analysis

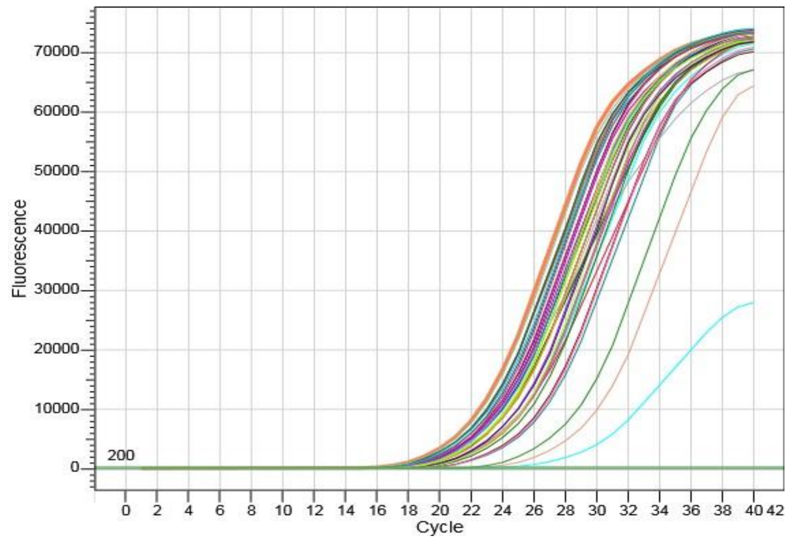
Total RNA was isolated by Trizol reagent according to manufacturer instruction from cultured luteal cells. qRT- PCR analysis, testing of RNA integrity and purity and gene expression studies was done as per the method described earlier. The primer annealing temperature employed for various investigated factors were as follows: 56°C for PCNA; 58°C for βactin; 60°C for CYP11A1; 3β-HSD and 62°C for BAX.

3.11.5 Progesterone (P_4) assay

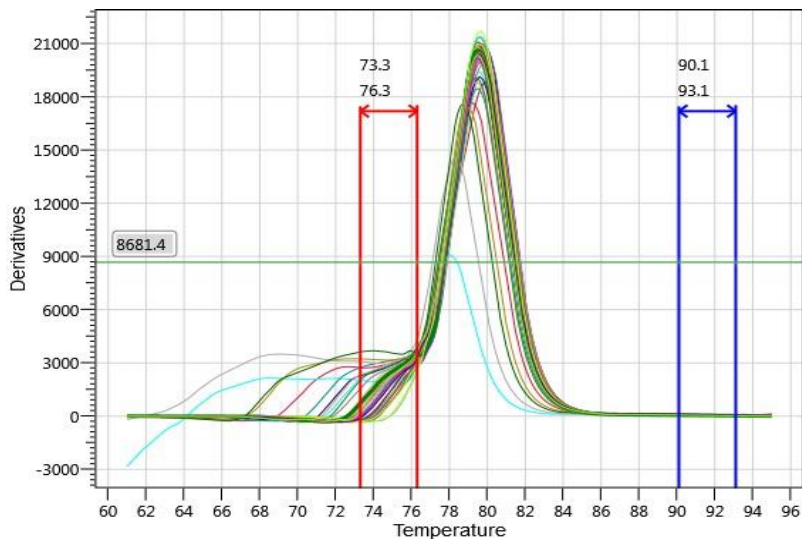
Concentrations of progesterone (P_4) in the spent culture media of luteal culture were estimated by Progesterone ELISA kit (Lot no.- #0289, Neogen) to confirm steroidogenic properties.

.12 Statistical Analysis

The data was analyzed by SPSS 16.0 software. All experimental data has been shown as mean ± SEM. The statistical significance of difference in mRNA expression of visfatin in CL, CYP11A1, 3β-HSD, PCNA, BAX and progesterone concentration in spent culture media was assessed by one-way ANOVA by the Tukey- Ducan as a multiple comparison test. Difference were considered if $P<0.05$



(A)



(B)

Fig 1: Amplification curve (A) and Dissociation curve (B) for visfatin

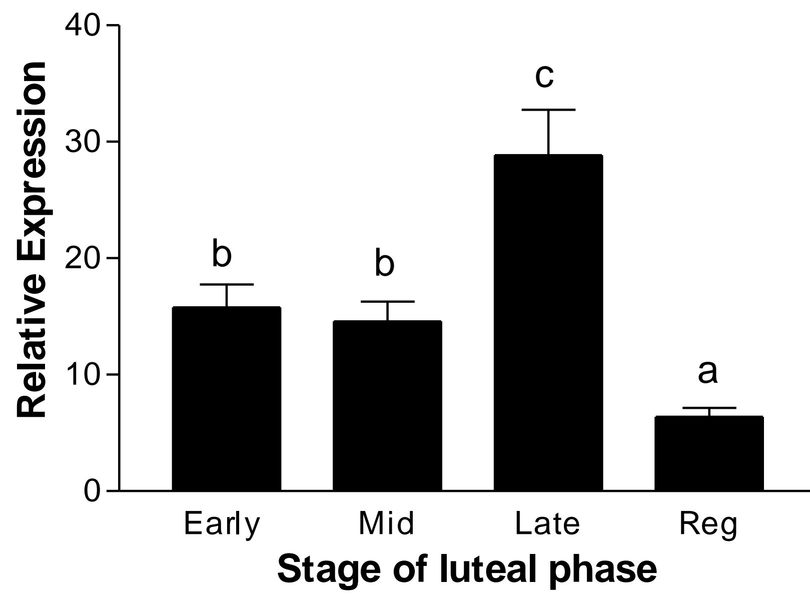


Fig 2 : Visfatin mRNA expression in stages of CL.

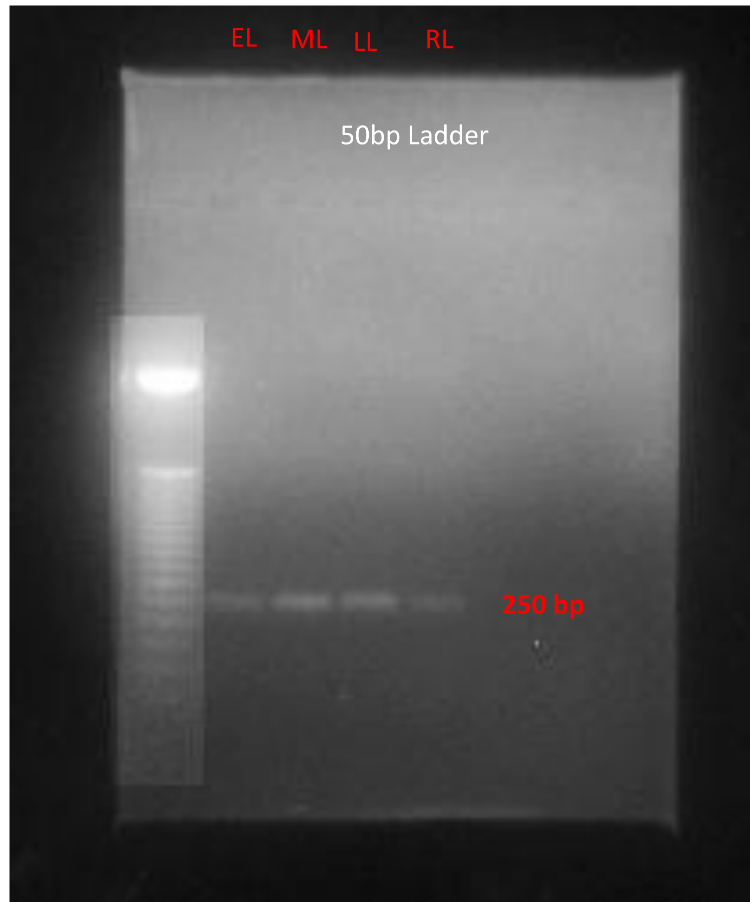


PLATE 1: 1.5% agarose gel showing 250 bp product for visfatin (EL- Early Luteal, ML- Mid Luteal, LL- Late Luteal and RL- Regressed Luteal)

RESULT AND DISCUSSION

The present study was conducted to find the mRNA and protein expression and localization of visfatin in CL of 40 buffalo ovaries and also the influence of visfatin on progesterone secretion.

4.1 Visfatin mRNA and protein expression in the corpus luteum of buffalo ovary

4.1.1 RNA integrity and purity

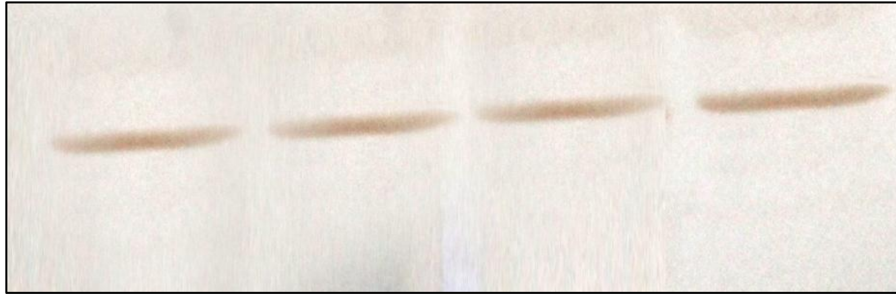
Total RNA was in good yield in all the 40 CL samples. The purity and concentration of total RNA was checked using nanodrop. The isolated RNA samples were free form protein contamination as the OD 260: OD 280 values were more than 1.8.

4.1.2 mRNA expression of visfatin in corpus luteum

We determined the expression of visfatin in corpus luteum of buffalo as shown in Plate1. We amplified one cDNA fragment of 250 bp by RT-PCR corresponding to visfatin. The tissue whose expression was lowest was used as calibrator for obtaining relative mRNA expression. Beta actin and RPL 15 was used as housekeeping gene. Amplification and dissociative curve are present in Fig 1(A) and (B) respectively. The analysis of visfatin mRNA by qRT-PCR showed that visfatin expressed at each stage and its transcripts varied through the luteal phase. We found that the transcripts of visfatin (mRNA expression) was low during early luteal stage (1-4 days) and mid luteal stage (5-10 days), increased and reached maximum in the late luteal stage (11-16 days) and then decreased in the regressed CL and is shown in Fig 2.

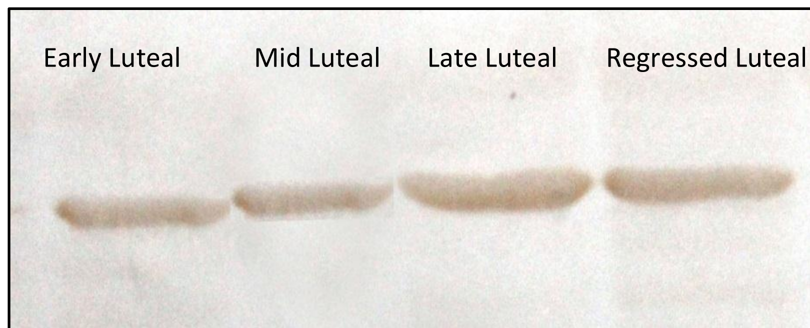
We found visfatin mRNA in all the stages and its transcripts varied throughout the luteal phase. Its expression was low during early, mid and regressed luteal stage and it reached maximum in late luteal stage. Reverchon *et al,* 2013 showed that visfatin is present in human ovarian follicular cells including granulosa, theca and cumulus cells and also in human oocytes. Chabrolle *et al*, 2007 and Madeja *et al*, 2009 have already found visfatin in oocytes of various species, including man in other adipokines such as leptin and adiponectin. Several studies also

found visfatin in follicular fluid and plasma, suggesting that not much visfatin is secreted by ovarian cells (Shen *et al,* 2010). Previous reports on NAMPT expression are shown in testies of pig (Chen *et al*, 2007), mice (Revollo *et al,* 2007) and avian species (Ocon-Grove *et al*., 2010). Detection of visfatin mRNA expression in adipose tissue and in adipocytes with normal and polycystic ovary syndrome (PCOS) women and visfatin mRNA expression in subcutaneous (sc) and omental (om) and also presence and significance of up regulated visfatin gene expression in adipose tissue depots in women with PCOS (Tan *et al.,* 2006). Chang *et al.,* 2010 suggested visfatin as a proinflammatory cytokine and a marker of adipose tissue associated with systemic insulin resistance and hyper lipidemia. The mRNA level of Visfatin/PBEF/NAMPT macrophages specific marker CD68 and proinflammatory gene were measured predominantly in paired visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) and no difference was found in between the level of mRNA visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) and neither were associated with the measures of obesity but were positively correlated with steady state plasma glucose concentration. Ma *et al.,* 2010 observed a significantly higher expression of visfatin mRNA in placenta of gestational diabetic mellitus (GDM) women than those with control groups and positively correlated the serum visfatin level with mRNA expression in placenta whereas in SAT and VAT it did not associated with it's mRNA expression. Seow *et al.,* 2011 investigated the visfatin expression in tissue from two different sources, i.e. omental adipose tissue and peripheral blood mononuclear cells (PBMCs) in women with polycystic ovary syndrome (PCOS) and in controls and expressed higher visfatin mRNA in both omental adipose tissue and PBMCs in women with PCOS, it indicated that mononuclear cells are a source of secreted visfatin in addition to adipose tissue in women with PCOS. Choi *et al*., 2012 investigated expression of visfatin mRNA from two groups of differently aged mice (6–11 and 26–31 weeks) and observed that visfatin was expressed in the ovary regardless of the age of female mice. Its expression was increased with advancing follicular development in both aged and young mice after PMSG administration. However, expression of visfatin mRNA was significantly decreased in aged mice compared with young mice. mRNA expression of visfatin in ovarian cells (granulosa and theca cells) in turkeys (*Meleagris gallopavo)* and mRNA expression of visfatin was higher in theca cells compared to granulosa cells in both F1 and F3/4



52kDa

VISFATIN



BETA - ACTIN

41kDa

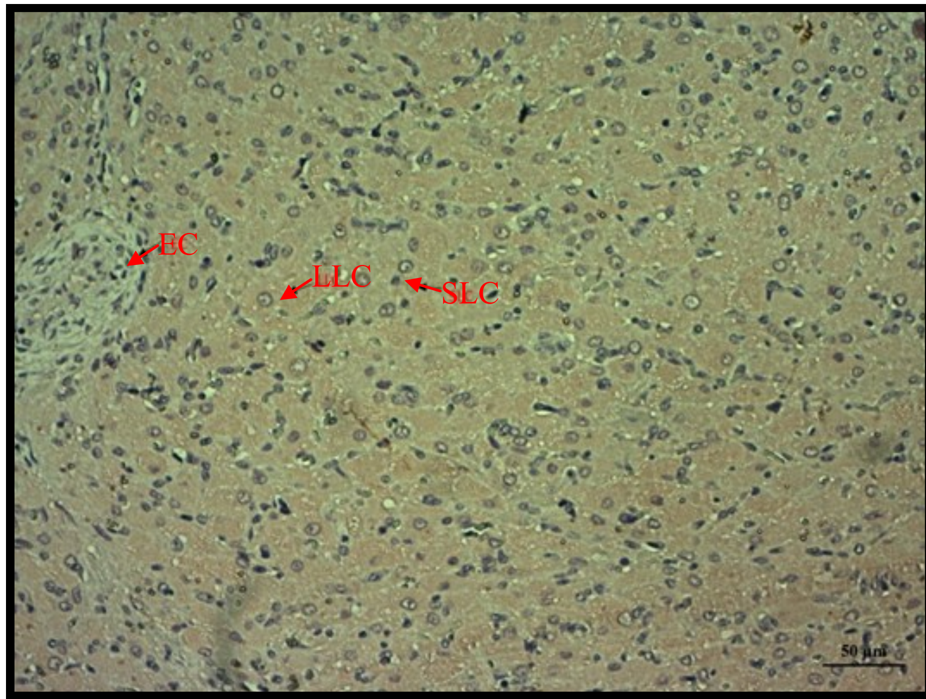
PLATE 2 : Western blot for Visfatin and Beta Actin

hierarchical follicles (Diot *et al.,* 2015a). NAMPT is present in ovarian cortex and in granulose and theca cells from hierarchical follicles in hen. Significantly higher mRNA expression was observed in granulosa cell in F1 and F2 hierarchical follicles and the level of expression was similar in granulosa and theca in F3 hierarchical follicle (Diot *et al*., 2015b). Novak *et al.,* 2015 found the physiological values of serum visfatin concentration and visfatin mRNA expression in prepubertal healthy boys and visfatin mRNA was higher in subcutaneous adipose tissue (SAT) when compared with visceral adipose tissue (VAT). Reverchon *et al*., 2016 found NAMPT/ Visfatin is highly expressed in large follicle (LF) than the small follicle (SF) of granulosa cells (GC) and used adipose tissue and mammary gland as positive controls and observed that visfatin protein is present in bovine CL and different follicular cells. They investigated the effect of FSH, IGF-I and Visfatin itself on NAMPT mRNA expression from small follicle (SF) in primary bovine granulosa cell and showed significant increase in NAMPT mRNA expression after 48 hour of stimulation by IGF-1 and Visfatin but not by FSH

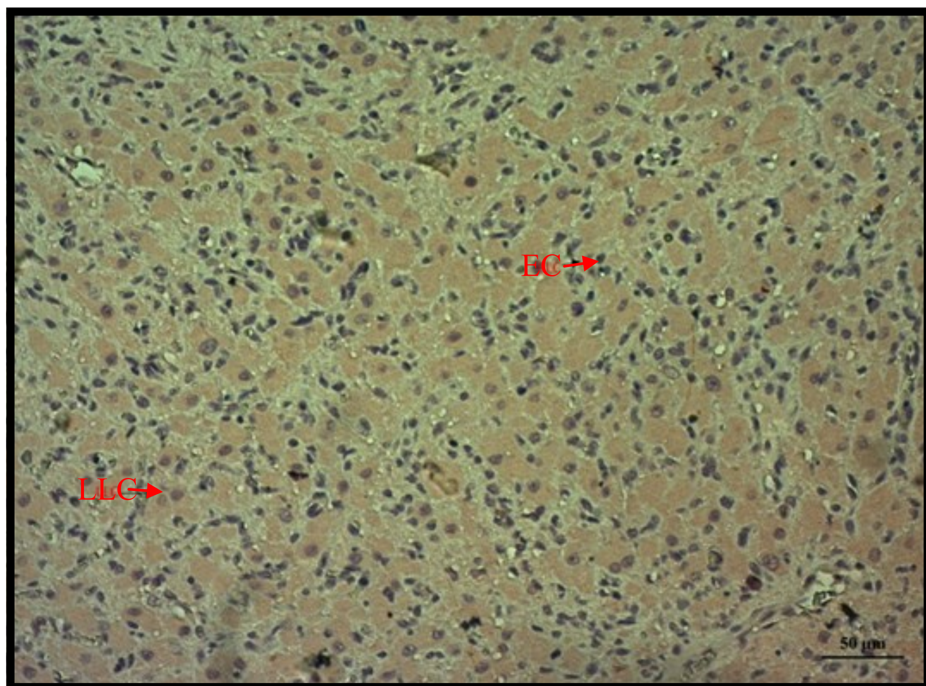
4.1.3 Protein expression analysis by western blot in Corpus luteum

The presence of visfatin protein expression during different stages of buffalo CL was verified by western blot. Proteins of visfatin and Beta actin were localized by immunoblotting. The protein bands of visfatin (52 KD) and Beta actin (41KD) are shown in Plate 2. The results showed that expression of proteins was low during early luteal stage and mid luteal stage, increased and reached maximum in late luteal stage and then decreased in the regressing CL. These results were similar in the pattern as observed for mRNA expression.

Expression of visfatin protein by immunoblotting revealed its presence in fresh human granulosa cells (hGCs) and in human ovarian granulosa-like tumour cell line (KNG) (Reverchon *et al*., 2013). Moreover, Ma *et al. (*2010) observed a significantly higher expression of visfatin protein in placenta of gestational diabetic mellitus (GDM) women and positively correlated the serum visfatin level with protein expression in placenta whereas in SAT and VAT it did not associated with it's protein expression. For detection of NAMPT protein in chicken testis and whether the NAMPT protein quantity differs between prepubertal and adult chickens, it was concluded that the testicular NAMPT protein quantity were not significantly different between adult and prepubertal chickens. (Ocon-Grove *et al*., 2010). Choi *et al*. (2012) investigated expression of visfatin protein from two

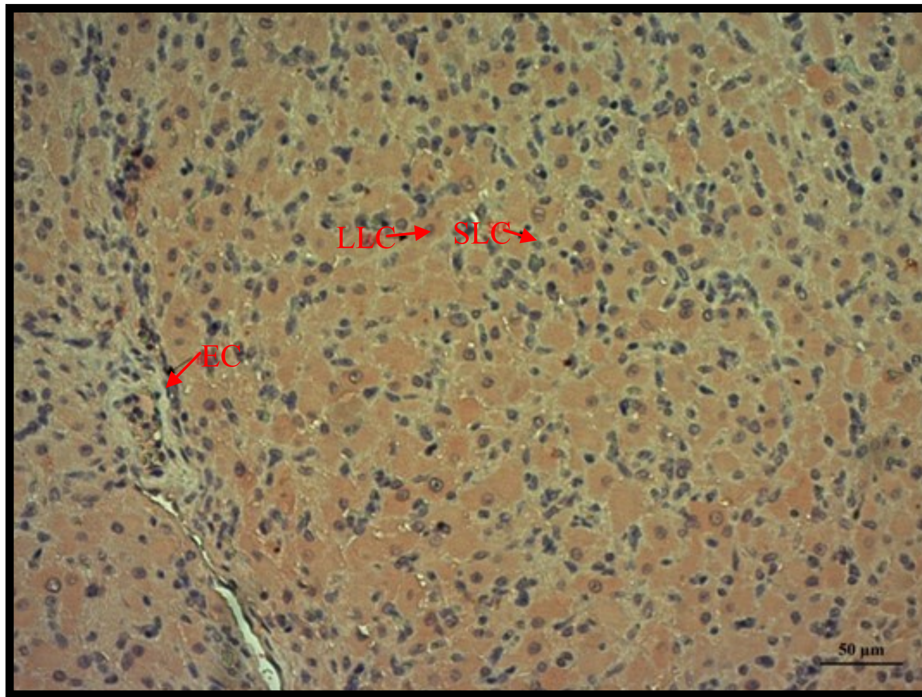


(A) Early Luteal Stage

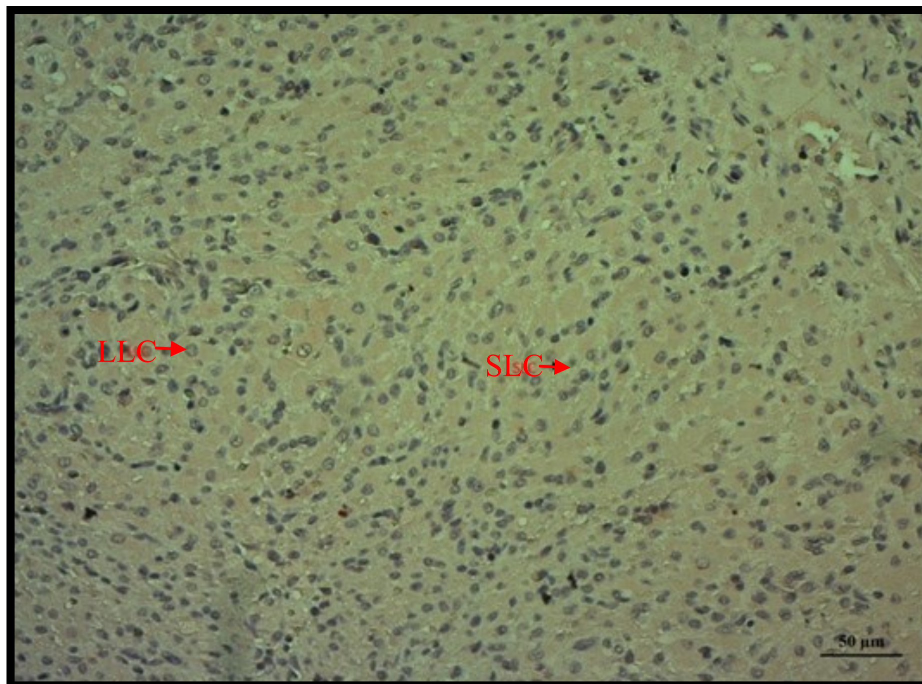


(B) Mid Luteal Stage

PLATE 3 : Distribution of visfatin immunoreactive cells in different stages of CL. Observed under 40 X
The distribution of visfatin positive cells in
(A) Early Luteal stage (arrow), LLC (Large Luteal cell), SLC (Small Luteal cell) and EC (Endothelial cell)
(B) Mid Luteal stage (arrow), LLC (Large Luteal cell) and EC (Endothelial cell)



(C) Late Luteal Stage

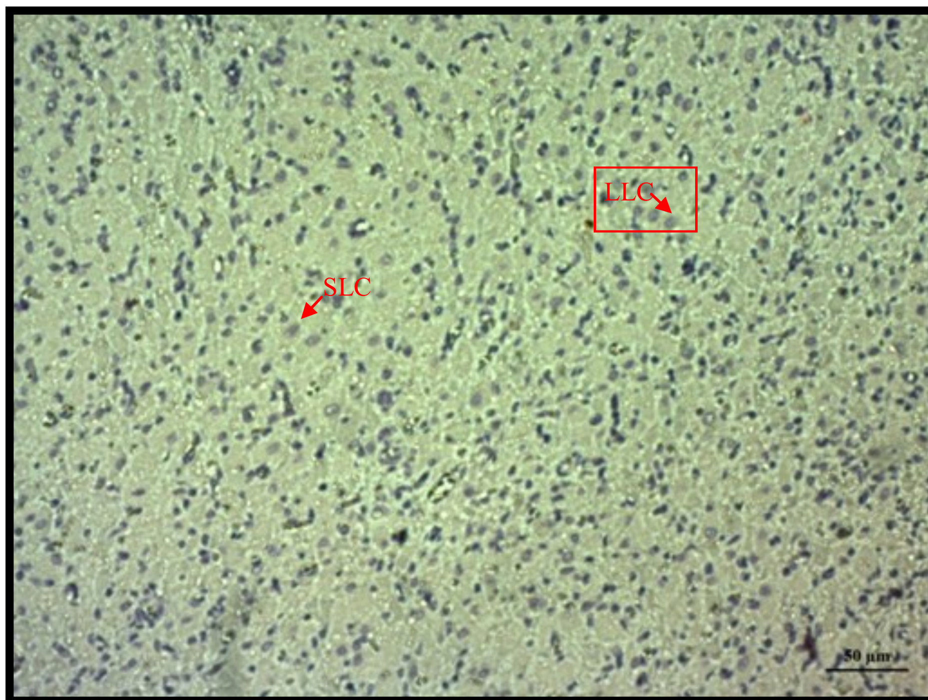


(D) Regressed CL

Distribution of visfatin immunoreactive cells in different stages of CL. The distribution of visfatin positive cells in

(C) Late Luteal stage (arrow), LLC(Large Luteal cell), SLC(Small Luteal cell)

(D) Regressed CL (arrow), LLC(Large Luteal cell), SLC(Small Luteal cell)



(E) Control

Distribution of visfatin immunoreactive cells in different stages of CL. The distribution of visfatin positive cells in (E) Control LLC (Large Luteal cell) and SLC (Small Luteal cell)

groups of differently aged mice (6–11 and 26–31 weeks) and observed that visfatin was expressed in the ovary regardless of the age of female mice. Its expression was increased with advancing follicular development in both aged and young mice after PMSG administration. However, expression of protein was significantly decreased in aged mice compared with young mice. Expression of visfatin was showed by Gurusubramanian and Roy (2014) in the diabetic rat testis with a significant decrease in visfatin expression as compared with control testis by western blot analysis. Previously Diot *et al.* (2015a) showed protein expression of visfatin in ovarian cells (granulosa and theca cells) in turkeys (*Meleagris gallopavo)* and the protein levels were confirmed by immunoblotting. Diot *et al*. (2015b) concluded that the NAMPT is present in ovarian cortex and in granulose and theca cells from hierarchical follicles in hen. Also investigated NAMPT protein expression in hen during follicle development in both granulosa and theca cells.

4.2 Localization of visfatin by immunohistochemistry

Firstly the CL morphology was evaluated by using haematoxylin and eosin staining and the luteal cells were identified on the basis of their shape and localization. As observed under microscope, the cells were large, round and with central nucleus. Small and flat endothelial cells were localized around the inner layer of capillary. In the surrounding of outer layer of capillary oval shaped pericytes were co- localized with endothelial cells. By using immunohistochemistry, immunoreactivity of visfatin (Plate3) was observed and the difference in reactivity was stage specific. The localization of visfatin protein was in various cell types in the CL sections of different stages. The positive cells were influenced by the different stages of estrous cycle and immunostaining intensity varied from phase to phase. The immunoreactivity was greater in late luteal stage and was found exclusively found in the cytoplasm of luteal cells. The negative controls without primary antibody showed only weak background staining.

Ocon-Grove *et al*. (2010) localized NAMPT in prepubertal and adult chicken testis by using immunohistochemistry and observed that in prepubertal chicken testis NAMPT was localized in the nucleus of myoid cells, sertoli cells and in leydig cells. However, in adult chicken NAMPT immunostaining was observed in the cytoplasm of leydig cells, sertoli cells, primary spermatocytes,

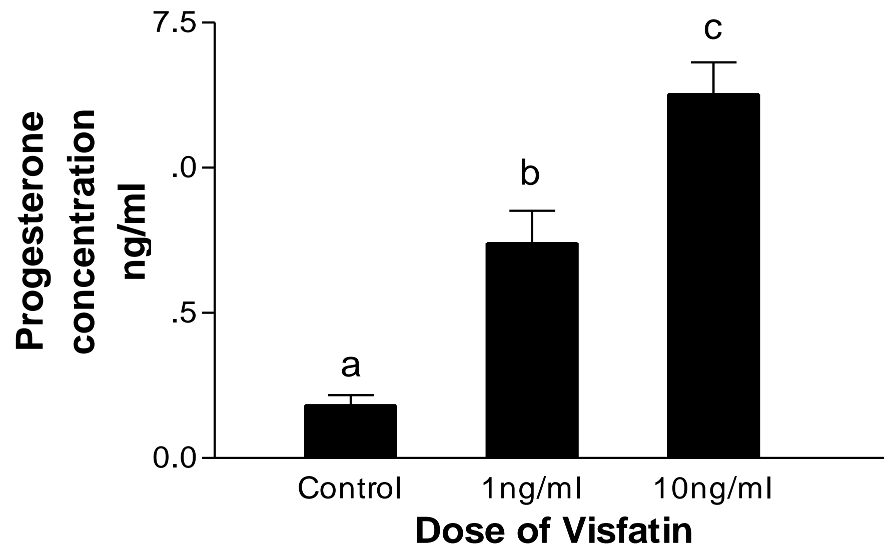


Fig 3: Concentration of progesterone in visfatin treatment at 2 different dose rate in cultured luteal cell

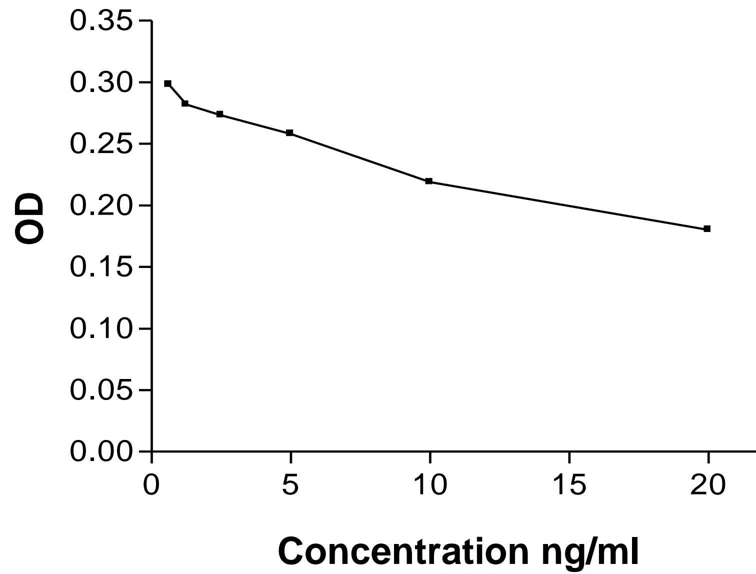


Fig 4: Standard curve for progesterone.

secondary spermatocytes, round spermatids and elongated spermatids but not in spermatogonial cells. Choi *et al*. (2012) localized visfatin expression in mice ovary by immunohistochemistry and showed decreased expression of visfatin in aged mice compared with young during Immunostaining of ovary sections. The expression was localized in granulosa cells, cumulus cells, stromal cells, and endothelial cells. The immunoreactivity of visfatin revealed more in granulosa and cumulus cells of young mice compared with aged mice**.** Moreover, Reverchon *et al*. (2013) localized visfatin in human ovarian follicle by immunochemistry. Song *et al*. (2014) verified the correlation between visfatin and reproduction by observing the round and oval visfatin immunoreactive cells in different developmental stages (primary follicle and secondary follicle) of mouse ovarian follicles that also specially in the granular layer of growing ovarian follicle. Gurusubramanian and Roy (2014) localized visfatin in diabetic rat testis and observed that affected seminiferous tubules showed mild immunostaining in comparison with normal tubules of diabetic and control rat testis. While in normal seminiferous tubules of both diabetic and control rat showed a similar pattern of visfatin. Visfatin was observed in Leydig cells, spermatids and sperm while faint staining was observed in Sertoli cells and primary spermatocytes of normal seminiferous tubules of control and diabetic rat testis and visfatin was clearly seen in cell membrane of round spermatids. The diabetic rat with affected tubules showed giant cells with strong immunostaining. Reverchon *et al.* (2016) performed immunohistochemistry with ovarian sections from bovine follicles and confirmed that the finding of immunoblot in the theca cells but less in granulosa cells.

4.3 Effect of visfatin on progesterone secretion, its intermediatory proteins and apoptotic markers in cultured luteal cells

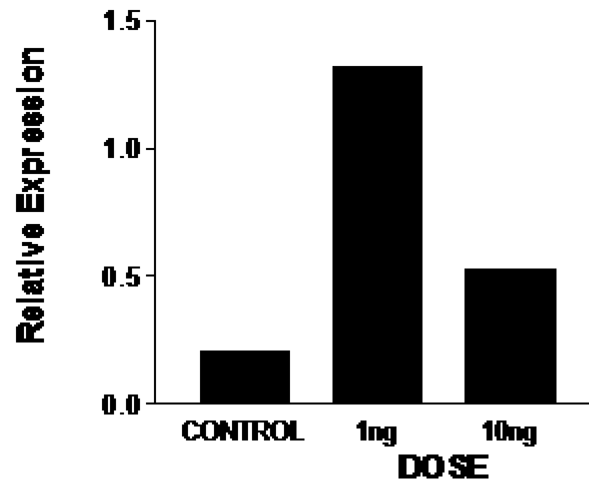
4.3.1 Effect of visfatin on progesterone secretion

Luteal cells from CL of late luteal stage were cultured after attaining 75-80% confluency for 48 h, without (control) or with increasing doses of visfatin (1 and 10 ng/ml). Concentration of progesterone (P_4) in control culture decreased. Progesterone concentration increased (P<0.05) with visfatin treatment in a dose dependent manner. Concentration and standard curve of progesterone are presented in Fig 3 and 4 respectively.

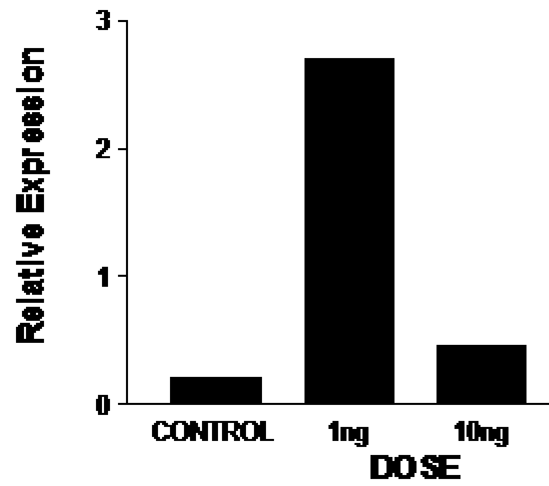
Effects of TFN-α on visfatin expression in BeWo cells were determined by Ma *et al.,* 2010. Visfatin expression was measured in a dose dependent way and interestingly at 48 h incubation with TNF-α, visfatin secretion from BeWo cells was increased at a concentration of 100 ng/ml when compared to non stimulated controls. Reverchon *et al.* (2013) determined the effect of human recombinant visfatin on basal and IGF-I or FSH- induced progesterone production by human ovarian granulosa-like tumour cell line (KGN) and human granulosa cells (hGCs) cells. In basal state visfatin treated with 10ng/ml was increased by 2 fold secretion of progesterone and no effect was observed in the presence of FSH. Diot *et al.* (2015b) investigated the effect of recombinant human NAMPT (rhVisf) on progesterone secretion in granulosa cells from hierarchical follicles in hen. *In vitro* treatment with human recombinant NAMPT (100 ng/ml) for 48 h halved basal and IGF1-induced progesterone secretion and this was associated with reduction in STAR and HSD3B protein levels and MAPK3/1 phosphorylation levels in granulosa cells. These effects were abolished by the addition of FK866, a specific inhibitor of NAMPT enzymatic activity. It was concluded that NAMPT is present in hen ovarian cells and inhibits progesterone production in granulosa cells. Reverchon *et al.* (2016) observed that IGF-I and visfatin (10 and 100 ng/ml) but not FSH increased mRNA expression levels of NAMPT after 48 hours of stimulations in the bovine granulosa cells and also observed that human recombinant visfatin increased the release of progesterone secretion and this was associated with an increase in the protein level of STAR, the HSD3B activity, and the phosphorylation levels of IGF-1 R and MAPK ERK1/2 in the presence or absence of IGF1. All these effects were abolished when NAMPT was knocked down and when the sirtuin pharmacological inhibitors CHIC-35 and EX-527 were pre incubated in bovine granulosa cells. Thus, in cultured bovine granulosa cells, visfatin improves basal and IGF1- induced steroidogenesis and IGF1 receptor signaling through SIRT1.

4.3.2 <u>Effect of visfatin on mRNA expression of CYP11A1 and β- HSD in cultured luteal cells</u>

Luteal cells were treated with 2 different doses (1 ng/ml and 10 ng/ml) of visfatin after attaining 75-80% confluency. mRNA expression from the harvested cells showed that CYP11A1 and3β– HSD increased non significantly at 1ng/ml dose of visfatin as compared to control (Fig 5 (A) and (B) respectively).

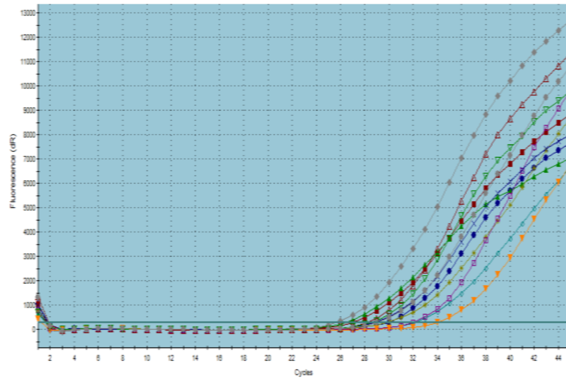


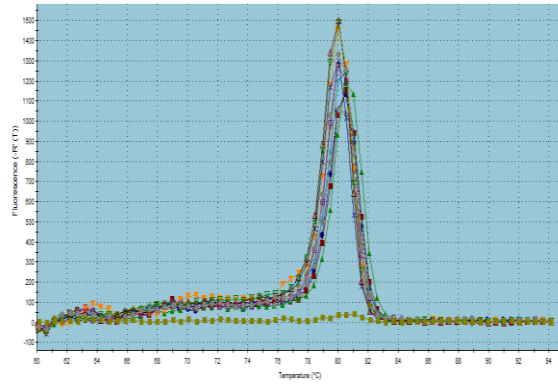
(A)



(B)

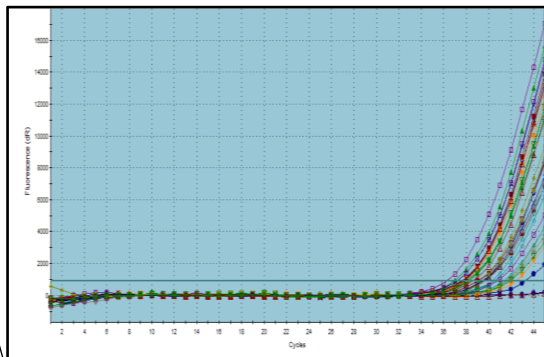
Fig 5: mRNA expression of intermediate proteins CYP11A1 (A) and 3β-HSD (B)

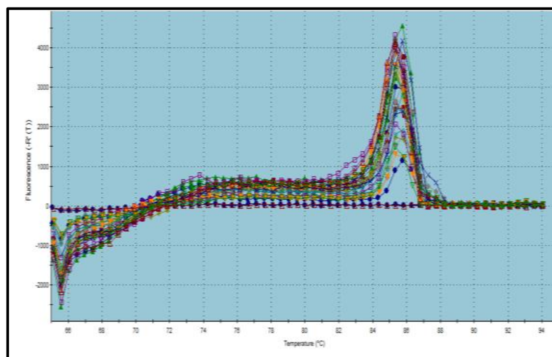




(A) (B)

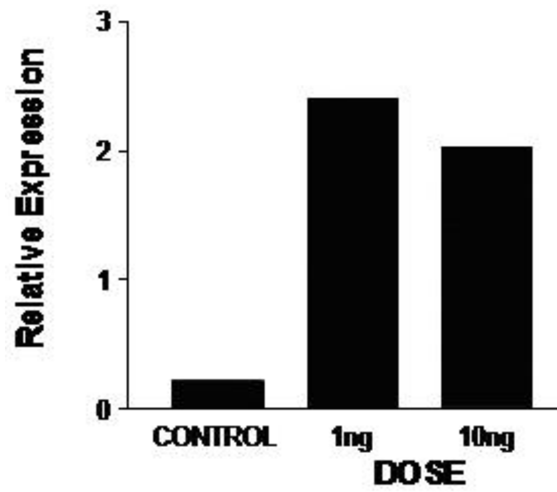
Fig6 : Amplification curve (A) and Dissociation curve (B) for CYP11A1



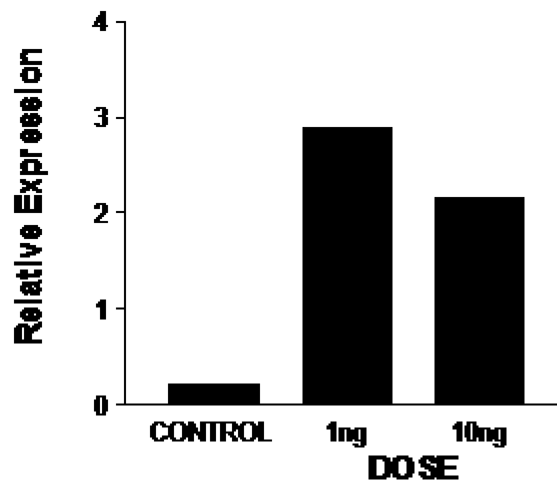


(A) (B)

Fig7 : Amplification curve (A) and Dissociation curve (B) for 3β-HSD

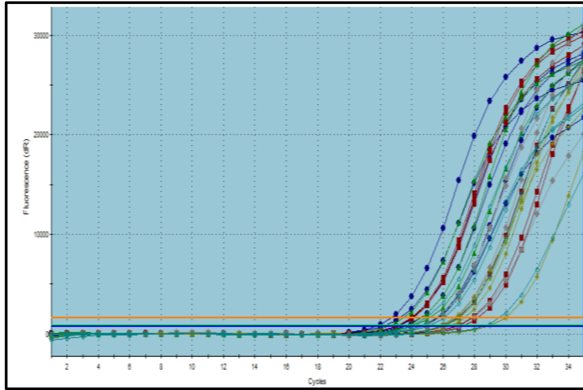


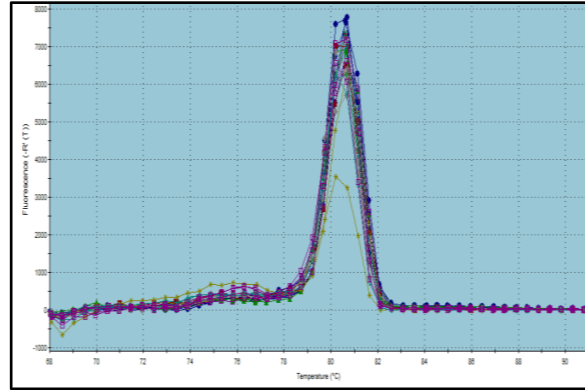
(A)



(B)

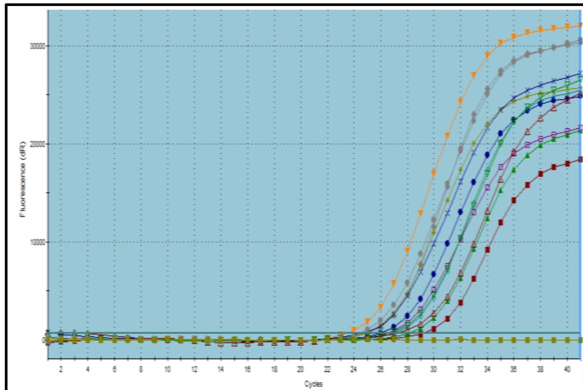
Fig 8: mRNA expression of apoptotic markers BAX (A) and PCNA (B)

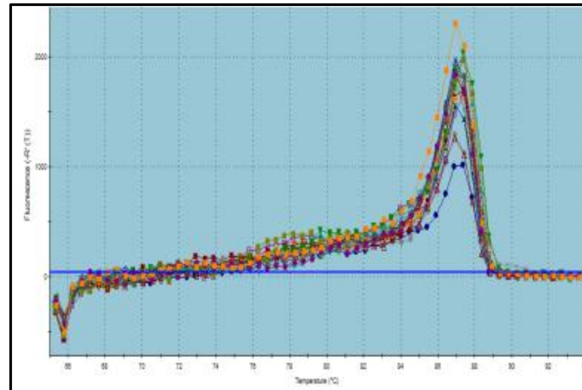




(A) (B)

Fig9 : Amplification curve (A) and Dissociation curve (B) for BAX





(A) (B)

Fig10 : Amplification curve (A) and Dissociation curve (B) for PCNA

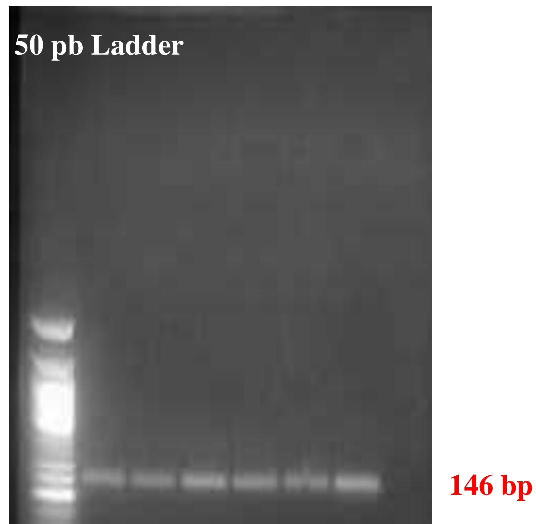


PLATE 4: 1.5% agarose gel showing 146 bp product for CYP11A1

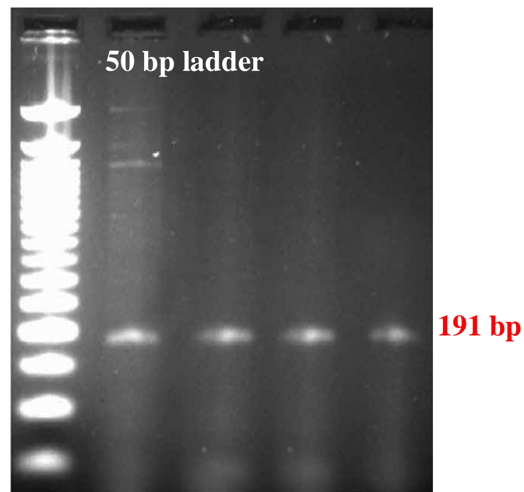


PLATE 5: 1.5% agarose gel showing 191 bp product for 3β-HSD

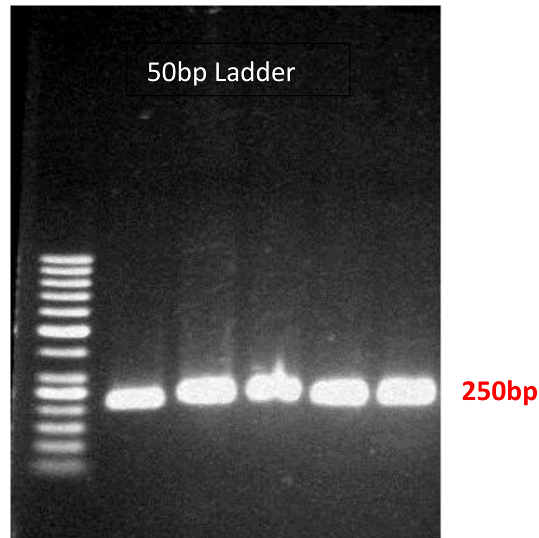


PLATE 6: 1.5% agarose gel showing 250 bp product for BAX

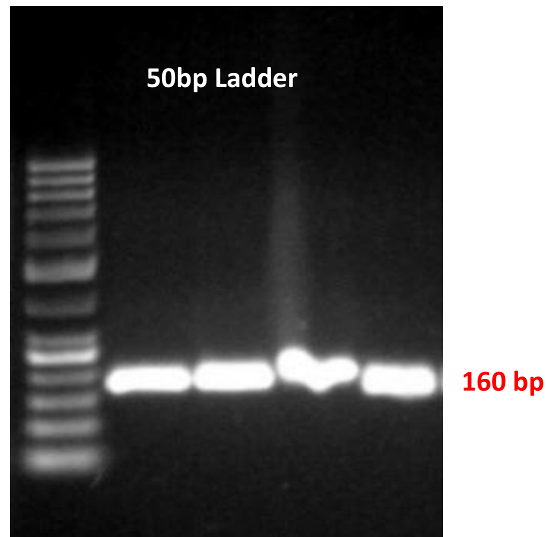


PLATE 7: 1.5% agarose gel showing 160 bp product for PCNA

Amplification curve (A) and dissociation curve (B) for CYP11A1and 3β-HSD are presented in Fig 6 and 7 respectively and the gel product for CYP11A1and β- HSD are shown in Plate 4 and 5 respectively.

4.3.3 <u>Effect of visfatin on MRNA expression of BAX and PCNA in cultured luteal cells</u>

Luteal cells were treated with 2 different doses (1ng/ml and 10ng/ml) of visfatin after attaining 75-80% confluency. mRNA expression from the harvested cells showed that the apoptotic markers BAX and PCNA increased non significantly at 1ng/ml dose of visfatin as compared to control (Fig 8 (A) and (B) respectively). Amplification curve (A) and dissociation curve (B) for BAX and PCNA are presented in Fig 9 and 10 respectively and the gel product for PCNA and BAX are shown in Plate 6 and 7 respectively.

An *in vitro* luteal cell culture was used to evaluate the effect of visfatin on intermediate proteins (CYP11A1 and 3β-HSD) and apoptotic markers (PCNA and BAX). The mRNA expression of progesterone secretion intermediate (CYP11A1, 3β-HSD) and apoptotic markers (PCNA and BAX) was non significantly higher in cultured cells treated with 1 ng/ml of visfatin as compared to control cultured cells. The stimulatory effect of visfatin on progesterone secretion was associated to an increase in 3β-HSD activity as it is responsible for the conversion of pregnenolone to progesterone. Diot *et al. (*2015b) showed that recombinant human NAMPT significantly reduced level of intermediate protein 3β-HSD in primary granulosa cells of hen. Reverchon *et al. (*2016) investigated the effect of hVisf on the activity of β-HSD and concluded that the increase in steroid secretion in response to hVisf may be due to an increase in the amount of 3β-HSD activity in bovine granulosa cells.

SUMMARY AND CONCLUSIONS

The buffalo is an economically important livestock species in many Asian, Mediterranean and Latin countries. Its genetic improvement, especially in reproductive performance stands high amidst agricultural research requirements of these countries. However, a better understanding of luteal dynamics and ovulation may be essential for augmenting conception rate and for minimizing early embryonic losses. The failure in conception may often arise due to defects in the process of transformation of follicular structure into luteal structure that are essential for maintenance of pregnancy in these animals. Follicle development in domestic ruminants involves the growth of a cohort of ovarian follicles, out of which only one is selected to undergo pre-ovulatory maturation, while the rest of follicles usually termed subordinate ultimately end in atresia. In buffalo, as in cattle, the dominant follicle is typically the first member of the cohort to reach 9 mm in diameter. It will be the only follicle to continue growing to ovulatory size (about 14-20 mm).

The ovarian cycle of domestic species is characterized by repeated patterns of cellular proliferation, differentiation and transformation of ovarian steroidogenic cells (granulosa, theca and luteal cells) that accompany follicular and luteal development. Recent reports provided involvement of visfatin in ovarian functions. Visfatin is a recently discovered hormone and the present study was carried out to explore the involvement of this peptide hormone in luteal and follicular development and functions in the ovary of buffalo.

In first part of study, expression of visfatin mRNA and protein in corpus luteum of buffalo ovary was performed. CL were classified into early luteal stage (days 1-4), mid luteal stage (days 5-10), late luteal stage (days 11-16) and regressed luteal stage (days >17) of estrous cycle. Luteal tissue was frozen in liquid nitrogen and stored until RNA and protein isolation. qPCR and western blot were applied to investigate mRNA and protein expression. Beta actin was used as a control for mRNA and protein expression calculation. The results confirmed the hypothesis that visfatin is found in buffalo CL during estrous cycle. The mRNA and protein expression of visfatin in buffalo CL were consistent throughout the estrous cycle. Their relative expression however varied according to the

stage of estrous cycle. It was higher in late luteal phase and lower in early, mid and regressing phases.

In second part of study localization of visfatin in CL was done by immunohistochemistry. Visfatin immunoreactivity was observed in cytoplasm of small and large luteal cells. The intensity of immunostaining and number of luteal cells observed containing visfatin protein in the present study was increased during maturation and decreased during regression of CL. These observations indicated the autocrine and paracrine role of visfatin in CL functions and development.

In the third part of study, for further confirmation of visfatin's role in CL *in vitro* luteal cell culture was done. Luteal cells were isolated from 3rd stage CL (days 11-16) and were cultured *in vitro* and treated with visfatin at dose 1 and 10 ng/ml concentrations for 48 h after obtaining 75-80% confluency. Progesterone concentration was checked in spent media. Progesterone concentration increased with visfatin treatment in a dose dependent manner. mRNA expression was checked for intermediate proteins (CYP11A1 and 3β-HSD) and apoptotic markers (PCNA and BAX) from harvested luteal cells. The mRNA expression of progesterone secretion intermediates (CYP11A1, 3β-HSD) and apoptotic markers (PCNA and BAX) was non significantly higher in cultured cells treated with 1ng/ml of visfatin as compared to control cultured cells. The stimulatory effect of visfatin on progesterone secretion was associated to an increase in β-HSD activity as it is responsible for the conversion of pregnenolone to progesterone.

Conclusions

It can be concluded from the results of the present study that the dynamics of mRNA and protein expression of visfatin in corpus luteum of buffalo during estrous cycle exists indicating the presence and role of visfatin in CL. The localization of visfatin was done by immunohistochemistry and it is indicating the role of visfatin in CL development and functions of buffalo ovary. Visfatin has a stimulatory role on progesterone secretion in luteal cell culture of buffalo ovary.

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APPENDIX

Solutions used for SDS-PAGE and western blotting

1 Resolving Buffer (pH 8.8) -

Tris	18.17 g
SDS	0.40 g
Distilled water was added to make the final volume to 100 ml	3.025 g

2 Stacking Buffer (pH 6.8) –

Tris	0.40 g
Distilled water was added to make the final volume to 100ml.	

3 SDS-PAGE

Resolving/Running Gel	**12%**
Acrylamide (40%)	2.4 ml
Resolving buffer	2 ml
Water	3.5 ml
10% APS	80 µl
10% SDS	60 µl
TEMED	8 µl
Stacking Gel	
Acrylamide(40%)	0.30 ml
Stacking buffer	0.75 ml
Water	1.92 ml
10% APS	30 µl
10%SDS	30 µl
TEMED	3 µl

4 10% Ammonium per sulphate

APS	1.0 g
Distilled water	10 ml

Stored at 4ºC

5 Gel staning solution

Coomassie brilliant blue	0.25 g
Methanol: deionized water (1:1)	90 ml
Glacial acetic acid	10 ml

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

6 Gel distaining solution

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

7 Transfer buffer

Tris base	242 g
Glycine	11.95 g
SDS	0.185 g
Methanol	100 ml

Distilled water was added to make the final volume up to 500 ml

8 Blocking buffer

PBS	1000 ml
BSA	1.5%

9 Phosphate buffered saline (PBS)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g

KH_2PO_4	0.24 g

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

Reagents used for Agarose Gel Electrophoresis

1 Tris acetate- EDTA (TAE) buffer 50X

Tris base	242 g
Glacial acetic acid	57.1 g
Sodium EDTA (pH 8.0)	37.2 g

Distilled water was added to make up to final volume of 1000 ml. A working solution of 1 X was used.

2 Safe view DNA stain

3 µl of safe view DNA stain was added in 40 ml 1.5% agarose solution.

Reagents used for Immunohistochemistry

1 1.5% BSA in PBS

2 Citrate Buffer 10 Mm

Trisodium citrate	2.9 g
Distilled water	1 L

Mixed to dissolve, pH adjusted to 6.0 with 1 N HCl and 0.5 ml Tween 20 added.

3 3% H_2O_2 in Methanol

VITA

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THESIS ABSTRACT

a)	Title of the thesis	**EXPRESSION AND LOCALIZATION OF VISFATIN IN CORPUS LUTEUM OF BUFFALO AND ITS INFLUENCE ON PROGESTERONE SECRETION**
b)	Full name of student	**ANKITA THAKRE**
c)	Name and address of Major Advisor	**Dr. J.P. KORDE** **Associate Professor and Head,** **Dept. of Veterinary Physiology,** **Nagpur Veterinary College,** **Nagpur**
d)	Degree to be awarded	M.V.Sc
e)	Year of award of degree	2018
f)	Major subject	Veterinary Physiology
g)	Total number of pages in the thesis	32
h)	Number of words in the abstract	295
i)	Signature of Student	
j)	Signature, Name and Address of forwarding authority	

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ABSTRACT

The present research was conducted to investigate the expression and localization of a regulatory protein, visfatin in corpus luteum and to evaluate the *in vitro* role of visfatin on steroid production inluteal cell culture of buffalo ovary. To determine the expression and localization of visfatin, corpus luteum were

classified to following stages as days 1–4 (early), 5–10 (mid), 11–16 (late) and >17 (regressing stage) of estrous cycle. The qPCR, RT-PCR, western blot and immunohistochemistry techniques were applied to investigate mRNA, protein expression and localization. Beta actin and RPL15 was used as a control for mRNA expression and beta actin for protein expression calculation. The results of the present study indicates that visfatin is present in buffalo CL. However, the relative expression of visfatin varied according to the developmental stage of CL. Expression of visfatin was higher in late luteal phase and lower in early, mid and regressing phases ($P < 0.05$). Result of Immunohistochemistry showed that visfatin was localized in cytoplasm of large and small luteal cells with highest immunoreactivity observed in late luteal stage. For confirmation of steroid stimulating role of visfatin in CL luteal cells from 3rd stage CL were cultured *in vitro* and treated with visfatin each at 1 and 10 ng/ml concentrations for 48 h after obtaining 75-80% confluence. At a dose of 1 and 10 ng/ml of visfatin, there was significant ($P < 0.05$) increase in progesterone concentration from spent media of treatment group as compared to control group. Progesterone concentration increased with visfatin treatment in a dose dependent manner. The mRNA expression of progesterone secretion intermediate (CYP11A1and 3β-HSD) and apoptotic markers (PCNA and BAX) was non significantly higher in cultured cells treated with 1 ng/ml of visfatin as compared to control and 10 ng/ml cultured cells.

प्रबंधसारांश

अ.	प्रबंधाचे शिर्षक	:	म्हैशीच्या विस्फारित बिजकोषातील ल्युटियल पेशींमध्ये व्हिस्फॅटिनचा अविर्भाव व स्थान निश्चिती तसेच त्याचा पेशींतील प्रोजेस्ट्रॉनच्या स्त्रावावर प्रभाव
ब.	विद्यार्थ्याचे पुर्ण नांव	:	**अंकिता ठाकरे**
क.	मार्गदर्शकाचे नांव व पत्ता	:	डॉ. जे.पी. कोरडे सहयोगी प्राध्यापक व विभाग प्रमुख पशु शरिरक्रियाशास्त्र विभाग नागपूर पशुवैद्यक महाविद्यालय नागपूर
ड.	प्रदान करण्यात येणारी पदवी	:	**एम.व्ही.एस.सी**
इ.	पदवी प्रदान करण्याचे वर्ष	:	२०१८
फ.	मुख्य विषय	:	**पशु शरीरक्रियाशास्त्र**
ग.	प्रबंधातील एकूण पृष्ठे	:	३२
ह.	सारांशातील एकूण शब्द	:	२०२
ई.	विद्यार्थ्याची सही	:	
ज.	अग्रेषित करणाऱ्या अधिकाऱ्याची सही, नांव आणि पत्ता	:	सहयोगी अधिष्ठाता नागपूर पशुवैद्यक महाविद्यालय नागपूर

सारांश

सदर संशोधन म्हैशीच्या विस्फारित झालेल्या बिजकोषामध्ये व्हिसफॅटिन नामक नियामक प्रथिनाचा (रेग्युलेटरी प्रोटिन) अविर्भाव व ल्युटीयल पेंशीमध्ये त्यांचे स्थान निश्चिती तसेच व्हिस्फेटिनचा ल्यूटीयल पेंशीतील प्रोजेस्टेरॉनचा स्त्रावावरच्या

प्रभावाचे अध्ययन करण्यात आले आहे. व्हिस्फेटिनचा अविर्भाव, स्थान निश्चिती व प्रमाण निर्धारित करण्यासाठी कॉर्पस ल्युटीयम चे कार्यात्मक व रचनात्मक विकासानुसार चार टप्प्यात वर्गीकरण करण्यात आले. जसे की प्रारंभिक विकासात्मक टप्पा (बिजकोश विस्फारित झाल्यानंतर १–४ दिवस), मध्ये (५–१० दिवस), शेवटचा विकासात्मक टप्पा (११–१६ दिवस) आणि प्रतिगमीत कॉर्पस ल्युटीयम (१७ दिवसानंतर) वर्गीकृत केलेल्या कॉर्पस ल्युटीयम मध्ये व्हिस्फेटिनचा अविर्भाव स्थान व प्रभावाचे विश्लेषण करण्यासाठी क्यु. आरटी–पि.सी.आर, आरटी.पी.सी.आर, वेस्टर्न ब्लॉटींग, इम्युनोहिस्टोकेमिस्ट्री ई. तंत्रपध्दतीचा वापर करण्यात आला. व्हिस्फेटिनचा अविर्भाव बिटाएक्टीन संप्रेरक ठेवुन एमआरएनए व प्रथिन्याच्या प्रमाणावरून करण्यात आला. संशोधनाच्या परिणामातुन असे आढळले की म्हैशीच्या कॉर्पस ल्युटीयम मध्ये व्हिस्फेटिनचा अविर्भाव तुलनात्मक दृष्टया बिजकोश विस्फारित झाल्यानंतर शेवटच्या विकासात्मक टप्प्यात (११ ते १६ दिवस) अधिक व इतर विकासात्मक टप्प्यामध्ये कमी प्रमाणात आहे. व्हिस्फेटिन हे ल्यूटियल पेशींच्या सायटोप्लाझम मध्ये अधिक प्रमाणात केंद्रीत असल्याचे इम्युनोहिस्टोकेमेस्ट्री द्वारे निदर्शनास आले व हे प्रामुख्याने शेवटच्या विकासात्मक टप्प्यातील (११–१६ दिवस) लहान व मोठया पेशींमध्ये आढळले शेवटच्या विकसात्मक टप्प्यातील पेशींची ७५ ते ८० टक्के कृत्रिम वाढ पुर्ण झाल्यानंतर ४८ तासात व्हिस्फेटिनची १ व १० नॅनोग्रॅम प्रति मिली मात्रा दिल्यावर प्रोजेस्ट्रॉनचे प्रमाण आकडेवारीत्मक (पी<0.05) वाढल्याचे दिसून आले. तसेच व्हिस्फेटिनच्या वाढत्या मात्रेनुसार प्रोजेस्ट्रॉनच्या प्रमााणात वृध्दी दिसुन आली.