EVALUATION OF OREXIN SYSTEM IN BUFFALO OVARY AND ITS *IN VITRO* EFFECT ON STEROIDOGENESIS

THESIS

Submitted

in partial fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

IN

VETERINARY PHYSIOLOGY

BY

SARDAR VIKAS MURLIDHAR
Enrolment No.: V/02/745

Nagpur Veterinary College, Nagpur

MAHARASHTRA ANIMAL AND FISHERY SCIENCES UNIVERSITY, NAGPUR, 440 001
(INDIA)

2021

DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled "**EVALUATION OF OREXIN SYSTEM IN BUFFALO OVARY AND ITS *IN VITRO* EFFECT ON STEROIDOGENESIS"** 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.

Date:

Place: Nagpur

Signature

SARDAR VIKAS MURLIDHAR
Enrol.No.: V/02/745

(Dr. J. P. Korde)
Chairman,
Advisory Committee with date

DECLARATION OF ADVISORY COMMITTEE

Shri SARDAR VIKAS MURLIDHAR has satisfactorily prosecuted his course of research for a period of not less than two semester and that the thesis entitled "**EVALUATION OF OREXIN SYSTEM IN BUFFALO OVARY AND ITS *IN VITRO* EFFECT ON STEROIDOGENESIS**" submitted by him is the result of research work sufficient to warrant its presentation to the examination in the subject of **Veterinary Physiology** for the award of **Doctor of Philosophy** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur. We also certify that the thesis or part there of has not been previously submitted by him of a degree of any other University.

Place: Nagpur

Date:

Dr. J. P. Korde
Advisor/ Guide
Associate Professor & Head
Dept. of Veterinary Physiology
Nagpur Veterinary College, Nagpur

Name and Designation of Advisory Committee

	Name	Designation	Signature
1.	**Dr. J. P. Korde (Chairman)**	Associate Professor & Head Dept. of Veterinary Physiology Nagpur Veterinary College, Nagpur	________________
2.	**Dr. K. B. Bahiram (Member)**	Assistant Professor Dept. of Veterinary Physiology Nagpur Veterinary College, Nagpur	________________
3.	**Dr. S. W. Bonde (Member)**	Professor & Head Dept. of Veterinary Biochemistry Nagpur Veterinary College, Nagpur	________________
4.	**Dr. S. B. Kawitkar (Member)**	Professor & Head Dept. of Animal Nutrition Nagpur Veterinary College, Nagpur	________________
5.	**Dr. D. S. Raghuwanshi (Member)**	Professor ARGO, & DDR(CBF) Nagpur Veterinary College, Nagpur	________________

CERTIFICATE

This is to certify that the thesis entitled "**EVALUATION OF OREXIN SYSTEM IN BUFFALO OVARY AND ITS *IN VITRO* EFFECT ON STEROIDOGENESIS"** submitted by **SARDAR VIKAS MURLIDHAR** to Maharashtra Animal and Fishery Sciences University, Nagpur in partial fulfillment of the requirement for the degree of Ph.D. has been approved by the Student's Advisory committee after examination in collaboration with the External Examiner.

Name & Signature of External Examiner	Signature with Seal Head of Department	**Dr. J. P. Korde** Advisor/ Guide Associate Professor & Head Dept. of Veterinary Physiology Nagpur Veterinary College, Nagpur

Name and Designation of Advisory Committee

	Name	Designation	Signature
1.	**Dr. J. P. Korde (Chairman)**	Associate Professor & Head Dept. of Veterinary Physiology Nagpur Veterinary College, Nagpur	________________
2.	**Dr. K. B. Bahiram (Member)**	Assistant Professor Dept. of Veterinary Physiology Nagpur Veterinary College, Nagpur	________________
3.	**Dr. S. W. Bonde (Member)**	Professor & Head Dept. of Veterinary Biochemistry Nagpur Veterinary College, Nagpur	________________
4.	**Dr. S. B. Kawitkar (Member)**	Professor & Head Dept. of Animal Nutrition Nagpur Veterinary College, Nagpur	________________
5.	**Dr. D. S. Raghuwanshi (Member)**	Professor ARGO, & DDR(CBF) Nagpur Veterinary College, Nagpur	________________

Associate Dean
Nagpur Veterinary College,
Nagpur

ACKNOWLEDGEMENT

On the execution of the present study, I would like to take this opportunity to extend my deepest sense of gratitude and words of appreciation towards those who helped me during the pursuit of this study. I deem to a proud privilege and feel immense pleasure to acknowledge all those who are directly or indirectly involved.

Firstly, I would like to express my sincere gratitude to my guide **Dr. J .P. Korde**, Associate Professor and Head, Department of Veterinary Physiology, Nagpur Veterinary College Nagpur for the continuous support of my Ph.D study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study.

Words are not sufficient to express my sincere sense of gratitude, but with full honor I express sincere thanks to my co-guide, **Dr. Mahesh Gupta,** Assistant Professor, Department of Veterinary Physiology, Nagpur Veterinary College Nagpur, for his sagacious guidance, invaluable suggestions, judicious supervision, meticulous planning, keen interest, constructive criticism, excellent co-operation, constant encouragement and generous contribution of his knowledge during the entire period of my research work.

I owe a debt of gratitude to all the members of the advisory committee **Dr. K. B. Bahiram** Assistant Professor, Department of Veterinary Physiology, **Dr. S. W. Bonde**, Professor, Department of Veterinary Biochemistry, **Dr. S. B. Kawitkar**, Professor and Head, Animal Nutrition, **Dr. D. S. Raghuwanshi**, Professor, Department of ARGO & DDR, CBF, NVC, Nagpur for their painstaking guidance and generous help during the entire period of my study.

It is my pleasure to express my sincere thanks to **Dr. Bhanubhakode**, Professor and Head Department of Veterinary Anatomy, **Dr. N. V. Kurkure,** Professor and Head, Department of Veterinary Pathology and **Dr. S. W. Kolte**, Professor and Head, Department of Veterinary Parasitology and **Dr. S. P. Choudhary,** Professor and Head Department of Veterinary Public Health Epidemiology and Centre for Zoonoses, for providing me scientific gazettes required for the analysis study of the work.

I express my sincere thanks to ***Dr. S. B. Kawitkar****, Associate Dean, Nagpur Veterinary College, Nagpur for providing necessary facilities required for this project to carry out the research work.*

Without the expertise help ***of Dr. Shashank Dudhe & Dr. Swapnil Magar (JRF)*** *the important section of thesis could not be completed. He deserves my whole hearted respect, thanks and gratitude.*

My sincere thanks to ***Shree. D. V. Patil,*** *Assistant Professor, Department of Animal Genetics and Breeding for his kind cooperation throughout the period of work.*

My deepest gratitude goes to my close friends ***Dr. S. P. Awandker*** *for their moral support, uncountable kindness and help during entire research work.*

I also want to thank to ***Shree S.S.Chavan kaka & Anil Kamble Kaka*** *for their co-operation for completion of my research.*

I wish to express my deep sense of gratitude to ***Dr. Karuna Bambode*** *for her unforgettable help during research work.*

I really thankful to my colleagues ***Dr. Ashish Shende, Dr. Kishor Rathod, Dr.Umesh Mayande, Dr. Shekhar Badhe, Dr. Vijay Basunathe and Dr. Suresh Jadhav*** *for their unfaltering help and inspiration.*

My vocabularies utterly fail in expressing my accolade to my Parents Shree Murlidhar Sardar and Smt. Kusum in molding my future and warmly recollect the love and affection. I fondly recall with love, the emotional support, consistent encouragement and inspiration exhibited by my Sister ***Rasika & sister in laws Umesh Surwade.***

Last but by no means least it gives me immense pleasure to thank my wife ***Sonal and My daughter Akshaya and Nilaya.*** *They supported me mentally while staying at Nagpur during my PhD work.*

"Dedicated To My Parents"

Date:

Place: Nagpur **Sardar Vikas Murlidhar**

TABLE OF CONTENTS

	CHAPTER	PAGE NO.
I	INTRODUCTION	1-5
II	REVIEW OF LITERATURE	6-18
III	MATERIALS AND METHODS	19-36
IV	RESULTS AND DISCUSSION	37-45
V	SUMMARY AND CONCLUSIONS	46-50
VI	PROPOSED AREA OF FUTURE RESEARCH	51
A)	BIBLIOGRAPHY	i-x
B)	APPENDIX	xi
C)	VITA	xii
D)	THESIS ABSTRACT	xiii-xv
E)	प्रबंध सारांश	xvi-xviii

LIST OF TABLES

Table No.	PARTICULARS	Page No.
3.1	Target gene, primer sequence, amplicon length and accession number for qRT-PCR used in the study	**20**
3.2	Classification of Corpus Luteum	**22**
3.3	Reaction mixture for cDNA synthesis	**26**
3.4	Reaction mixture for optimization of primers	**27**
3.5	Cycling conditions for optimization of primers	**27**
3.6	Reaction mixture for qRT-PCR	**29**
3.7	Cycling steps for qRT-PCR	**30**

LIST OF FIGURES

Sr.No.	Title	After Page
4.1	Amplification cure (A) and Dissociation curve (B) for Prepro-orexin (PPO), OX1R and OX2R.	**38**
4.2	Relative mRNA expression of PPO, OX1R, and OX2R in CL and agarose gel showing its product.	**38**
4.3	Concentration of progesterone (P_4) in orexin treatment at three (3) different doses rate in luteal cell culture.	**40**
4.4	Standard curve for progesterone (P_4)	**40**
4.5	Expression of steroidogenic gene StAR (A), CYP11A1 (B) and 3B-HSD (C) mRNA in luteal cell culture on orexin treatment for at 3 different dose rate (n=4 CL) in buffalo.	**40**
4.6	Amplification curve (a) and Dissociationcurve (b) for StAR (A), CYP11A1 (B) and 3β-HSD (C) in cultured luteal cell.	**42**
4.7	Relative mRNA expression of PPO, OX1R, and OX2R in GC and TIC in buffalo.	**42**
4.8	Concentration of estradiol (E_2) in orexin treatment at three (3) different doses rate with FSH and IGF-I in GC culture.	**44**
4.9	mRNA expression of aromatase CYP19A1 in GC culture.	**45**

LIST OF PLATES

Sr. No.	Title	After Page
4.1	1.5% agarose gel showing 179bp, 144 bp&77 bp product for pre-pro orexin (PPO), OX1R and OX2R.	**38**
4.2	Distribution of orexin A in immunoreactive cells in different stages of CL. observed under 20X. The distribution of orexin positive cells in CL1, CL2, CL3, CL4 & Negative Control (arrow) LLC (Large Luteal Cell), SLC (Small Luteal Cell) and EC (Endothelial Cell).	**40**
4.3	Distribution of OX1R in immunoreactive cells in different stages of CL. observed under 20X. The distribution of orexin positive cells in CL1, CL2, CL3, CL4 & Negative Control (arrow) LLC (Large Luteal Cell), SLC (Small Luteal Cell) and EC (Endothelial Cell).	**40**
4.4	Distribution of OX2R in immunoreactive cells in different stages of CL. observed under 20X. The distribution of orexin positive cells in CL1, CL2, CL3, CL4 & Negative Control (arrow) LLC (Large Luteal Cell), SLC (Small Luteal Cell) and EC (Endothelial Cell).	**40**
4.5	1.5% agarose gel showing 84bp, 191bp and 146bp product for StAR (A), 3β-HSD (B) and CYP11A1(C).	**42**
4.6	1.5% agarose gel showing 179bp ,144 bp, 77 bp,100bp &140bp product for PPO, OX1R, OX2R, β-actin and RPL 15 in ovarian follicles.	**42**
4.7	Distribution of orexin A in immunoreactive cells in different stages of Follicular cell observed under 20X. The distribution of orexin positive cells in F1, F2, F3, F4 & Negative Control (arrow) GC (Granulosa Cell), TIC (Theca Interna Cell)	**44**
4.8	Distribution of OX1R in immunoreactive cells in different stages of Follicular cell observed under 20X. The distribution of orexin positive cells in F1, F2, F3, F4 & Negative Control (arrow) GC (Granulosa Cell), TIC (Theca Interna Cell)	**44**
4.9	Distribution of OX2R in immunoreactive cells in different stages of Follicular cell observed under 20X. The distribution of orexin positive cells in F1, F2, F3, F4 & Negative Control (arrow) GC (Granulosa Cell), TIC (Theca Interna Cell)	**44**

ABBREVIATIONS

%	:	Percentage
<	:	Lesser than
>	:	Greater than
°C	:	Degree Celsius
µg	:	Microgram
µl	:	Mililiter
3β-HSD	:	3β-hydroxysteroid dehydrogenase
ANOVA	:	Analysis of variance
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	:	Cytochrome p450 A (cholesterol side chain cleavage)
DAPI	:	4,6-diamidono-2-phenylindole
DEPC	:	Diethyl pyro-carbonate
DF	:	Dominant follicle
DMEM/F12	:	Dulbecco Modified Eagle and F-12 Ham Medium
DNA	:	Deoxy ribonucleic acid
DW	:	Distilled water
EDTA	:	Ethylenediamine tetra acetic acid
FBS	:	Fetal Bovine serum

FF	:	Follicular fluid
Fig.	:	Figure
FSH	:	Follicle stimulating hormone
g	:	Gram
GC	:	Granulosa cell
h	:	Hour/s
H_2O_2	:	Hydrogen peroxide
HRP	:	Horse radish peroxidase
IGF-I	:	Insulin like growth factor
KDa	:	Kilo Daltones
LN2	:	Liquid nitrogen
M	:	Molar
Mg	:	Milligram
$MgCl_2$	:	Magnesium chloride
Min	:	Minutes
Ml	:	Milliliter
Mm	:	Milimolar
MM	:	Master mix
Mol. Wt.	:	Molecular weight
mRNA	:	Messenger ribonucleic acid
NFW	:	Nuclease free water
ng	:	Nanogram
NTC	:	No template control
OD	:	Optical density
OX	:	Orexin
OX1R	:	Orexin Receptor 1

OX2R	:	Orexin Receptor 2
OXA	:	Orexin A
OXB	:	OreXin B
P_4	:	Progesterone
PBS	:	Phosphate buffered saline
PCNA	:	Proliferating cellular antigen
PCR	:	Polymerase chain reaction
Pmol	:	Pico mole
qPCR	:	Quantitative PCR
qRT-PCR	:	Quantitative real-time PCR
RNA	:	Ribonucleic acid
RPL 15	:	Ribosomal Protein L 15
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SD	:	Standard deviation
SDS	:	Sodium dodecyl sulphate
Sec	:	Seconds
SEM	:	Standard Error of Mean
StAR		Steroidogenic acute regulatory protein
TAE	:	Tris-Acetate-EDTA buffer
TIC	:	Theca interna cells
β-Actin	:	Beta actin

INTRODUCTION

Buffalo (*Bubalus bubalis*) is one of the most important multi-purpose dairy animal and contributes capital role in rural economy and dairy industry in many developing countries across the world. Owing to its notable contribution, it is rightly designated as the 'Black Gold' of India (Das *et al.,* 2013). The world buffalo population is 204.34 million across 42 countries, of which 198.41 million (97.1 %) are in Asia (FAOSTAT, 2019). Throughout the last 10 years, the world buffalo population approximately increased by 1.49% annually, and in India the population increased by 1.53 %. Buffalo population in India is 109.85 million, which is first ranking in the world population (livestock census, 2020). It contributes 72 million tons of milk and 3 million tons of meat in world food production and this is mainly produced by small holder farmers (Singh and Barwal, 2010).

The country has made grand spectra on the world stage being top milk producer and leading meat exporter with crucial contribution of 71.3 million tons milk (53 % of total) and 86 per cent of the exported meat of buffalo alone. Buffalo meat export has earned foreign exchange to the tune of around three billion US dollars in 2019-20 (APEDA 2020, www.apeda.gov.in).

In spite of its economic importance in agriculture, dairy and meat industries, scanty information is available on their gene organization, development and transcriptional status, including the molecular regulation of ovarian functions.

Mammalian reproduction involve the process of development, differentiation and alteration of ovarian follicular cells followed by establishment and regression of the corpora lutea (CL) in a cyclic fashion (Evans and Fortune, 1997). In buffalo, the follicular growth is categorized by 2-3 follicular waves during estrous cycle. When the follicle attains about 14-20 mm size it either ovulates or begins regressing (Ginther *et al*., 2003). The length of the estrous cycle in buffalo is similar to that of cattle, ranging from 17 to 26 days with a mean of around 21 days (Jainudeen and

Hafez, 1993). In buffaloes, ovarian follicular dynamics during the estrous cycle is similar to that of cattle (Taneja *et al.,* 1996). Majority of buffalo have two waves of follicular activity during their estrous cycle (Warriach and Ahmad, 2007). The buffaloes have estrous cycles with 1, 2 or 3 follicular waves. The number of waves in a cycle are associated with the luteal phase and with estrous cycle length (Baruselli *et al*., 1997).

The major function of the ovarian follicle is to provide a suitable environment in the form of estradiol (E2) and local growth factors for development, maturation and ovulation of the ovum (Gougeon, 1996), whereas corpus luteum (CL) secretes progesterone (P4) which is very important to create a suitable uterine environment for implantation of the embryo and maintenance of pregnancy (Schams and Berisha, 2004). The CL is temporarily functioning organ and plays an important role in the regulation of the estrous cycle and in safeguarding of pregnancy. The safeguarding of pregnancy is carried out mainly by progesterone (P4), the major steroid hormone synthesized by CL (Stocco *et al.,* 2007). This process of CL formation, development and regression as well as function of CL is well regulated by pituitary gonadotropins. However, numbers of local factors have also been found to regulate luteal dynamics (Chouhan *et al.,* 2013, Gupta *et al.,* 2014, Gupta *et al*., 2019, Thakre *et al*., 2021).

The role of pituitary gonadotropins and growth hormone is well documented in the process of follicular and luteal dynamics. Still, recent evidences also proved the essential modulatory role by locally formed factors such as steroid hormones, peptides and growth factors like vascular endothelial growth factor (VEGF), IGF-I, fibroblast growth factor (FGF), angiopoietin, thrombopoietin, ghrelin in the development of follicle, CL and regulation of reproductive cycle (Ali *et al*., 2014, Chouhan *et al*., 2014, Gupta *et al*., 2014, Gupta *et al*., 2015, Mishra *et al*., 2016).

The orexin is a neuropeptide also known as hypocretin and it regulates arousal, wakefulness, and appetite. The orexin binds to two G-protein coupled receptors, orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R). OX1R had

shown affinity for orexin-A and OX2R had shown affinity for both orexin-A and orexin-B (Sakurai *et al*., 1998). Orexin–A is a peptide synthesized mainly by neurons with perikarya located within and around the lateral and posterior hypothalamus, found to modulate the activity of gonadotropin-releasing hormone (GnRH) neurons and gonadotropin-secreting pituitary cells (Rosati, 2015). Orexin A (OXA) and Orexin B (OXB), are neuropeptides derived by proteolytic cleavage from a 130 amino acid precursor, prepro-orexin (PPO), which was isolated from the rat hypothalamus (de Lecea *et al*., 1998). Orexin A and Orexin B are synthesized mainly by neurons with their soma located in the lateral hypothalamus and projections throughout the brain, including gonadotropin-releasing hormone neurons in the rat and in ovine hypothalamus (Sasson *et al*., 2006).

Orexin neuropeptides have been studied in many different areas of neuroscience research, including sleep regulation, feeding behavior, reward and motivation, anxiety and depression, cardiovascular regulation, pain, migraine, and neuroendocrine regulation, including reproduction (Peyron and Kilduff 2017; Bonnavion and de Lecea, 2010). Orexin neurons perform various roles in the coordination of emotion, energy homeostasis, reward system and arousal. It is regulated by peripheral metabolic cues, including ghrelin, leptin, and glucose concentration. It provides a link between energy homeostasis and arousal states. Further, orexin neurons sense the outer and inner environment of the body and maintain the proper wakefulness level of the animals for survival (Inutsuka and Yamanaka, 2013). Manipulation of the orexin ligand receptor system demonstrate therapeutically useful not only in the treatment of sleep disorders such as narcolepsy and insomnia, but also in the treatment of several medical and psychiatric disorders associated with sleep disturbance (Taheri and Bloom, 2001).

Evidences demonstrate a close link between orexin and reproductive functions in females. Although the general view has been that the principal effect of orexin are on the neuroendocrine component of reproduction in some species (Russele *et al*., 2001; Small *et al*., 2003; Silveyra *et al*., 2007a; Kaminski *et al*., 2010a). The

expression of orexin and its receptors have been located in rat, pig, dog and cat ovaries (Cataldi *et al*., 2012; Chojnowska *et al*., 2012; Nitkiewicz *et al*., 2010, 2014; Levanti *et al*., 2015; Basini *et al*., 2018; Ciccimarra *et al*., 2018). In rat and pig, it is reported that peptide expression of orexin and its active receptor OX1R and OX2R are involved in follicular development (Silveyra *et al*., 2007b; Cicimarra *et al*., 2018) and localization of both receptors has also been observed in the granulosa and theca cell layers of healthy follicles in pig (Chojnowska *et al*., 2012; Nitkiewicz *et al*., 2014; Ciccimarra *et al*., 2018). In rat ovary, expression of the orexin receptors was observed throughout the estrous cycle but the level is fluctuated with oestrous cycle and the highest level observed in pro-estrous phase (Silveyra *et al.*, 2007b).

Some studies have shown that orexin has crucial role in steroidogenesis. Progesterone and estradiol are important steroid hormones produced by the CL and ovarian follicles, respectively. The hormone progesterone is produced primarily by CL in the ovaries during the second half of the estrous cycle (Diep *et al*., 2015), that maintains ovarian function. The steroidogenic acute regulatory protein (StAR), cytochrome P450 (CYP11A1), and 3-beta-hydroxysteroid dehydrogenase (3β-HSD) play important role in transforming cholesterol in to steroid hormones, especially progesterone. StAR regulates cholesterol transfer within the mitochondria, which is the rate-limiting step in the production of steroid hormones (Miller and Strauss, 1999). CYP11A1 participates in the biosynthesis of progenenolone from cholesterol (Storbeck *et al*., 2007). These three proteins are essential for the biosynthesis of all steroid hormones including P4 (Lachance *et al*., 1992). Similarly, the enzyme in estrogen biosynthesis is cytochrome P450 aromatase, which convert theca cell derived androgen to estrogen (Luu-The, 2001).

As per the literature available, orexin has been shown to regulate gonadal functions in rats, pig, humans, dog and cat. However, no reports are available regarding existence of orexin in buffalo ovary. Therefore, the present study was proposed to investigate the evaluation of orexin system in buffalo ovary and its *in vitro* effect on steroidogenesis with following objectives.

1. To study the expression of orexin mRNA during different developmental stages of ovarian follicles and corpus luteum in buffalo ovaries.
2. To study the localization of orexin during different developmental stages of ovarian follicles and corpus luteum in buffalo ovaries.
3. To study the effect of orexin on estrogen and progesterone secretion in cultured granulosa cell and 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 orexin and its receptors mRNA

Johren *et al*. (2001) observed expression of prepro-orexin (PPO) and the orexin receptor subtypes OX1 and OX2 in peripheral rat tissues which was analyzed by using real-time quantitative RT-PCR. They detected significant amounts of prepro-orexin mRNA in testis, but not in ovaries. OX1 receptor mRNA was highly expressed in the brain and at lower levels in the pituitary gland. Only small amounts of OX1 receptor mRNA was found in other tissues such as kidney, adrenal, thyroid, testis, ovaries, and jejunum. Very high levels of OX2 receptor mRNA, 4-fold higher than in brain, was found in adrenal glands of male rats. Low amounts of OX2 receptor mRNA was present in lung and pituitary. Moreover, the sexually dimorphic expression of OX1 and OX2 receptors in the hypothalamus, pituitary, and adrenal glands suggested gender specific role of orexins in the control of endocrine functions.

Karteris *et al*. (2004) investigated the expression of prepro-orexin (PPO) and receptors in the human male reproductive system was analyzed by RT-PCR using a cDNA panel. Both OX1R and OX2R appear to be expressed in human testis, epididymis, penis, and seminal vesicle. Interestingly, prepro-orexin appeared to have a more confined expression only in epididymis and penis, whereas testis and seminal vesicle were void of prepro-orexin mRNA.

Silveyra *et al.* (2007a) reported that PPO, orexin1 and orexin 2 expression was determined in ovaries of adult rats at different stages of the estrous cycle and correlated to the endocrine status, dark-light cycle, and food consumption. Both orexinergic receptors vary along the estrous cycle, with a very marked increased

observed in the evening of pro-estrous (orexin 1 expression increases 3-fold, while orexin 2 increase 4-fold). The fact that the increase of both orexin receptors expression exclusively during the late afternoon and night of pro-estrus. Further they suggested that ovulation is related to particular hormonal status and not related to the sleep-wake cycle or food intake.

Ning *et al.* (2008) investigated expression of prepro-orexin and orexin receptor 1 (OX1R) in hypothalamus during estrous cycle of swine. The expression of orexin receptor 1(OX1R) mRNA levels were highest in the meta-estrous phase and lowest in the di-estrous phase of estrous cycle of swine.

Nitkiewicz *et al.* (2010) determined the mRNA and protein expression of orexin receptors (OX1R and OX2R) in the porcine ovary on 2-3, 10-12,14-16 and 17-19 days of oestrous cycle. Using real-time PCR, higher OX1R mRNA expression was detected in porcine CLs than in granulosa and theca cells. The expression peak of the OX2R gene occurred in granulosa cells. The OX1R protein content was higher in CLs isolated during the luteal phase in comparison with follicular cells. The OX2R protein level was more pronounced in CLs on days 10-12 and 14-16 than in the remaining periods of the cycle.

Kaminski *et al*. (2010a) detected expression of OX1R and OX2R in adenohypophysis (AP) and neurohypophysis (NP) of cycling pigs. The gene expression was detected in adenohypophysis on days 2-3 relative to days 10-12, 14-16 and 17-19. In NP OX1R mRNA level was elevated on days 10-12 compared to the remaining stages. OX2R gene expression in adenohypophysis was the lowest on days 10-12 and the expression peak occurred on days 17-19. In neurohypophysis the highest expression of OX2R mRNA was noted on days 17-19 in relation to the remaining periods. OX1R protein content in adenohypophysis was greatest on days 10-12, whereas in neurohypophysis it was greatest on days 2-3 and 14-16. In both cases the lowest OX1R protein expression was observed during follicular phase. OX2R protein in adenohypophysis was lower on days 2-3 and 14-16 compared to

days 10-12 and 17-19. In neurohypophysis the lowest expression of this protein was on days 17-19 and the highest on days 10-12. On these bases it was suggested that the OX1R and OX2R mRNA expressed in the pituitary of the pig and indicate the dependence of orexin receptor expression on the endocrine reproductive state.

Kaminski *et al*. (2010b) showed expression of OX1R and OX2R in porcine hypothalamic structure engaged in GnRH production and secretion, preoptic area (POA), mediobasal hypothalamus (MBH) and stalk median eminence (SME) on days 2-3,10-12, 14-16 and 17-19 of the oestous cycle. The highest OX1R gene expression in preoptic area was observed on days 17-19 of the cycle. Changes in the mRNA expression in mediobasal hypothalamus and stalk median eminence throughout the cycle were negligible. The expression peak of OX2R gene in preoptic area and stalk median eminence occurred on days 17-19 as well. There were no changes in the gene expression in mediobasal hypothalamus. OX1R protein content in preoptic area and stalk median eminence was also greatest on days 17-19 and OX2R protein expression was most pronounced in mediobasal hypothalamus and stalk median eminence during the same phase of the cycle. However, fluctuation of OX1R and OX2R, mRNA and protein content in pig hypothalamus depend on the phase of estrous cycle. Further, study suggested that orexin, influence on the hypothalamic-pituitary-ovarian axis and affect reproductive functions.

Nitkiewicz *et al*. (2012) studied the expression of prepro-orexin (PPO) gene in porcine endometrium and myometrium. The highest expression of PPO mRNA was observed in the endometrium and the myometrium on day 14-16 of the cycle. The expression of the PPO gene on days 2-3 was more abundant in the myometrium than in the endometrium, whereas on days 17-19 the gene expression was markedly higher in the endometrium. Further they suggested expression of prepro-orexin (PPO) gene in porcine endometrium and myometrium in uterus and their release dependent on the hormonal status of animals.

Cataldi *et al.* (2014) detected OX1R and OX2R mRNA expression in anterior pituitary cell treated with OXA and OXB. OXA and OXB treatment decreased the mRNA expression of OX1R. The effect on OX1R was not modified by blocking agent, but it was abolished in presence of antagonist. The blocking drugs alone did not modify expression. Neither OXA, and nor the blocking drugs, modified OX2R mRNA expression. OXB increased OX2R mRNA expression and only OX2R antagonist blocked this effect.

Nitkiewicz *et al*. (2014) reported that the expression of prepro-orexin mRNA was significantly higher in porcine theca interna cell on days 17-19 than in granulosa cells and corpora lutea isolated on day 2-3, 10-12 and 14-16 of the estrous cycle of pig.

Smolinska *et al*. (2014) investigated the expression of the prepro-orexin (PPO) gene increased in adenohypophysis and neurohypophysis during the estrus cycle. The highest PPO protein concentration in adenohypophysis (AP) were reported on 2 to 3 days of estrus cycle and in neurohypophysis (NP) on 10 to 12 and 17 to 19 days of estrus cycle. The expression of PPO mRNA was lower in adenohypophysis than in neurohypophysis, but PPO protein level were higher in adenohypophysis. The study suggests expression of the orexin gene and orexin proteins in the porcine pituitary and the correlations between expression levels and the phases of the estrous cycle.

Smolinska *et al*. (2015) noted that the expression of prepro-orexin (PPO), OX1R and OX2R was different in the conceptuses and trophoblasts during early pregnancy. Local orexin production and the presence of the specific orexin receptors suggest that the orexin system may participate in the control of porcine reproductive functions by exerting endocrine and auto/paracrine effects on the uterus, conceptuses and trophoblasts during early pregnancy. This study provides the first evidence for the presence of orexins and their receptors in the uterus, conceptuses and trophoblasts

in pigs during early pregnancy. The local orexin system is dependent on the stage of pregnancy.

Joshi and Singh (2017) determined the expression of orexin A and orexin 1R in mouse testis during different stage of postnatal development. The semi-quantitative RT-PCR analysis showed that orexin A and orexin 1R were expressed in the testis both at transcript and protein levels during different stages of postnatal development. The expression of orexin A and orexin 1R increased progressively from day of birth (0 dpp) until adulthood (90 dpp), with maximal expression at 90 dpp.

Basini *et al*. (2018) reported expression of OXA and its receptor OX1R and OX2R in swine corpus luteum. The expression of PPO and OXA and its receptors levels are closely associated with the animal hormonal status as they vary throughout the estrous cycle. Particularly, corpora lutea, they showed that the highest intensity occurred during the mid-luteal phase (days 10-12 in estrus cycle).

2.2 Localization of orexin and its receptors by Immunohistochemistry

Johren *et al*. (2001) investigated localization of OX2 receptor mRNA in zona glomerulosa and reticularis by *in situ* hybridization, indicating a role in adrenal steroid synthesis and / or release. OX1 receptor mRNA in the pituitary and OX2 receptor mRNA in the adrenal gland were much higher in male than in female rats. In the hypothalamus, OX1 receptor mRNA was slightly elevated in female rats. The differential mRNA expression of orexin receptor sub-types in peripheral organs indicates discrete peripheral effects of orexins in the existence of a peripheral orexin system.

Dall'Aglio *et al*. (2010) investigated presence and distribution of orexin A in the pancreas of domestic (Cattle, sheep and pigs) animals. In score of immunohistochemistry, the endocrine cell was positive for OXA in the presence of cytoplasmic staining. The OXA positive cells were oval-or round shaped and contained many small densely grouped at perinuclear granules. Further they

suggested that the OXA cells were arranged in clusters and mainly distributed around blood capillaries.

Tafuri *et al*. (2010) investigated the orexin A immunoreactivity in sertoli cells (round shaped) and spermatids (oblong shaped) of rat testis. Hematoxylin-counterstained sections revealed orexin A positive spermatids in the stages of the germinal epithelium cycle ranging from the VII to the XIVth stage.

Nitkiewicz *et al*. (2012) conducted experiment on localization of orexin A and orexin B in porcine uterus, the orexin A signal intensity was highest on days 2-3 in the luminal epithelium and on days 2-3 and 10-12 in the stroma. In circular muscles of the myometrium, the highest immune-reactivity was found on days 2-3. Orexin B immune-reactivity was highest on days 10-12 in longitudinal muscles, on days 17-19 in glandular epithelium and stroma, and on days 10-12 and 14-16 in luminal epithelium. They suggested that orexin A and B are produced in the porcine uterus and that their release independent on the hormonal status of animals.

Nitkiewicz *et al*. (2014) reported orexin A and B was localized in cells of the porcine ovary (corpora lutea, granulosa and theca interna cells of ovarian follicles) during the estrous cycle. The highest immunoreactivity of orexin A in CL was observed on days 10-12 than 14-16 of the cycle. In theca intern cell the signal intensity of immune-reactivity of orexin A was higher than granulosa cells on days 17-19 of the estrous cycle. The immune-reactive orexin B intensity was highest in CL on day 14-16 than day 2-3 and 10-12 of the cycle. Signal intensity of immune-reactive orexin B was similar in granulosa and theca interna cells of ovarian follicles.

Smolinska *et al*. (2014) observed that localization of orexin A and B in the porcine pituitary during the estrous cycle was in adenohypophysis (AP), orexin A immunoreactivity was higher on days 10 to 12 and 14 to 16. In neurohypophysis (NP), the highest content of the analyzed protein was observed on days 10 to 12 and the lowest level on days 14 to 16 and 17 to 19. OXB immunoreactivity in

adenohypophysis reached the highest level on day 2 to 3, and the lowest level was determined on days 10 to 12 and 17 to 19.

Levanti *et al*. (2015) explained the role of orexin A in female genital system of dog and cat and demonstrated the presences of orexin A in the ovary of dog and cat through immunofluorescence technique. The positivity for the antibody tested was demonstrated in all the developmental follicular stages. In cat, ovary positivity for anti-orexin A was detected in primary follicles at the level of oocyte localized in cortical zone. In secondary follicles, positivity was observed in the oocyte and in thecal cells. In dog ovary, reactivity was observed in all the developmental stages too. In primary follicles, positivity was present in oocyte and in follicular flattened cells. In secondary follicles, oocyte and thecal cell were positive. In the tertiary follicle, a faint immune-reactivity was present only at the level of cumulus oophorus cell, showing a different pattern in comparison with cat.

Liguori *et al*. (2017) reported the presence of orexin B and orexin 2 receptor by means of immunohistochemistry. Orexin B immune-reactivity (IR) was found in the tubular compartments of the testis and particularly in leydig cells and round and elongated spermatids. They suggest that orexin B and its receptor 2 play a functional role both in the interstitial and tubular compartments of the alpaca testis.

Joshi and Singh (2017) localized orexin A and its receptor in mouse testis by immunohistochemistry. They observed OXA and OX1R in both the interstitial and tubular compartments of the testis throughout the period of post-natal development. In testicular secretion on day 0 of postpartum (dpp), gonocytes, sertoli cells and foetal leydig cells showed OXA and OX1R immune-positive signals. At 10 dpp, sertoli cell, spermatogonia, early spermatocytes and leydig cells showed immune-positive signals for both the ligand and the receptor. On 30 and 90 dpp, the spermatogonia, sertoli cell, spermatocytes, spermatids and leydig cells showed the OXA and OX1R immune-positive intense signals. At 90 dpp, strong OXA positive signals were seen in leydig cells, primary spermatocytes and spermatogonia, while OX1R-

immunopositive intense signals were observed in leydig cells and elongated spermatids.

Basini *et al*. (2018) reported expression of orexin A and its receptor OX1R and OX2R in swine corpus luteum. Localization immunohistochemistry (IHC) revealed intense immunostaining of cytoplasm and nucleus of luteal cells for OXA and OX2R, whereas the immunoreactivity (IR) for OX1R was mostly associated with the nuclear envelop. OXA and OXR2 in luteal cells resembled in immunohistochemistry (IHC) despite a lesser intensity being observed for OXR2, whereas the IR for OXR1, besides being weakly diffused in the cytoplasm, was predominantly associated with the nuclear envelopes, as the positive signals for the IR overlapped the one for DAPI. Immunoreactivity for OXA and its receptors was clearly observed in some corpora lutea, specially in the septa derived by the folding of the theca. Endothelial cells of blood vessels and small elongated or irregular shaped cells in the interstitium clearly also shown IR for OXA and its receptors. Further, OXA and its receptors are co-localized in these cells, both at the level of the nuclear envelop and in the cytoplasm. Frequently, the cytoplasmic IR had a granular shape.

Ciccimarra *et al.* (2018) conducted an experiment on the localization of orexin A (OXA) and its receptors by immunochemistry in different ovarian follicles. The intense cytoplasmic positivity was observed for OXA in granulosa and thecal cells of swine ovarian follicles. In granulosa cells and in the corona radiata cells of follicles the signal intensity of OXA was faint but clear by localized in their cytoplasm. In the theca interna, number of cells for OXA and its receptors was small and of two different types. First type was represented by irregular shaped cells, second type cell was roundish with cytoplasm uniformly immunoreactive only for OXA. In the interstitial tissue underlying large follicles, very large number of irregular-shaped cells shown strong immunoreactivity for OXR1 and OXA. In granulosa cell, the localization of OXR1 appeared related to the nucleus, whereas OXR2 showed a clear cytoplasmic and diffuse localization.

Ragionieri *et al*. (2018) reported experiment on localization of OXA and its receptors in porcine ovary, orexin A and its receptors localization by double immunofluorecent method. OXA and OX2R appeared strong associated to cytoplasm vesicles, while OX1R occurs overlapped nucleus in cytoplasm in DAPI staining. The immunoreactivity for OX1R was more intense nuclear envelope and to a lesser extent in cytoplasm. Immunoreactivity of OXA and OX2R was clearly detected in cytoplasm.

Li *et al*. (2019) investigated the immunolocalization of granulosa cells by immunofluorescence in ovian ovary. The cell morphology of the granulosa cells was shown by using haematoxyline and eosin staining of cell sections and giemsa staining showed that the edge was clear, resembling a triangular cone or in irregular star. The cytoplasm was pink and the nucleus was dyed blue colour.

2.3 Estrogen and Progesterone secretion from granulosa and theca interna cells.

Porkka-Heiskanen *et al*. (2004) studied both orexin A and B concentration in hypothalamus of rat. The orexin A concentration in the hypothalamus of young cycling rats was higher on the day of pro-estrus 5-6 h after the lights were switched on, than on the other days of the estrous cycle at the same circadian time. Orexin B concentration was higher on both the day of pro-estrus and the day of estrus as compared with the days of diestrus. The hypothalamic concentrations of both orexin A and B in the non-cycling middle-aged rats were lower than those in cycling rats on the days of proestrus and estrus.

Silveyra *et al*. (2007a) demonstrated the prepro-orexin (PPO), orexin 1 Receptor (OX1R) and orexin 2 receptor (OX2R) expression in different stage of estrous cycle of rats. Both orexinergic receptors vary with the estrous cycle, in evening there was marked increased in OX1R with 3-fold and in OX2R increased with 4-fold during pro-estrous phase.

Silveyra *et al.* (2007b) reported that expression of orexin receptor and prepro-orexin (PPO) in rat hypothalamus was detected that orexin receptor 1 (OX1R) and Orexin receptor 2 (OX2R) gene expression was increased higher at three-fold in anterior hypothalamus and medio-dorsal hypothalamus during pro-estrous phase of cycle.

Cataldi *et al*. (2012) investigated the effect of orexins on the mRNA expression of both receptors, by quantitative RT-PCR on luteal cells from super-ovulated rat ovaries and granulosa cells from diethylstilbestrol treated rat ovaries. *In vitro* treatment of orexin-A (1 nM) or orexin-B (1nM) in luteal cells decreased progesterone secretion. Orexin A treatment increased expression of both orexin-1R and orexin-2R mRNA. The effect on orexin-1R mRNA expression was abolished by an orexin-1R selective receptor antagonist SB-334867 and the effect on orexin-2RmRNA expression was abolished by a selective orexin-2R antagonist JNJ-10397049. Orexin-B did not modify orexin-1R mRNA expression, but increased orexin-2R mRNA expression. The effect of orexin-B on orexin-2R was abolished by a selective orexin-2R antagonist. Neither the expression of orexin receptors nor progesterone secretions by granulosa cells were affected by orexins. FSH, as positive control, increased both steroid hormones secretion, but did not induce the expression of orexin receptors in granulosa cells isolated from late pre-antral /early antral follicles. In ovaries obtained immediately after sacrifice of rats, the expression of orexin-1R and orexin-2R was higher in super-ovulated rat ovaries compared to control or diethylstilbestrol treated rat ovaries. A selective presence and function of both orexinergic receptors in luteal and granulosa cells is described, suggesting that the orexinergic system have a functional role in the ovary.

Chojnowska *et al*. (2012) studied the effect of orexins on secretion of progesterone and estradiol by FSH-induced porcine ovarian granulosa cells harvested from large follicles of pigs ovaries. The cells were cultured in the presence of orexin A or B (1-100 nM) added alone or in combination with orexin receptor type 1 antagonist (SB, 1 μM) or receptor type 2 blocker (JNJ, 1 μM). Both orexins did not

influence progesterone secretion compared to the control group. However, orexin A and B higher dose (100 nM and 10 nM, respectively) used together with their receptor blockers inhibited progesterone release. They suggest both orexins suppressed estradiol production by granulosa cells and which influences the steroidogenesis in porcine granulosa cells.

Cataldi *et al.* (2014) demonstrated the gonadotropins secretion into culture media of anterior pituitary cells incubated with orexin A and B; whereas orexin A increased FSH and LH secretion. The effects of the neuropeptide on FSH and LH were suppressed by the blocking agent, OX1R antagonist SB-334867 and OX2R antagonist JNJ-10397049, when used alone or combined. OXB shows a similar effect. The blocking drugs by themselves had no action on gonadotropins release in to the culture medium.

Nitkiewcz *et al*. (2014) observed *in vitro* effect of orexins on the secretion of steroid hormones by porcine luteal, granulosa and theca interna cells. The highest expression of prepro-orexin mRNA was observed in theca interna on days 17-19 of the estrous cycle, and they examined dose (0.1; 1; 10nM), orexins A and B did not change non-stimulated progesterone secretion by luteal, granulosa and theca interna cells, estradiol release from granulosa and theca interna cell as well as androstenedione and testosterone secretion by theca cells. Under *in-vitro* conditions, at the concentration of 10 nM, orexins A and B inhibited FSH- induced estradiol secretion by granulosa cells. Further they suggested that orexins can affect porcine reproductive functions through modulation of ovarian steroidogenesis.

Smolinska *et al*. (2014) investigated that in porcine pituitary, enhanced level of OX1R mRNA during the luteal phase and higher concentration of prepro-orexin (PPO) and OX2R mRNAs during the follicular phase of the cycle imply that OX2R gene expression is stimulated by progesterone, whereas the expression of prepro-orexin (PPO) and OX2R genes are influenced by estradiol.

Salim *et al.* (2016) observed that mean relative mRNA expression of CYP19A1 was significantly increased in the hypothalamus of androgenized compared to non-androgenized female rats futher they observed that hypothalamic cells were cultured and treated with orexin (2, 4 and 8 µg/mL) which decreased significantly the hypothalamic CYP19A1 mRNA level of androgenized rats compared to androgenized control group. Further, they suggested orexin exert inhibitory effect on the gene expression CYP19A1 in the hypothalamus of female rats.

Kiezun *et al*. (2017) investigated OXA effect on oestradiol (E2) and oestrone (E1) secretion by porcine endometrial and myometrial explant and gene expression of key steroidogenic enzymes cytochrome P450c17 (CYP17A1) and P450 aromatase (CYP19A3) on day 10-11, 12 to 13, 15 to 16 and 27 to 28 of pregnancy and on days 10-11 of the estrous cycle. OXA enhanced CP17A1 and CYP19A3 expression on days 15-28 of pregnancy, whereas decreased their expression on day 10-13. In the myometrium, OXA increased E1 secretion on day 10-16 of pregnancy, whereas, inhibited the release of E2 on days 27-28 and 12 to 13 of pregnancy. The expression of both the genes was suppressed on days 10-11 and 15-16 of pregnancy ($P<0,05$). Further they suggest that OXA via its influence on steroidogenesis play a regulatory role in the uterus.

Dobrzyn *et al.* (2018) investigated *in vitro* effect of progesterone (P4) on orexin system in porcine uterine tissues during pregnancy, in the endometrium, progesterone (P4) enhanced orexin A secretion on days 10 to 11 of gestation and orexin B secretion on days 12 to 13. In the myometrium, progesterone inhibited the secretion of both orexins on days 15 to 16 and OXB secretion also on days 12 to 13. In the endometrium, progesterone inhibited the expression of OX1R at nearly all times analyzed, whereas, the expression of OX2R was inhibited only on days 15 to 16 of gestation. In the myometrium, progesterone stimulated OX1R expression on days 12 to 13 and 15 to 16 of gestation and inhibited OX1R expression on days 27 to 28. The expression of OX2R in the myometrium increased on days 12 to 13 and

decreased on days 10 to 11 and 15 to 16. Further, they suggest that progesterone (P4) could regulate the expression of the orexin system in the porcine uterus during early pregnancy. It also play an important role in the regulation of maternal metabolism during pregnancy and contribute with the regulation of the reproductive system during pregnancy.

Ciccimarra *et al.* (2018) conducted the experiment on orexin system in swine ovarian follicles, in that orexin A effect on granulosa cell viability evaluated as ATP content, was significantly increased without different concentrations of OXA (0.1,1 and 10 nM). Whereas, the highest OXA concentration (10 nM) displayed a stimulatory effect. The evaluation of steroidogenic activity by RIA assay revealed that estrogen (E2) production was increased by all OXA concentration while the substance was ineffective in modulating progesterone (P4) production.

Kisielewska *et al.* (2019) studied effect of orexin B on the activity of 3β-HSD gene expression in endometrial and myometrial tissue and observed that OXB increased the expression of 3β-HSD at dose 1nM and 100nM in endometrium and in myometrium during pregnancy. The author observed that the increased 3β-HSD expression due to orexin B stimulation may stimulate the progesterone production during pregnancy

Li *et al*. (2019) investigated the effect of orexin A on progesterone secretion in granulosa cells from follicles in sheep. *In vitro* treatment with different concentration of orexin (1nM, 10 nM, 58nM, 100nM and 145 nM) with varying period of time (0 h, 24 h, 48 h and 72 h) promoted the secretion of progesterone (P4) from granulosa in ovine ovary as compared with the control. The expression of StAR, 3 β HSD and P450 (CYP11) gradually increased, and then decreased with increasing concentrations of orexin A but the expression of P450 (CYP11) decreased with the increase of time. Thus, they concluded that orexin A promotes the secretion of progesterone (P4) by regulating the expression of StAR, 3 β HSD and P450 (CYP11).

MATERIALS AND METHODS

The present study was conducted in the Department of Veterinary Physiology, Nagpur Veterinary College, Nagpur using number of materials and techniques which are described in this section. The geographical location of Nagpur is situated at an altitude of 307.42 meters above the mean sea level on 21.1503°N latitude and 79.0244°E longitude. Climate of Nagpur follows the same seasonal monsoons pattern wherein the hottest months are May and June. Monsoons pour in from July to September while chilling winter in December and January. The experiment was carried out from Jan. 2019 to Dec. 2020 for a period of 1 years and 11 month. 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-dt15 primer, PCR nucleotide Mix were from Promega, USA. The chemicals like Tris, Chloroform, Isopropanol, Ethanol, Agarose, DEPC, RIPA, buffer, Bradford reagent, bovine serum albumin Fraction V (BSA) from Himedia; 50 bp DNA ladder from BioLavs, New England; Trizol reagent from Invitrogen; DAB form Roche, Germany; GoTaq® Green Master Mix kit (Cat. No. AEC 101) from Sigma; FSH (Cat. No. F 2293) Sigma Aldrich, IGF-I (Cat. No. TC 300) Himedia Lab. India and Estrogen, Progesterone kits Cat. No. 402110, Cat. No. 402310 respectively from Neogen Life Sciences, USA.

3.2 Antibodies

3.2.1 Primary antibodies

Orexin A (KK09) (Lot – L1318) SC-80263 mouse monoclonal IgG_1, Santa Cruz Biotechnology.

Orexin R 1/2 (Lot- L0711) SC-166111 mouse monoclonal IgG_1, Santa Cruz Biotechnology.

3.2.2 Secondary antibodies

M-IgG k BP- HRP (Lot- J3118) M-IgG k BP- HRP SC- 516102 HRP conjugated, Santa Cruz Biotechnology.

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 3.1: Target gene, primer sequence, amplicon length and accession number for q RT-PCR used in the study

Target Gene	Primer sequence 5'-3'	Amplicon length (Bp)	Accession No. / Reference
Prepro-Orexin	For: ATC TCT CCC CCT TTC CCG TC Rev:AGGAGACCTTTGTAGAGGAAGGAT	179	XM_006055754.1
Orexin 1R	For: TCC CAG AGC CAA CAG AAG GT Rev: CAC TAA GGC TGA CGG GCA T	144	XM_006054498.1
Orexin 2R	For: CCGTGGCCGCTGAAATAAAG Rev: AAACACCAAGAGCACCACCA	77	XM_019985477.1
β- actin	For: TCT CAC GGA GCG TGG CTA CAG Rev:CTGCTCGAAGTCCAGGGCCACGTA	100	NM_001290932.1
RPL 15	For: TGGGCTACA AGGCCA AACAA Rev: GCTTCGAGCAAACTTGAGCTGG	140	MG969348
3β-HSD	For: GATCATCTGCCTGTTGGTGGA Rev: GTGGATGACCACTGAGGTGC	191	Kumar et al.,2012
CYP11 A1	For:AGTTCGAGGGATCCTACCCAGA Rev: AGCCATCACCTCCGTGTTCAG	146	Gupta *et al.,* 2014
CYP19 A1	For: CGTCCTGGTCACCCTTCT Rev: ACGCACCGACCTTGCAA	57	U18447.1

Target genes: Prepro-orexin A, Orexin 1 R, Orexin 2R, beta actin and RPL 15 are used for mRNA expression; 3β-HSD, CYP11A1 and CYP19A1 are used for *in vitro* mRNA expression.

3.4 Glasswares and Plasticwares

For RNA work, RNase-free plasticwares and glassware ware 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 RAase 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:

Deep freezer -20 ^{o}C (Hoshizaki), Agarose gel electrophoresis apparatus (Cat no-#MGU-202T, C.B.S. scientific), Real-Time q PCR System instrument (Insta Q 96, Himedia), 96 well Thermal cycler (Thermofisher Scientific), Nanodrop (Eppendorf, USA), Cell counter (Hemocytometer), Gel documentation analysis system (Bio Rad), Homogenizer (Cole Parmer Lab GEN 7, USA), Incubator (humid CO_2 incubator recommended), Inverted microscope, Laminar flow, Power Pac V 200 Bio-Rad, Refrigerated centrifuge (BioEra), Refrigerator, Double distillation apparatus, Spinner (BioEra), Sterilizer/autoclave and Microwave Oven.

3.6 Collection and classification of corpus luteum (CL)

Buffalo ovaries were collected from native slaughter house after slaughter and were transported on ice to the Laboratory. Forty ovaries, each with corpus luteum were used to extract 10 CL per group for RNA extraction and immunohistochemistry/immunolocalization studies. The CL was classified in to following stages: Early luteal phase, mid luteal phase, late luteal phase and regressing CL as described by Sarkar *et al*. (2010). The standard criteria used for classification of CL is presented in Table 3.2. Luteal tissue was stored at -20°C until the RNA and protein isolation.

Table 3.2: Classification of Corpus Luteum

STAGE	External appearance on ovary	Appearance on CL	Photo
Early Luteal phase (day 1-4)	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 (days 5-10)	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 herd >1cm in diameter	
Regressed CL (day >17)	Regressing CL	Surrounded by yellow lutein (white arrow head)	

3.7 Collection of follicles and preparation

Whole reproductive tract from buffaloes were collected from local slaughterhouse and were transported on ice to laboratory within 10-20 min after slaughter. The stage of the estrous cycle was defined by macroscopic observation of the ovaries (colour, consistency, corpus luteum stage, number and size of follicles) and the uterus (colour, consistence and mucus) as described previously (Sarkar *et al*., 2010). From the morbid ovary, only follicles which appeared healthy (well vascularized and having transparent follicular wall and fluid) and with diameter more than 3mm were used. Large follicles (>14 mm) were collected only from regressed CL, with signs of mucus production in the uterus and cervix, and were considered to be pre-ovulatory.

For RNA extraction, follicles were dissected out from ovarian stroma. The adjacent tissue (theca externa) was carefully removed with forceps under a stereo zoom microscope as previously described (Sarkar *et al*., 2010, Gupta *et al.,* 2014) and their surface diameter was determined. After aspiration of follicular fluid (FF), each follicle was bisected and its inside wall was gently scraped and flushed with Ringers solution to separate out the granulosa cells (GC) and the remaining follicles were flushed after GC separation from the theca interna cells (TIC). GC and TIC isolated from each follicle were transferred into separate tubes and labeled. The GC in the flushing solution was centrifuged at 3000 x g for 10 min at 4 ^{o}C. The TIC and GC pellet were separately snap frozen in liquid nitrogen and stored at -80 ^{o}C until RNA and protein isolation. The FF was stored at -20°C until determination of progesterone (P4) and estrogen (E2). Since healthy follicles have relatively constant P4 levels in FF, only follicles with P4 below 100 ng/ml FF were used for the evaluation, to exclude atretic follicles (Sarkar *et al*., 2010, Gupta *et al.,* 2014).

3.7.1 Follicle classification

Follicle		Size in mm	E2 conc. ng/ml in FF
F1	Small	4-6 mm	<0.5
F2	Medium	7-9 mm	0.5-5
F3	Large	10-13 mm	5-40
F4	Pre-ovulatory	>14 mm	>180

Forty (40) ovaries, each with follicles, were used to extract 10 follicles each per group of RNA extraction and immunohistochemistry studies.

3.8 Hormone determination

Concentrations of P4 and E2 in the FF and spent culture media of luteal cells and GC culture were estimated using P4 ELISA kit (Cat. No. 402310, Neogen Life Sciences, USA) and E2 ELISA kit (Cat. No. 402110, Neogen Life Sciences, USA) supplied by Immunotech, Czech Republic as per manufactures instruction. The measurable range was 0.05-50 ng/ml for P4 and 6-5000 pg/ml for E2. The FF was diluted accordingly with PBS. The intra- and interassay coefficients of variation were 6.5% and 7.2% for P4 and 12.1 % and 11.2 % for E2, respectively.

3.9 Primers

For primer design the Fast PCR (Version: 6.2.73) software was used. The details of the primers used are shown in Table 3.1.

3.10 Quantitative RT-PCR

Total RNA was isolated from different stage of CL (10 CL per group) and follicles (GC and TIC collected from 10 follicles per group) by Trizol reagent (Invitogen, USA) 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 ^{TM}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 qPCR reactions.

3.10.1 Total RNA extraction from CL, GC and TIC Procedure

Total RNA extraction from CL, GC and TIC was done using Trizol reagent (Invitrogen) described as bellow:

1. The CL was isolated aseptically by dissecting the ovary, GC and TIC were isolated from follicle and fast freeze in liquid N_2 and stored at -20°C for further analysis.
2. Approximately 100mg of the luteal tissue was chopped / crushed.
3. The fine crushed luteal tissue, GC and TIC pellet was taken in 0.1 % DEPC treated eppendorf tube containing 1 ml Trizol reagent, homogenized with the help of homogenizer (Cole Parmer Lab GEN 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 500µl 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 minutes to precipitate the RNA as pellet.
8. The supernatant was removed completely and the RNA pellet 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 dissolved in 30µl of nuclease free water (NFW) (Lot. No. 0000213084, Promega, Madison, USA)

3.10.2 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 for the protein contamination as the OD 260: OD 280 values were more than 1.8.

3.10.3 Synthesis of cDNA

The first strand cDNA was synthesized for the isolated RNA. RT-PCR was done using 5X Reaction Buffer, MgCl2 PCR Nucleotide Mix, RNase Inhibitor, Reverse Transcriptase, Oligo-(dt 15) Primer, RNA template, NFW. Reverse transcription was carried out in 20µl reaction mixture depicted in Table3.

Table 3.3 Reaction mixture for cDNA synthesis

Components of reaction mixture	Quantity	Final Conc.
5X reaction Buffer	4 µl ()	-
$Mgcl_2$	3 µl	25 mM
PCR Nucleotide Mix	1 µl	10 mM
RNase Inhibitor	0.5 µl	-
Reverse Transcriptase	1 µl	-
Oligo-(dt 15) Primer	1.5 µl	0.75 µg
RNA template	1 µl	1 µg (Total Conc, RNA Temp + NFW)
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.

3.10.4 Gradient PCR/ End point PCR optimization

PCR was standardized to amplify buffalo gene sequence using RT-PCR Quantam Biotech s-96. Factor specific primers were used for the amplification of genes. The annealing temperature was standardized using cDNA prepared from mRNA of buffalo CL, GC and TIC by PCR. The reaction was carried out at different annealing temperatures. 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 are presented in Table 4.

Table 3.4: Reaction mixture for optimization of primers

Components of Reaction Mixture	Quantity	Final Conc.
Gotaq® Green Master Mix	10 µl	-
Forward primer	0.5 µl	0.5µM
Reverse primer	0.5 µl	0.5µM
DNA template	1.0 µl	50ng/ 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 (Bio Era). 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 3.5: 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 orexin and RPL15

3.10.5 Agarose gel electrophoresis

The verification of amplification of specific RT-PCR amplicon was done by agarose gel (Appendix) electrophoresis. 1.5% agarose (Cat. No. MB229, Himedia) was mixed with 1X TAE buffer and melted in a heating mantle. When the molten gel cooled to about 42°C, ethidium bromide was added to make final concentration 0.5 µl/ml. 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. No. 0000255933, Promega, USA) and loaded into the wells. For the comparison, a 50 bp or 100 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 (Bio Rad).

3.10.6 Determination of efficiency of primers

Efficiencies were determined by running a standard curve for each assay prior to processing experimental samples. A standard curve was obtained by serial dilution of the cDNA containing the template and regression line equation in relation to the threshold values ("Ct") was formulated. To obtain best accuracy level 6 serial dilutions of cDNA were taken in triplicate. Slopes were calculated using qPCR software Instaq96. (INSTA Q 96, Himedia)

3.10.7 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 minimized. 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, optically clear caps were used.1µl of cDNA was taken and the master mix used for reaction of qRT-PCR is presented in Table 6.

Table 3.6: Reaction for mixture qRT-PCR

Components of Reaction Mixture	**Quantity**	**Final Con.**
Gotaq® Green Master Mix	7.5 µl	-
Forward primer	0.5 µl	0.5µM
Reverse primer	0.5 µl	0.5µM
DNA template	1.0 µl	50ng/ 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 3.7: 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	

3.10. 8 Gene Expression Analysis

Beta actin and RPL15 were used as housekeeping gene. Efficiency corrected relative quantification of mRNA was obtained as described earlier by $2^{-\Delta\Delta ct}$. For this, efficiency of primers was determined by serial dilution of template cDNA sample and running in triplicate.

Constant amount of 1 µg of total RNA from CL, GC and TIC (N=10/group) were reverse transcribed using iScript ™ select cDNA Synthesis Kit (Bio-Rad) Laboratories, Hercules, CA, USA) as previously described. The qRT-PCR for each cDNA and the housekeeping gene β-actin and RPL15 was performed in duplicate using an Insta q96 Real-Time qPCR system instrument as per manufactures' instructions. PCR templates containing 0.5 µl reverse transcribed total RNA were added to 0.20µl forward primer (0.2 mM), 0.20 µl reverse primer (0.2 mM) and 5 µl of SyBR green super mix and NFW (4.1 µl) to make final volume of 10 µl and were subjected to the general real-time PCR protocol for all investigated factors. The following general real-time PCR protocol was employed for all investigated factors: denaturation for 30 sec at 95°C 40 cycle of a three segmented amplification and quantification program (denaturation for 10 sec at 95°C, annealing for 10 sec at the primer specific temperature (56°C for orexin R1and R2, 58°C for beta actin and 62°C

for orexin), elongation for 15 sec at 72°C), a melting step by slow heating from 61 to 95°C with a range of 0.58°C/sec and continuous fluorescence measurement, and a final cooling down to 4°C. After the run ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by showing the "cyber green (with dissociation curve)" method of the real time machine (Insta q 96, Himedia).

Real time PCR efficiencies were determined by amplification of a standardized dilution series, and slopes were obtained. The specificity of desired products was documented using analysis of melting temperature, which is product specific and high-resolution gel electrophoresis to verify that transcripts were of exact molecular size and further confirmed by sequence analysis. Negative control PCR containing all components except template were included for each sample to check out the formation of primer dimer. For qPCR analysis of each gene including the house keeping gene, ten replicates of GC and TIC extracted from each group of follicle were used.

3.10.9 Calculation of Relative Expression

Optical data were collected at end of each extension step, and relative expression of PCR product was determined by the $2^{-\Delta\Delta ct}$ 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.

3.11 Immunohistochemistry

CLs of different stages were separated from ovary and were fixed with 10% neutral buffer formalin (NBF) then dehydrated through a series of graded alcohols, paraffin-embedded, cut in to serial sections (5µm), mounted on Mayer's albumin coated slides and dried at 37°C overnight. Five representative CLs from 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, then 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, nonspecific background was eliminated by blocking with 5 % BSA (dissolved in 1xPBS) for 2 h at 37°C. Subsequently, sections were probed with mouse IgG of against Orexin A (KK09) and Orexin R 1/2 in 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. Again, 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. The slides were counter with Meyer’s hematoxylins for one minute. Sections were washed with distilled water and observed under microscope (Axio Lab A1from Zeiss). Images were captured using installed camera of Axio Cam ERC 5C.

The assessment of immunoreactivity was carried out in a semiquantitative manner. For this, at least 12–15 microscopic high-power fields (40X objective) were viewed in the 5 tissue sections processed for immunohistochemistry from each stage. In these fields, approximately 1,000 cells were counted. Then, the immunopositive cells were expressed as a percentage. The scoring pattern was as follows: 0: no immunoreactivity, 1: 60% immunopositive cells. Accordingly, every image was given a score as per its intensity. For grading, a 4-tire scoring system, that is, strong positive (3), moderately positive (2), low positive (1), and negative (0) was used.

3.12 Cell culture, Gene expression and hormone estimation

3.12.1 Preparation of stock for orexin

All the stock and working solutions required for Luteal and Granulosa cell culture were prepared using autoclaved distilled water. Orexin stock solution was prepared by reconstituting 100 µg of lyophilized desiccate of Orexin (Lot. No. L1318) in 1% BSA-PBS to yield working solutions of 0.1, 1.0 and 10 ng/ml made in aliquots and stored at -20°C until used. The working solutions were added @ 1µl/ml of DMEM/F12 media (containing serum FBS at 1%) so as to yield final treatment concentrations respectively of 0.1, 1 and 10 ng Orexin /ml media. The above three treatment concentrations were made fresh from the working solutions immediately before the treatment of luteal and GC cells in wells.

3.12.2 Luteal Cell Culture and Identification

A) Luteal Cell Culture

In order to evaluate effect of the orexin on luteal cell function, a luteal cell culture model was developed with cells isolated from fresh CL. Luteal cells were cultured as described in earlier studies (Robinson *et al*., 2008 and Gupta *et al*., 2014). Briefly, ovaries were collected from a native slaughterhouse and transported to the laboratory in 1XPBS at 37°C. The second stage CL (days 5-10) were used for luteal cell culture and these were selected based on criteria applied previously (Gupta *et al*., 2014). The CL was removed from the ovary with its all connective tissue and blood clot (if present) 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 an 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 to remove non dissociated tissue fragments. The filtrate was washed twice by centrifugation for 5 min at 250×g with DMEM/F12 media (Lot 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 re-suspended in DMEM/F12 medium (Lot. No. 0000313737, Himedia, India.) Containing 10% Fetal Bovine Serum (FBS) (Lot. No. 42G5176K, Gibco life technologies, South America) and antibiotic & antimycotic solution (10,000 units penicillin, 10mg streptomycin, 25µg amphotericin B per ml) (Lot. No. #067M4754V, Sigma, USA). Cell viability, was 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 (total volume: 1ml containing 10% Fetal Bovine Serum) in a humidified CO_2 (5%) incubator at 37.5°C. The cells were allowed to attach and grow till 75-80% confluence was obtained with replacing of the media at every 48h. and there after the media was replaced with fresh media containing different concentration (0.1,1 and 10ng/ml) of orexin and were maintained for 48 h. The doses of the orexin were selected based on the earlier report (Cataldi *et al*., 2012, Nitkiewicz *et al*., 2014). Control cells were grown in media without orexin. 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.

B) Identification of luteal cell:

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 brown colour under a phase contrast microscope.

3.12.3 Granulosa cell culture and Identification

A) Granulosa cell culture

Granulosa cells (GCs) were cultured as per protocol mentioned by Gupta *et al*., 2015. Briefly, ovaries were washed properly with physiological saline solution and GCs were collected from pre ovulatory follicles separately by aspiration of FF using a needle (18 gauge) and syringe (plastic, 10 ml) Aspirated contents were transferred to a 60-mm dish under sterile conditions containing 0.1% solution of PBS, and all cumulus oocyte complexes were recovered. The remaining cells and fluids were centrifuged in 15 ml conical tubes at 300 ×g for 5 min and the GC pellet was resuspended in 10 ml of 1X PBS prior to a second centrifugation. Finally, GCs were resuspended and washed in culture medium with DMEM/F12 media (supplemented with 10% FBS, antibiotic and antimycotic solution). The number of viable cells were counted using trypan blue exclusion. The cells were centrifuged, resuspended and 1.5×10^5 viable cells were transferred per well in a 24-well plate (total volume: 1ml containing 10% Fetal Bovine Serum (Gibco) and Antibiotic & Antimycotic solution (10,000 units Penicillin, 10mg Streptomycin, 25µg Amphotericin B per ml (Sigma Aldrich) in a humidified CO_2 (5%) incubator at 37.5°C. The cells were allowed to attach and grow (75-80% confluent) for 48h and thereafter the media was replaced with fresh media containing different concentrations (0.1, 1 and 10 ng/ml) of orexin and were maintained for 48h. The dose of the orexin was selected based on the earlier report (Cataldi *et al*., 2012, Nitkiewicz *et al*., 2014). Control cells were grown in media without orexin. The cells were allowed to attach for 24 h and after a further 24 h period the medium was changed. Then after 48 h the medium and cells were collected for mRNA isolation. Each treatment was tested in triplicate wells in each experiment.

3.12.4 Primers

For primer design the Fast PCR (Version: 6.2.73) software was used. The details of the primer used are shown in Table 3.1.

3.12.5 Quantitative RT-PCR and gene expression analysis

Total RNA was isolated from cultured luteal cells and Granulosa cell by using Trizol reagent (Invitrogen, USA) according to manufacturer instruction. qRT- PCR analysis, testing of RNA integrity and purity and gene expression studies were done as per the method described earlier (Gupta *et al*., 2019). The primer annealing temperature employed for various investigated factors were as follows: 60°C for 3β-HSD and RPL15, and 62°C for β-actin.

3.12.6 Progesterone (P4) and Estrogen (E2) assay:

Concentrations of P4 and E2 in the spent culture media of luteal and granulosa cell culture were estimated by P4 ELISA kit (Cat. No. 402310, Neogen Life Sciences, USA.), E2 ELISA kit (Cat. No. 402110, Neogen Life Sciences, USA) and FSH (Cat. No. F2293, Sigma Aldrich) at 30ng/mL, IGF-I (Cat. No. TC 300, Himedia Laboratories, India) at 10 ng/mL to confirm steroidogenic properties.

3.13 Statistical Analysis

The data was analyzed by SPSS 16.0 software. All experimental data has been shown as mean ± SE. The statistical significance of difference in mRNA expression of orexin A and its receptors (OX1R and OX2R) in CL, GC, StAR, CYP11A1, 3β-HSD, CYP19A1, estrogen and progesterone concentration in spent culture media was assessed using one-way ANOVA by the Tukey- Ducan as a multiple comparison test. Difference were considered at $P<0.05$

RESULT AND DISCUSSION

The present study was conducted to find the mRNA expression and localization of orexin and its receptors (Orexin Receptor 1 and Orexin receptor 2) in corpus luteum (CL) and ovarian follicles of buffalo ovaries and also to study the influence of orexin on estrogen and progesterone secretion.

4.1. RNA integrity and purity

Total RNA was isolated by Trizol method. The purity and concentration of total RNA was checked using nanodrop. The isolated RNA samples were free from protein contamination as the OD 260: OD 280 values were more than 1.8. The integrity of RNA was checked by running it on 1% agarose gel. The two intact bands (18s & 28s) shows that RNA was intact (Fig.4.1)

4.2 Expression and localization of orexin and its receptors in corpus luteum

4.2.1 mRNA expression of orexin and its receptors (OX1R and OX2R) in CL

The expression of prepro-orexin (PPO) and orexin receptors (OX1R and OX2R) were determined in corpus luteum of buffalo as shown in Plate 4.1. A cDNA fragment of 179 bp, 144 bp and 77 bp was amplified by RT-PCR corresponding to PPO, OX1R and OX2R, respectively. The relative mRNA expression was calculated by $2^{-\Delta\Delta ct}$ method. The tissue in which mRNA expression found lowest, was used as calibrator for obtaining comparative mRNA expression. Beta actin and RPL 15 were used as housekeeping gene. Amplification and dissociative curve are presented in Fig 4.1 (A) and (B), respectively. The analysis of PPO and its receptors mRNA by qRT-PCR showed that PPO and orexin receptors were expressed at each stage and the level of transcripts varied through the luteal phase. The relative expression of PPO, OX1R and OX2R is presented in Fig. 4.2. We found that the transcripts of PPO and OX2R was significantly ($P<0.05$) higher in early (CL1) and regressing (CL4) stage as compared to mid (CL2) and late (CL3) luteal stage. The expression between CL1 and

CL4 as well as between CL2 and CL3 was comparable. The expression of OX1R was lower in late luteal phase as compared to early and regressing CL. The mRNA abundance of OX1R was comparable among CL1, CL2 and CL3.

It was found that the mRNA expression of orexin and OX1R & OX2R in buffalo corpus luteum was varied according to the stage of corpus luteum; it was higher in early and regressing phases and lower in mid and late luteal phases. Previously orexin system has been expressed in ovarian cells of pig (Nitkiewicz *et al*., 2010, Kaminski *et al*., 2010b, Nitkiewicz *et al*., 2014; Basini *et al*., 2018), in rat (Silveyra *et al*., 2007a, Cataldi *et al*., 2012) and in dogs and cat (Levanti *et al*., 2015). The present finding are in agreement with the earlier observation in pig (Nitkiewicz *et al*., 2010, 2014 and Kaminski *et al*., 2010a, 2010b) whereas, the greatest mRNA expression of orexin and its receptors was observed in regressing phage of oestrus cycle. Similarly, the expression of PPO gene increased in early and regressing stage of estrous cycle observed by Smolinska *et al*., (2014). Silveyra *et al*., (2007a) demonstrated in rat, that both OX1 and OX2 mRNA expression was greatest during pro-estrous stage, further they also described the expression depending on the stage of estrous cycle. Likewise, the expression of PPO mRNA was detected in cyclic stage of rat ovaries (Johren *et al*., 2001). Several studies also reported the detection of PPO, OX1R and OX2R mRNA expression in uterus of pig during estrous cycle (Nitkiewicz *et al*., 2012; Smolinska *et al*., 2015).

4.2 Localization of orexin A and its receptors in corpus luteam (CL)

Initially, the morphology of the CL sections was evaluated by using haematoxylin and eosin staining; slides were observed under 20X microscope, cells were identified according to shape and localization site of orexin A, a secretory protein synthesized from PPO & its receptors. The luteal cells were rounded, large with central nucleus. Small and flat endothelial cells were localized adjacent to the inner layer of capillary. In the surrounding of outer layer of capillary egg-shaped pericytes were co-localized with endothelial cells. The immunoreactivity of orexin A,

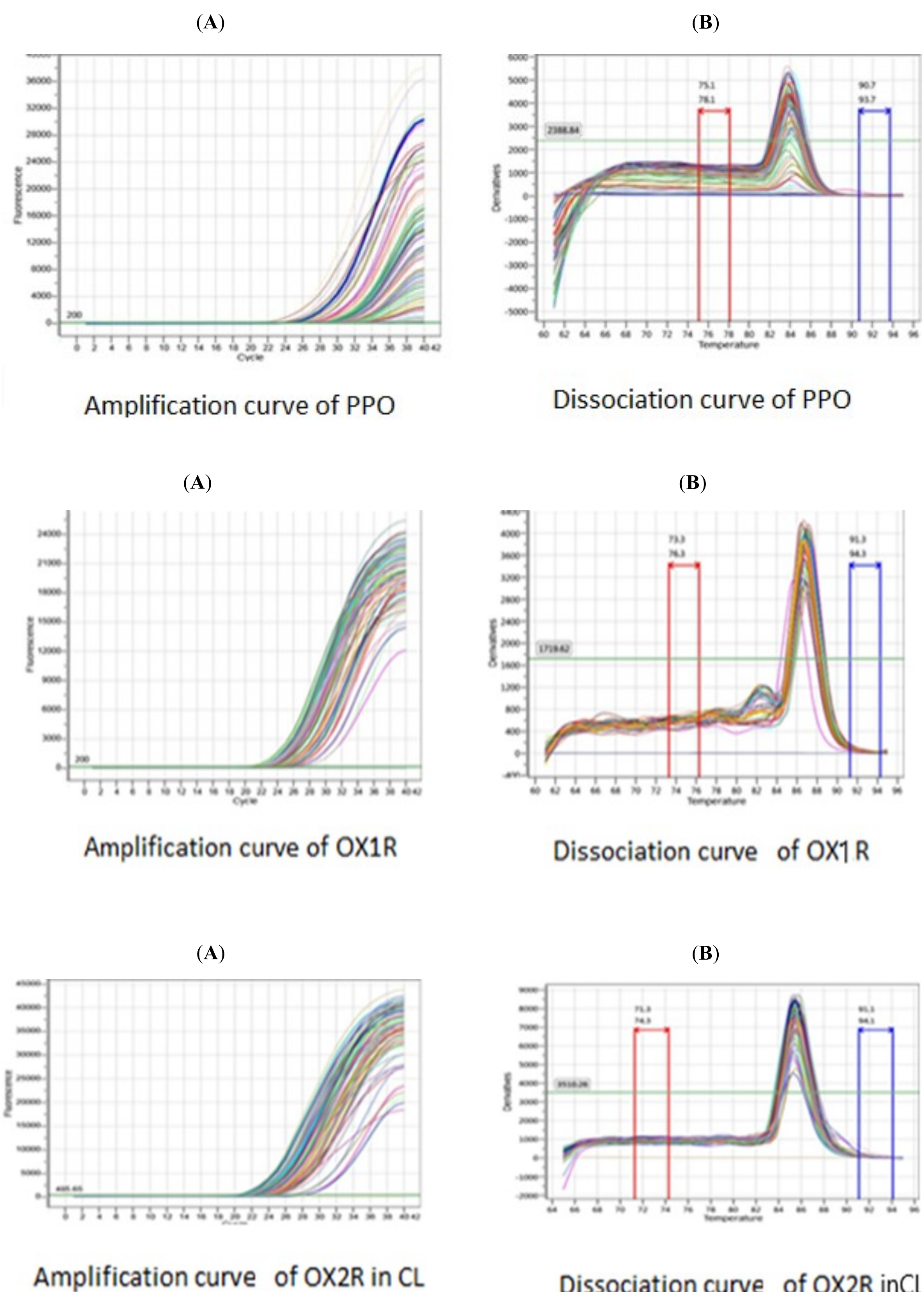


Fig. 4.1 Amplification (A) and Dissociation (B) curve in CL of PPO, OX1R and OX2R

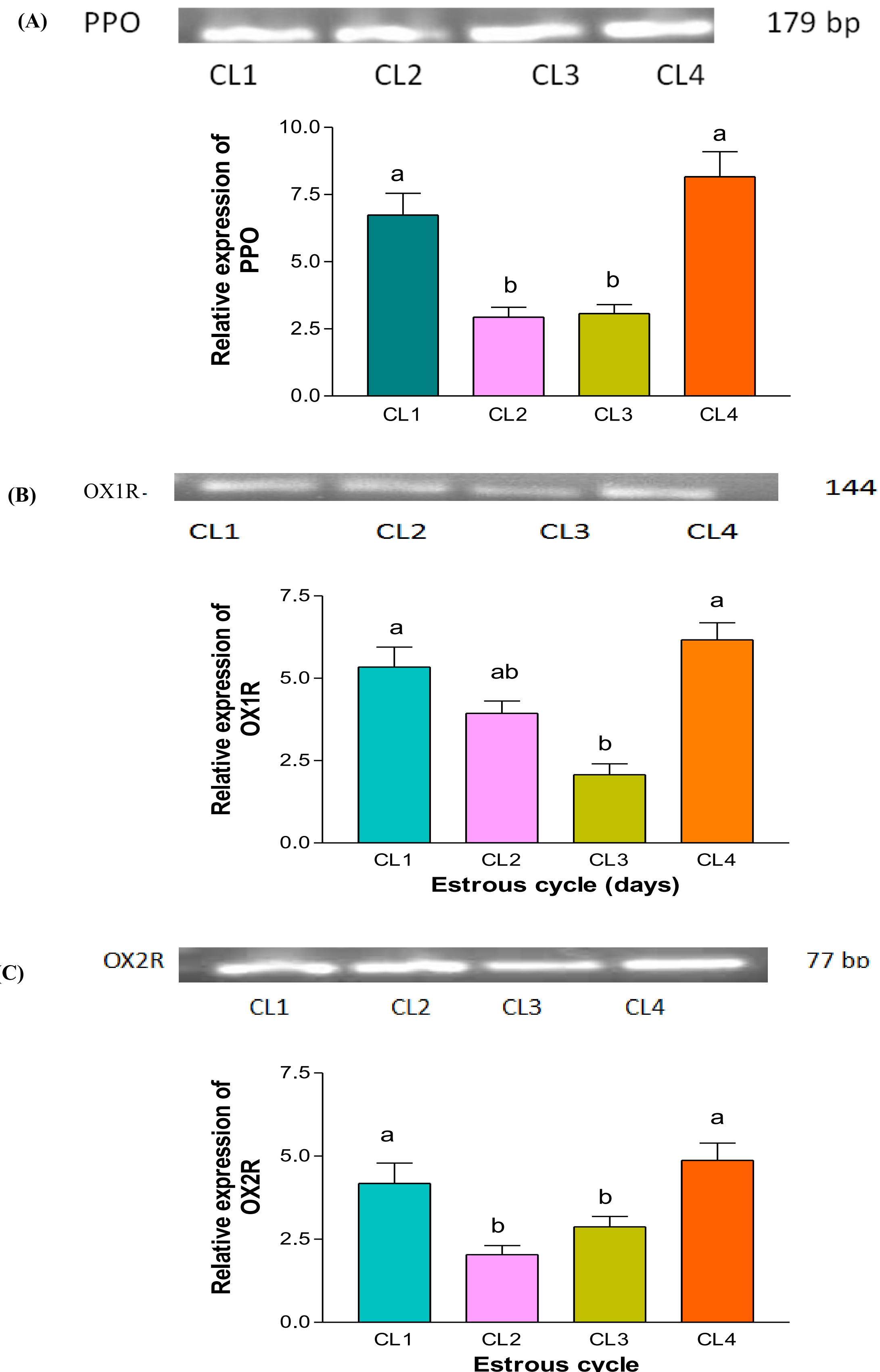


Fig. 4.2: Agarose gel showing 179 bp product for Pre pro-orexin (PPO) & Relative mRNA expression of in stages of CL (A). Agarose gel showing 144 bp product for orexin Receptor1 (OX1R) & Relative mRNA expression of OX1R in stages of CL (B). Agarose gel showing 77 bp product for orexin Receptor 2 (OX2R) & Relative mRNA expression OX2R in stages of CL (C). Different superscripts denote statistically different values ($P < 0.05$).

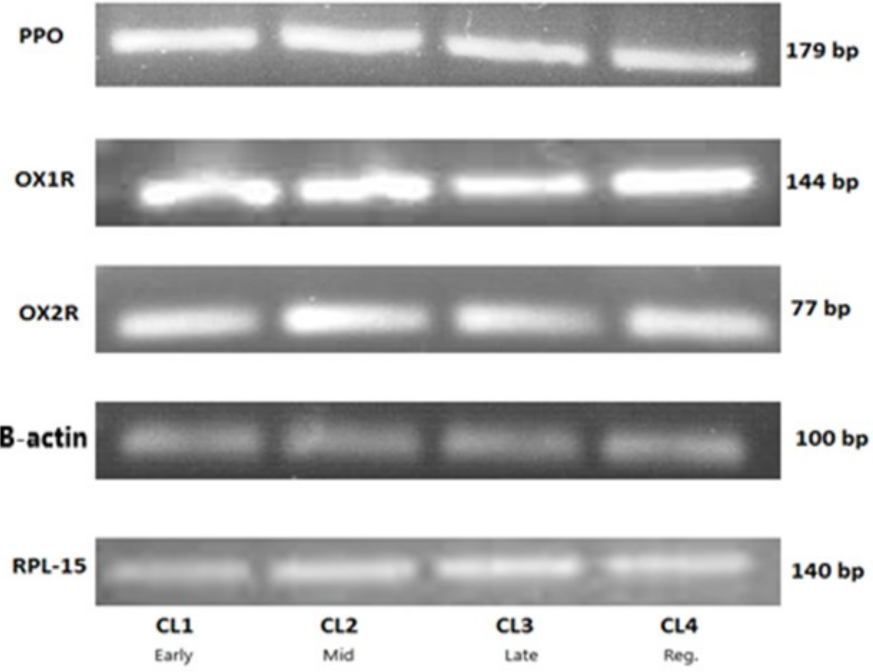


Plate 4.1 : 1.5% agarose gel showing Prepro-orexin (PPO):179 bp, OX1R: 144 bp, OX2R: 77 bp, b-actin:100 bp and RPL15:140 bp products at different stages of CL1(early luteal),CL2(mid luteal), CL3 (late luteal) and CL4 (regressed luteal).

OX1R and OX2R (Plate 4.2, 4.3 & 4.4) were observed using immunohistochemistry. The positive cells were influenced by the different stages of estrous cycle and immunostaining intensity varied from phase to phase. The immunoreactivity was moderate in early and regressing stage and was exclusively found in cytoplasm of luteal cells and also near periphery of nucleus. The negative controls containing isotype IgG presented only weak background staining. The intensity of immunohistochemistry was calculated using semiquantitative method and the immuno-positive cells were expressed in percentage. The scoring pattern was 0: no immunoreactivity, 1: <30%, 2: 30% - 60%, 3: > 60% immuno-positive cells. Accordingly, every image was given a score as per its intensity. For grading, a 4-tire scoring system was used as strong positive (3), moderately positive (2), low positive (1) and negative (0).

The result of immunohistochemistry is in agreement with observations of Basini *et al*., (2018) where they localized orexin A and orexin 1 receptor (OX1R) in swine corpus luteum (CL) by using immunohistochemistry and observed both orexin in cytoplasm and nucleus of luteal cells. Ragionieri *et al*. (2018) localized orexin A and its receptors expression in pig ovary by immunofluorescent methods, the immnunoreactivity of orexin A and OX2R appears in cytoplasmic vesicles and the immnunoreactivity for OX1R is often associated with the nuclear envelop and lesser extent in the cytoplasm of luteal cell. Moreover, Nitkiewicz *et al*., (2012) showed localization of orexin in pig uterus using fluorescent immunohistochemistry. Nitkiewicz *et al*., (2014) localized orexins (A & B) in pig porcine ovary by fluorescent immunohistochemistry and immunoreactivity was noted in corpora lutea during estrous cycle. The highest immunoreactivity of OXA was observed in CL on days 10-12 and 14-16 of cycle. The immnunoreactivity in OXB was highest in CL on days 14-16, 2-3 and 10-12 of cycle. Orexin was also observed in sertoli cells and spermatids in rat testis by Tafuri *et al*. (2010). Joshi and Singh, (2017) localized orexin A and orexin 1 receptor (OX1R) in mouse testis by immunohistochemistry.

The immunoreactivity observed in both the interstitial and the tubular compartment of testis throughout the period of postnatal development.

4.3 Effect of orexin on progesterone secretion in cultured luteal cells.

4.3.1 Effect of orexin on progesterone secretion

Luteal cells were harvested from early stage CL1 and were processed with RBC lysis buffer, collagenase, DNase, antibiotic, antimycotic solution and washed 3 times with culture media DMEM/F12. The cells were counted with eosin and 1.5 to $2x10^5$ cells were seeded in 12 well plate in triplicate for 48 h in DMEM/F12 + 10% FBS media. After attaining 75-80% confluency cells were acclimated with culture media with 1% FBS, the cells were treated without (control) or with increasing doses of orexin (0.1, 1.0 and 10 ng/ml). Standard curve of progesterone ELISA and P_4 concentration at different doses is presented in Fig. 4.3 and Fig. 4.4. Concentration of progesterone (P_4) in spent media of control culture was 9.34 ng/mL. Orexin at a dose 0.1 ng/ml the progesterone (P_4) concentration was comparable with control and at dose 1.0 and 10 ng/ml there was significantly ($P<0.05$) decline in P_4 concentration. Our result is in agreement with the findings obtained by Basini *et al*., (2018) and Nitkiewicz *et al*., (2014) in the swine, that at different doses of orexin (0, 0.1, 1 and 10 ng/mL) exerted inhibitory effects on P4 secretion by cultured luteal cells. In the present study inhibitory effect of orexin on progesterone secretion was observed.

4.3.2 Effect of orexin on mRNA expression of StAR, CYP11A1 and 3β-HSD in cultured luteal cells.

Luteal cells were treated with three different doses (0.1, 1.0 and 10ng/ml) of orexin after attaining 75-80% confluency, the spent media was separated and cells were treated with trizol to extract total RNA and reverse transcriptied to cDNA and analyzed in RT-PCR. Expression of StAR, CYP11A1 and 3β-HSD (Fig. 4.5 A, B and C respectively) in cultured cells varied significantly according to the given treatment. mRNA expression of StAR gene was comparable between control and 0.1 ng/mL dose of orexin, however significantly ($P<0.05$) decreased with increased doses of 1.0

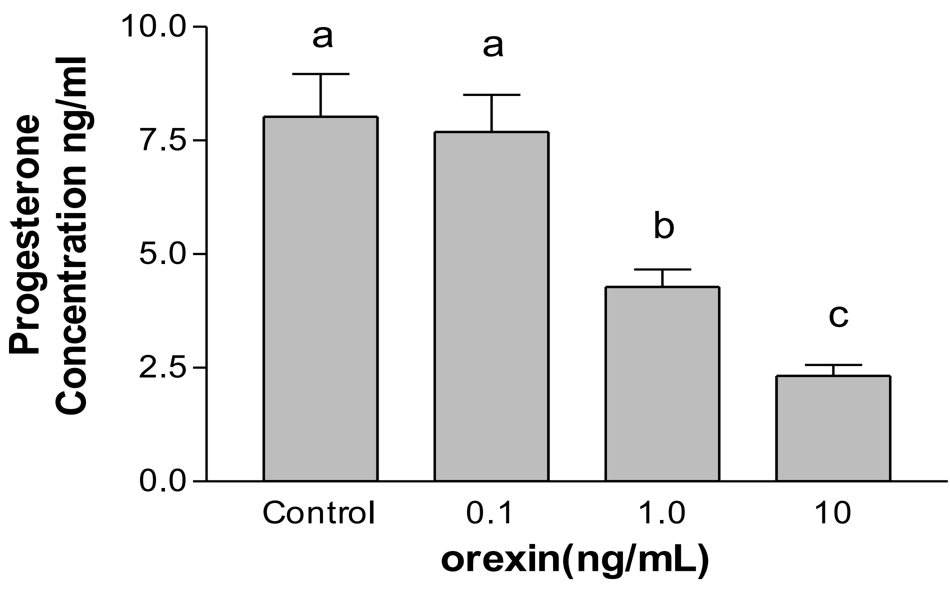


Fig.4.3 : Concentration of progesterone (P4) in culture media on orexin treatment at three different dose rate in culture luteal cell (n=4CL

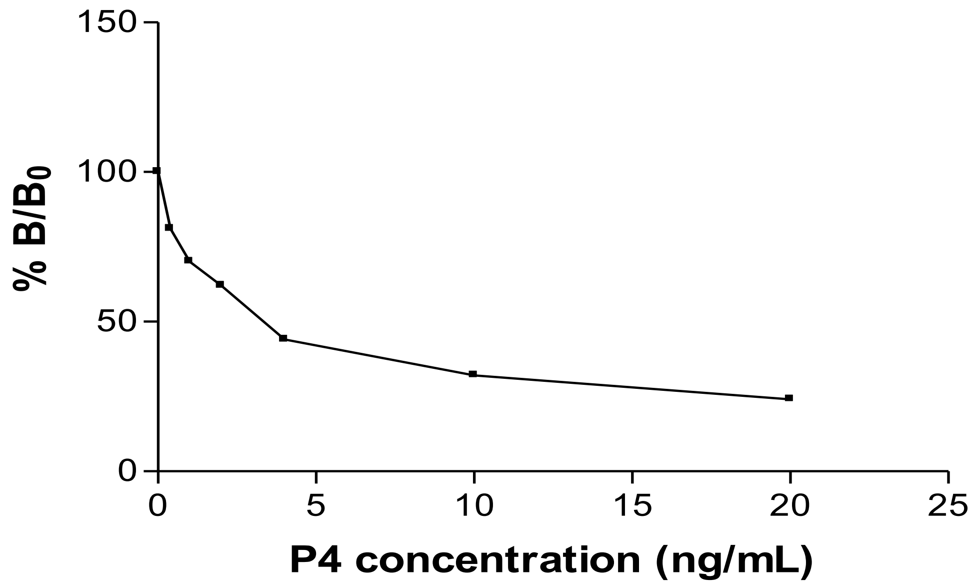
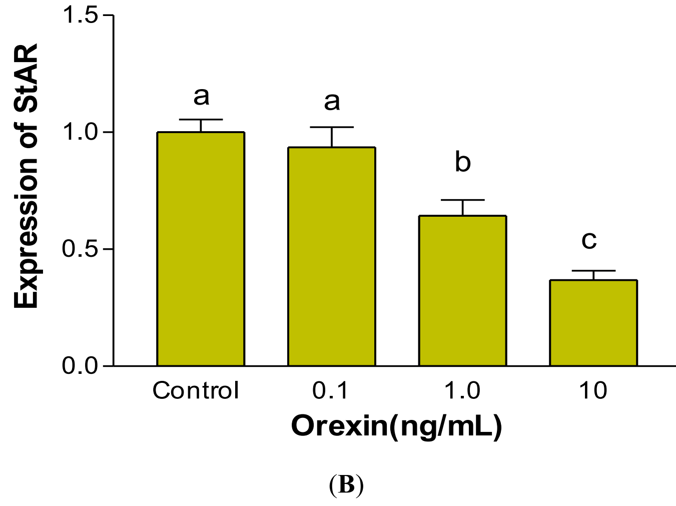
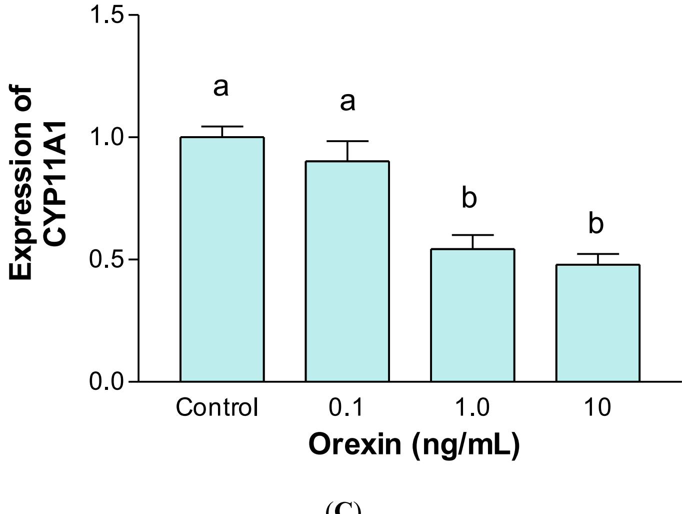


Fig. 4.4 : Standard curve for progesterone (P4)

(A)



(B)



(C)

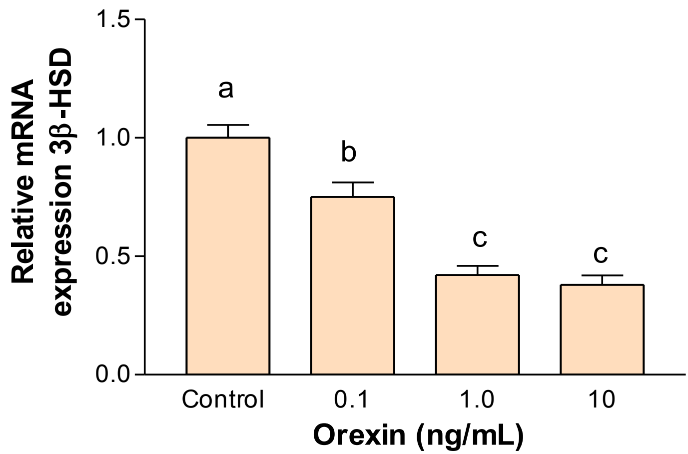


Fig. 4.5 : Expression of steroidogenic gene StAR (A), CYP11A1 (B) and 3B-HSD (C) mRNA in luteal cell culture on orexin treatment for at 3 different dose rate (n=4 CL) in buffalo. All values are shown as mean ± SEM. Different super-scripts denote statistically different values ($P<0.05$).

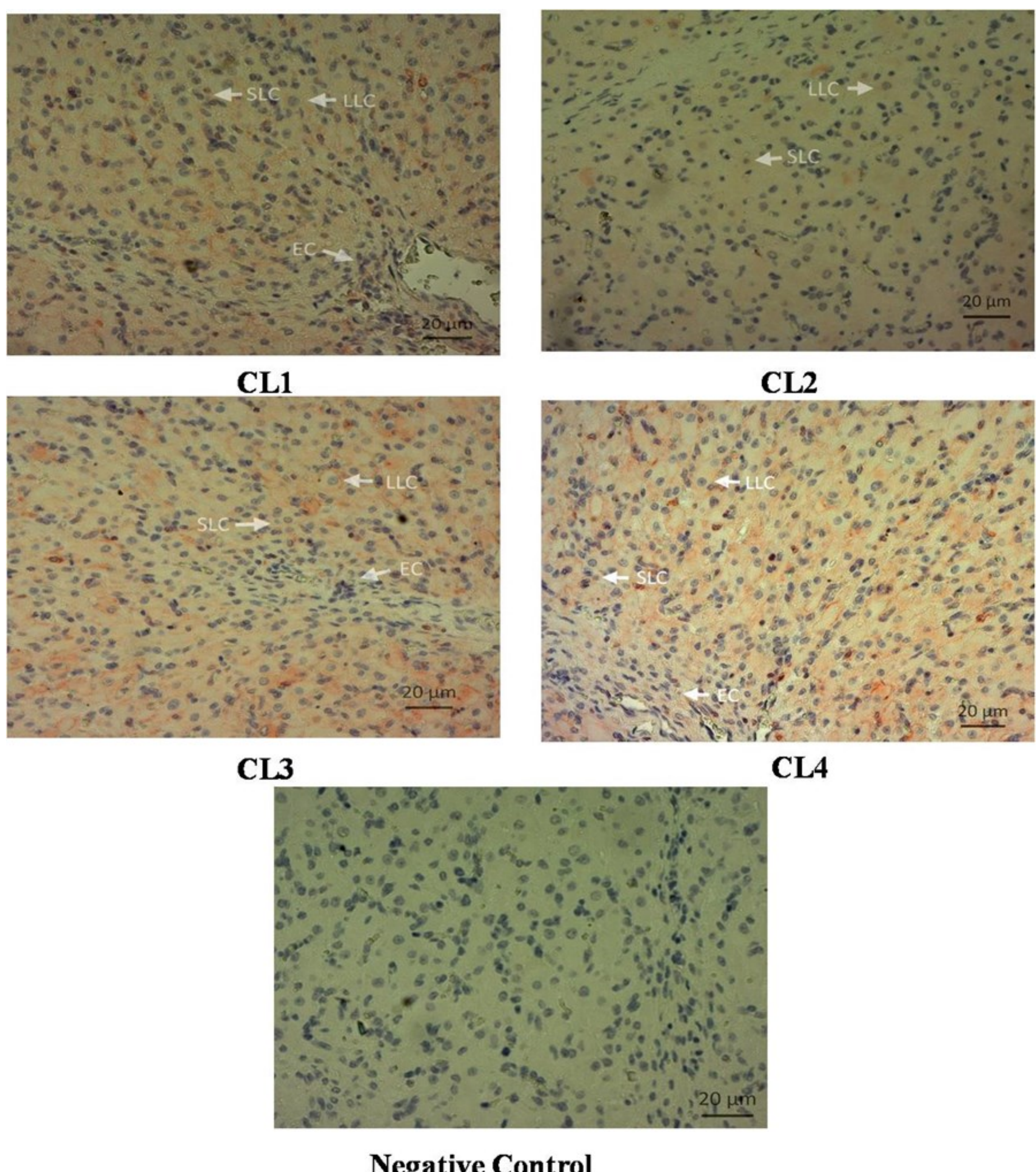


Plate 4.2: Distribution of orexinA in immunoreactive cells in different stages of CL. observed under 20X. The distribution of orexin positive cells in CL1, CL2, CL3, CL4 & Negative Control (arrow) LLC (Large Luteal Cell), SLC (Small Luteal Cell) and EC (Endothelial Cell).

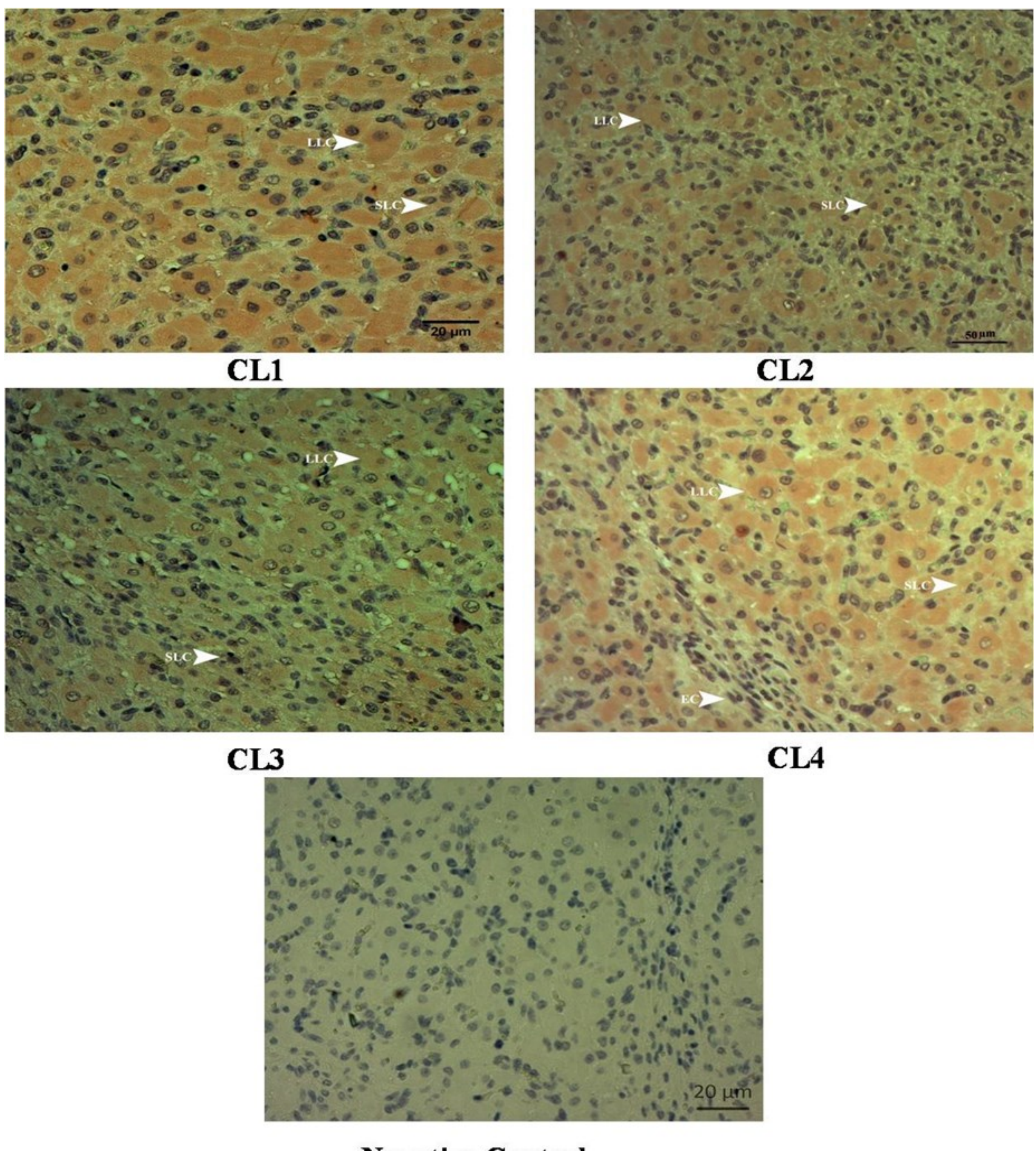


Plate 4.3: Distribution of orexin 1 Receptor (OX1R) in immunoreactive cells in different stages of CL. observed under 20X. The distribution of orexin positive cells in CL1, CL2, CL3, CL4 & Negative Control (arrow) LLC (Large Luteal Cell), SLC (Small Luteal Cell) and EC (Endothelial Cell)

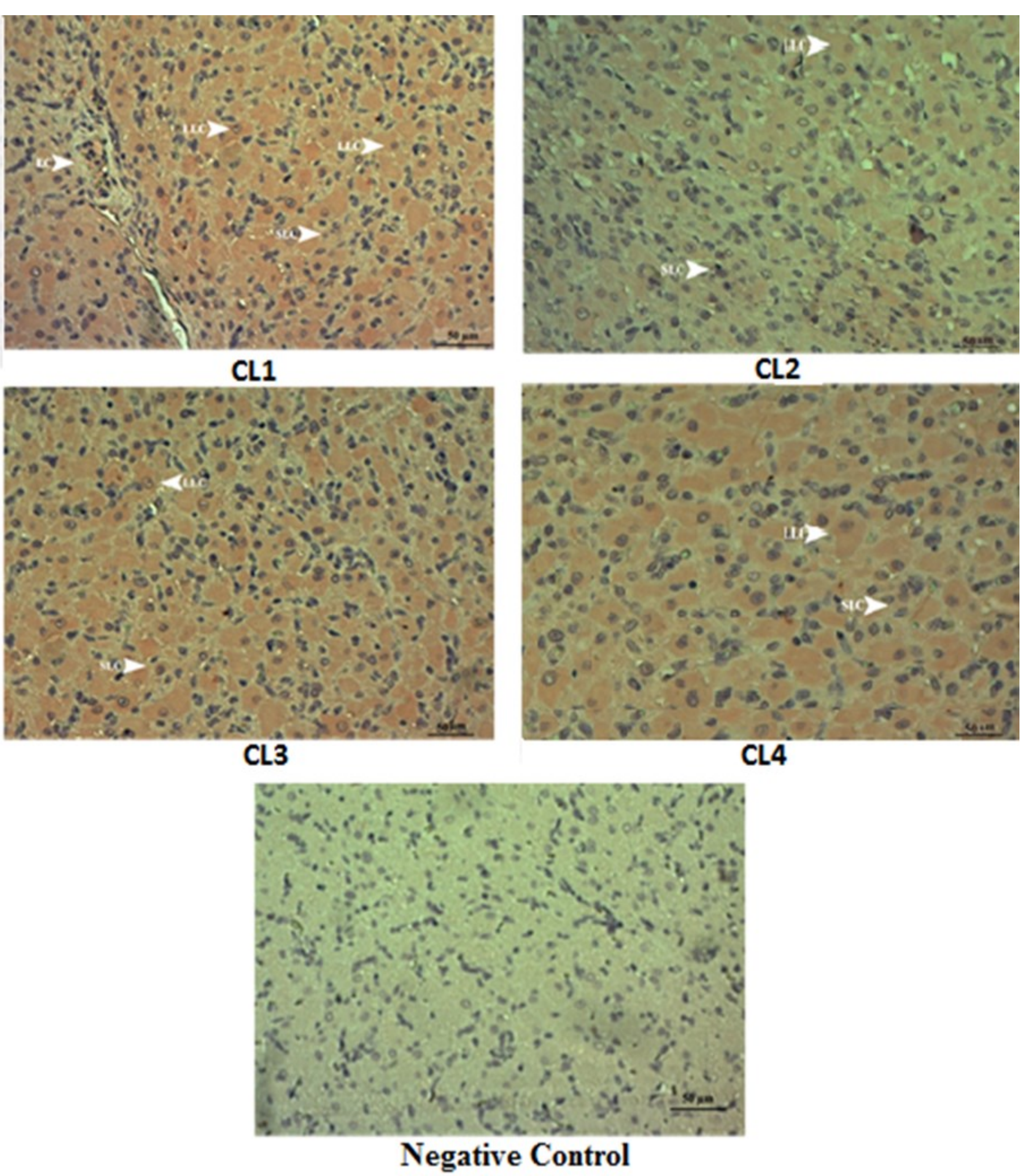


Plate 4.4: Distribution of orexin 2 Receptor (OX2R) in immunoreactive cells in different stages of CL. observed under 20X. The distribution of orexin positive cells in CL1, CL2, CL3, CL4 & Negative Control (arrow) LLC (Large Luteal Cell), SLC (Small Luteal Cell) and EC (Endothelial Cell)

and 10 ng/ml. The expression pattern CYP11A1 was similar to the expression of StAR for 0.1 and 1.0 ng/mL doses significantly ($P<0.05$) decreased, while no significant difference between 1.0 and 10ng/mL dose. The mRNA expression of 3β-HSD was significantly ($P<0.05$) declined in orexin treated cells at the dose 0.1, 1.0 and 10 ng/mL, where comparable at dose 1.0 and 10 ng/mL. Amplification curve (a) and dissociation curve (b) for StAR, CYP11A and 3β-HSD are presented in Fig 4.6 A, B and C respectively and the gel product for StAR, CYP11A and 3β-HSD are shown in plate 4.5 A, B and C, respectively.

The result of the present study are in agreement with the findings observed by Li *et al*., (2019) in ovine, that the expression of StAR, 3β-HSD and CYP11 decreased with increasing concentration of orexin. However, the present findings do not agree with observation of Kisielewska *et al*., (2019) in porcine species, in which dose of orexin increases the concentration of 3β-HSD. The decline in P4 secretion / production from luteal cells in response to orexin treatment is probably due to decrease in expression of genes associated with P4 synthesis (i.e StAR, CYP11A1 and 3βHSD).

4.4 Expression and localization of orexin and its receptors in ovarian follicles

4.4.1 mRNA expression of orexin and its receptors (OX1R and OX2R) in ovarian follicles

From the ovaries, the follicles of different physiological stages (small, medium, large and preovulatory) were separated and also from the follicles the granulosa cells (GC) and theca interna cells (TIC) were separated. The tissue whose expression was lowest was used as calibrator.

The RNA was reverse transcribed and cDNA was ambled in RT-PCR. The relative expression of PPO, OX1R and OX2R was calculated by $2^{-\Delta\Delta ct}$ method. Geometric mean of CT values of β-actin and RPL 15 was used as reference for calculation of relative expression. However, contrast result from same species indicate that the effect of orexin may be species specific / varies in different species. The amplification of PPO, OX1R, OX2R, β-actin and RPL15 by qPCR in GC and TIC is shown in Plate 4.6 A and B and the relative mRNA expression of

PPO, OX1R, and OX2R is shown in Fig. 4.7 A and B, respectively. The results showed that orexin system (PPO, OX1R and OX2R) is expressed in both GC and TIC of buffalo ovarian follicles at each stage. However, the level of gene expression of PPO and its orexin receptors (OX1R and OX2R) varied through the follicular phase in both GC and TIC. In GC, the mRNA expression of PPO and OX1R was significantly ($P<0.05$) higher in large and preovulatory follicles (F3 & F4) as compared to small and medium (F1 &F2) follicles. There was no significant variation in OX2R in GC among all stages of follicles. In TIC mRNA expression of PPO was significantly ($P<0.05$) high in F4 and was comparable to F1, F2 and F3 follicles. The expression of OX1R was significantly ($P<0.05$) higher in F4 followed by F3 and F2 and were comparable in F1 and F2. In TIC, the abundance of OX2R transcripts was significantly ($P<0.05$) highest in F4 follicles and comparable between F1, F2 and F3 with no significant difference.

The present study reported that the orexin mRNA expression level significantly ($P<0.05$) varied depending on follicle size and higher in large follicles. Our observations are in concurrence with findings of Chojnowska *et al*., (2012) and Ciccimarra *et al*., (2018) in which expression of orexin increased with follicle development and was highest in large follicles. The expression of orexin receptors in follicular cells has been reported in swine ovarian follicle (Nitkiewicz *et al*.,2010, 2014) which also support our observations. The highest expression for OX2R mRNA was noted in granulosa and theca cells on days 17-19 oestrus cycle in the study of Nitkiewicz *et al*., 2010. The expression of orexin receptors was found at higher levels of porcine hypothalamus (Kaminski *et al*., 2010a) and in pituitary (Kaminski *et al*., 2010b) during pre-ovulatory follicular phase (on days 17-19) in pig. Similarly, Silveyra *et al*., (2007a) in rat, demonstrated that both OX1R and OX2R expression are increased only during pro-estrous phase of estrous cycle. Expression of orexin receptors during the oestrous cycle was also noted in the highest branch of the HPG axis. Higher levels of OX1R and OX2R genes are detected in the mediobasal

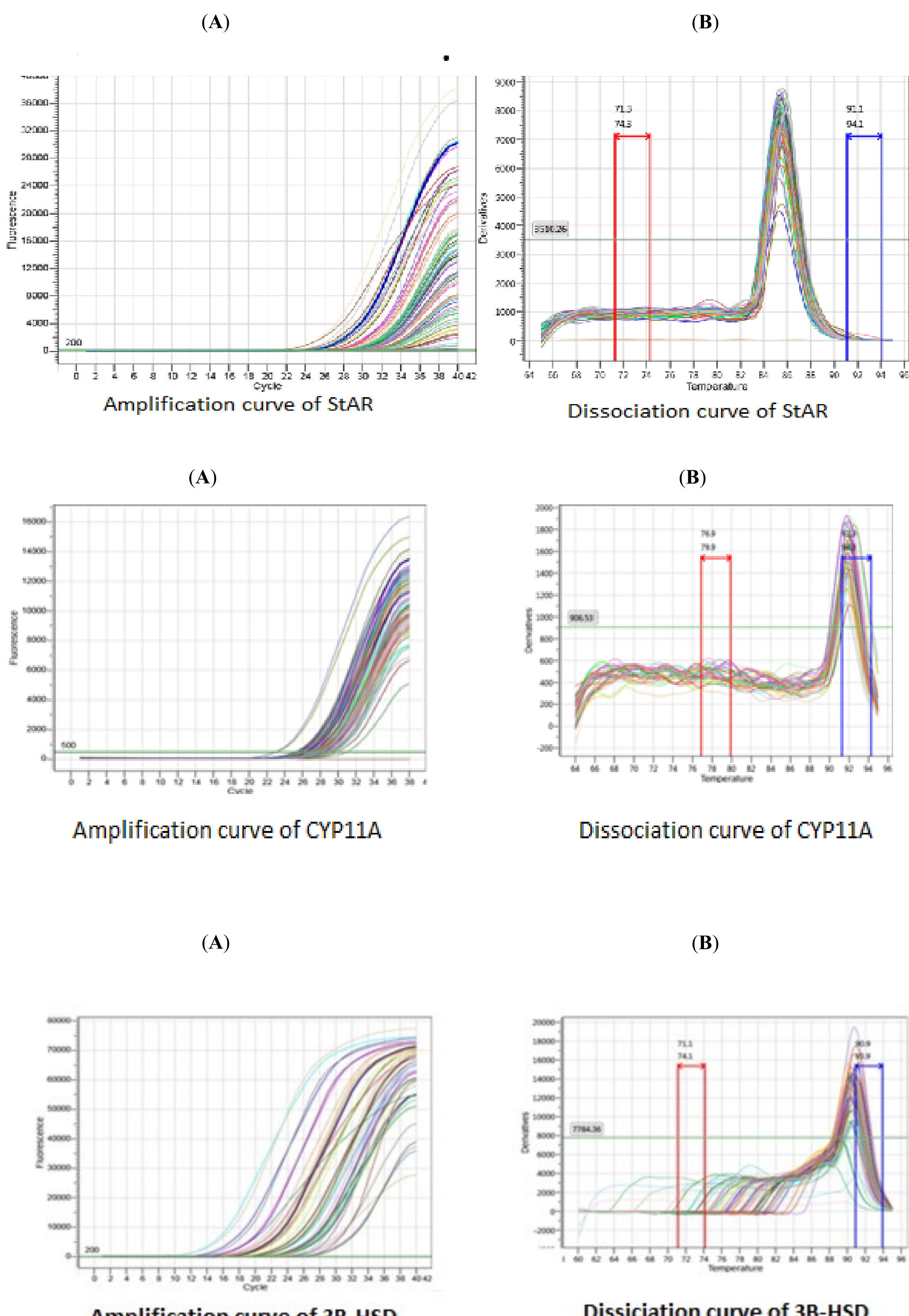


Fig. 4.6: Amplification (a)and Dissociation (b)curve of StAR, CYP11A, and 3β-HSD.

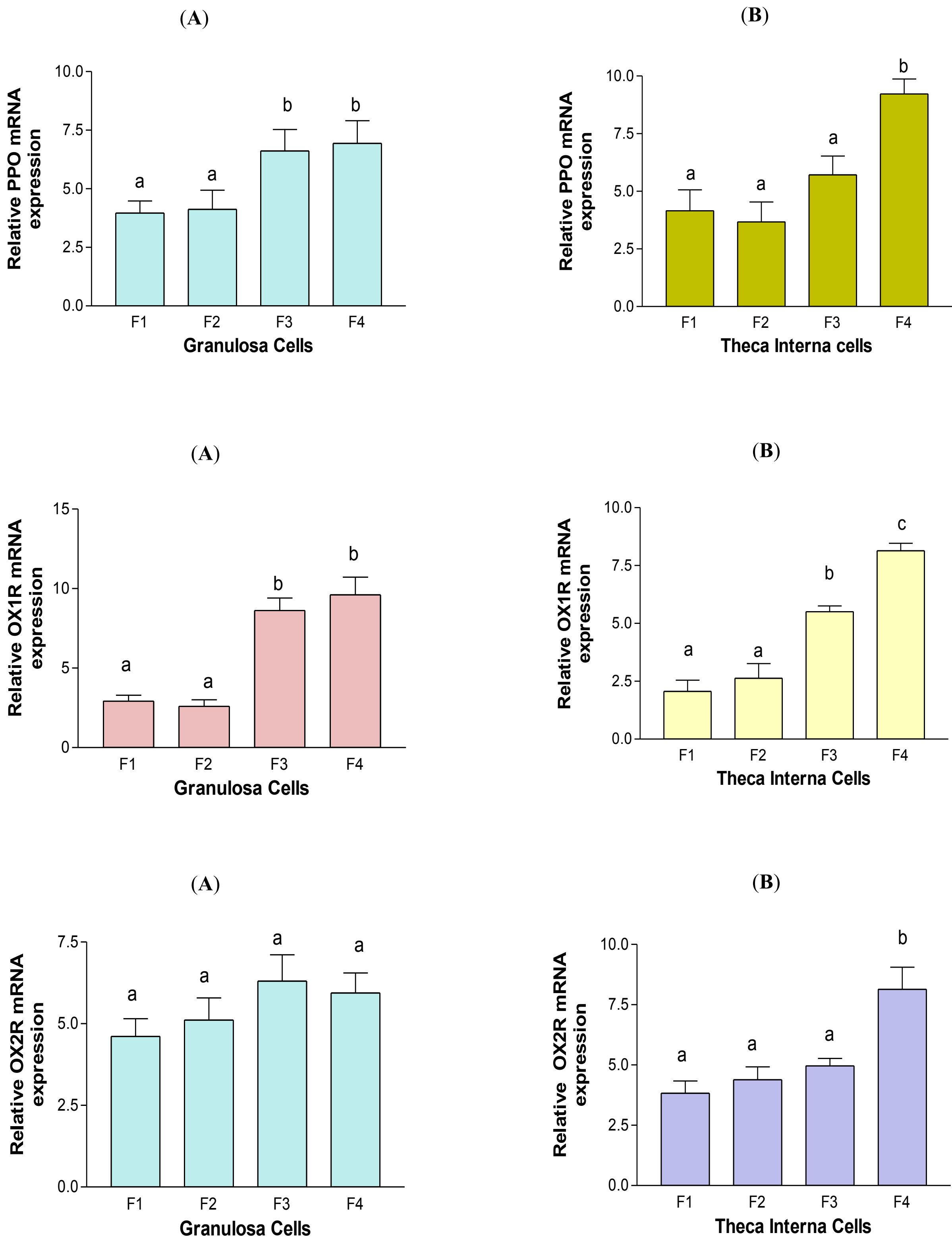
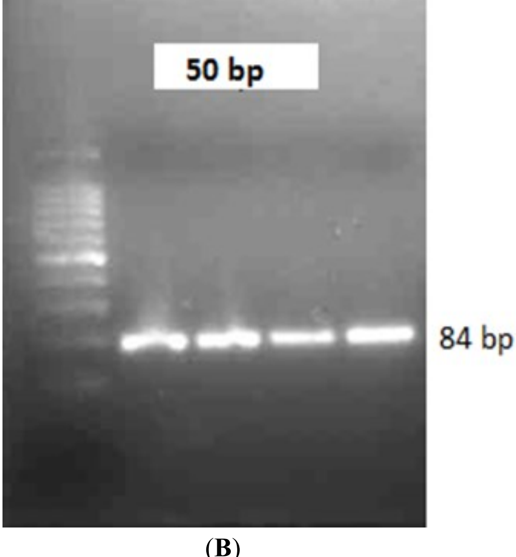
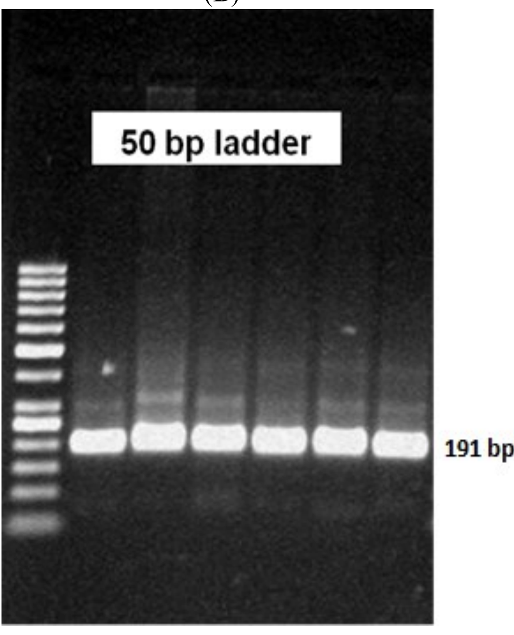


Fig.4.7: Relative amounts of PPO, OX1R and OX2R mRNA in granulosa cells(GC) and theca interna cells (TIC) cells of different follicle size in buffalo (A) PPO mRNA in GC, (B) PPO mRNA in TIC, (A) OX1R mRNA in GC, (B) OX1R mRNA in TIC, (A) OX2R mRNA in GC, (B) OX2R mRNA in TIC. Class F1 follicle was taken as calibrator. All the values are shown as means± SEM. Different superscripts denote differences in values (P<0.05). GC: granulosa cell; TIC: theca interna cell.

(**A**)



(**B**)



(**C**)

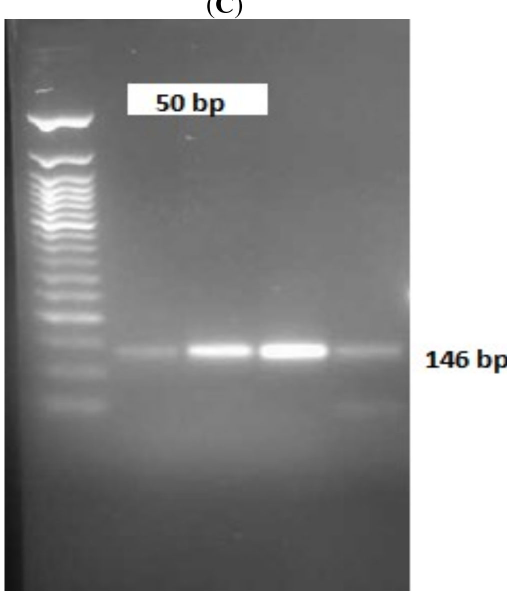


Plate 4.5: 1.5% agarose gel showing 84 bp product for StAR(A), 191 bp prod uct for 3β-HSD(B) and 146 bp product for CYP11A1(C).

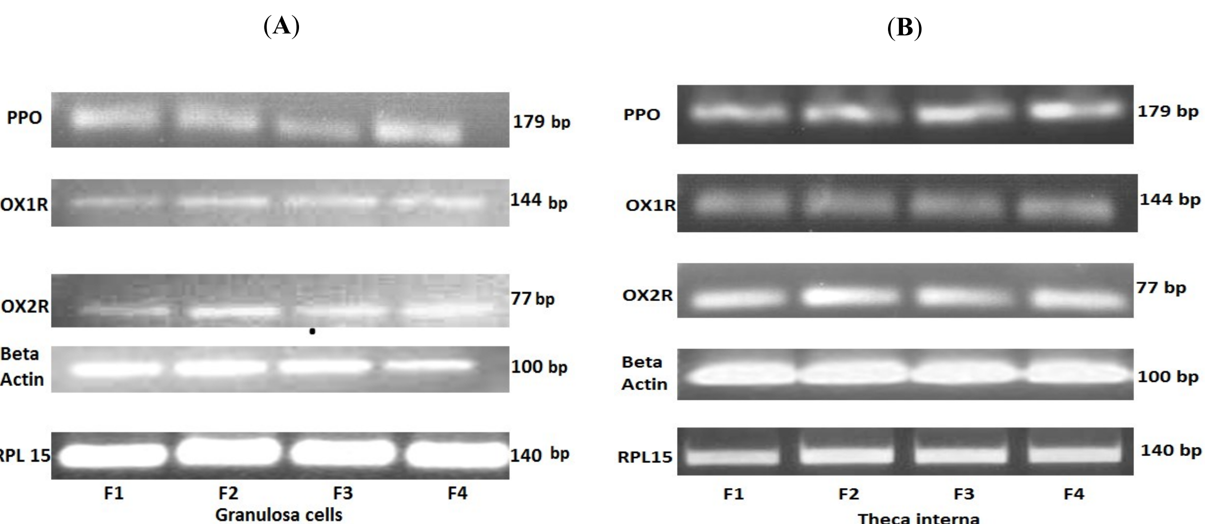


Plate 4.6: 1.5% agarose gel showing PPO: 179 bp, OX1R: 144 bp, OX2R: 77BP, Beta actin: 100 bp and RPL 15: 140 bp products at different follicular phase (F1, F2, F3 and F4) in GC and TIC

hypothalamus and anterior pituitary of rats during the pro-estrous (Silveryra *et al*., 2007b). Ning *et al*., (2008) verified that, OX1R mRNA levels in the porcine ovary were lowest in di-estrous and highest in the meta-estrous phase of the cycle. The present study supports the presence of orexin systems in buffalo ovarian follicles.

4.4.2 Localization of orexin A and its receptors (OX1R and OX2R) in ovarian follicles

The immunohistochemical localization of orexin A, OX1R and OX2R is presented in Plate 4.7, 4.8 and 4.9 respectively. The immunoreactivity of orexin A and its both receptors was present in GC and TIC cells of ovarian follicles and the reactivity varied with a stage-specific manner. The intensity of immunohistochemistry was calculated using semiquantitative method and immuno-positive cells were expressed in a percentage. The scoring pattern was 0: no immunoreactivity, 1: <30%, 2: 30% -60%, 3: > 60% immuno-positive cells. Accordingly, every image was given a score as per its intensity. For grading, a 4-tire scoring system was used as strong positive (+3), moderately positive (+2), low positive (+1) and negative (0). The intensity of immunostaining varied from phase to phase and the number of positive cells which was influenced by the different stages of the follicle size. The intensity of orexin was higher (+2) in F3 and F4 follicles than F1 and F2 follicles (+1). The intensity of orexin 1R and orexin 2R was +1 in F1 & F2 and +2 in F3 and F4 follicles. The negative controls, with isotype IgG and without primary antibodies, showed only a weak background staining.

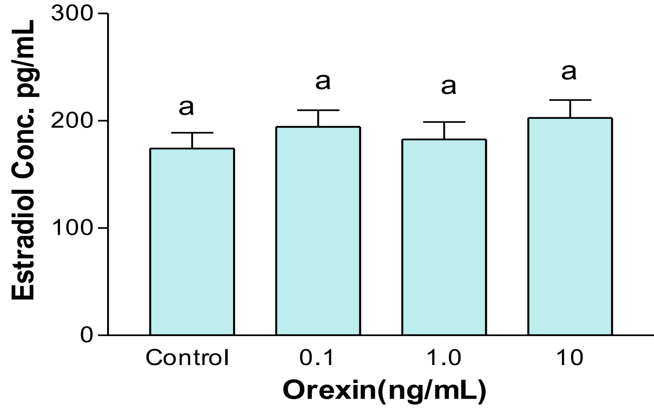
The result of the present study are in agreement with observations of Ciccimarra *et al*., (2018), where localization of OXA, OX1R, OX2R was noted in the granulosa cell and thecal cells of swine ovarian follicle by using immunohistochemistry. In addition, it was also detected that OXA and OX2R are localized in cytoplasm of GC and immunoreactivity of OX1R observed in nucleus, OX2R in cytoplasm of TIC and expression of PPO was shown in granulosa layer of large follicles. According to investigational data, ovarian orexin

localization found species-dependent: the occurrences of its mRNA was not detected in the rat ovary (Johren *et al*., 2001), whereas it was found in porcine pig (Nitkiewiez *et al*., 2014), the adult cat and dog ovary (Levanti *et al*., 2015). Similarly, Ragionieri *et al*., (2018) observed localization of orexin A and its receptors by immunofluorescent methods in follicular phase of swine ovary, in which OXA and OX2R were appeared associated to cytoplasmic vesicles and the immunoreactivity of OX1R was often associated with nuclear envelope and lesser extent in cytoplasm. The presence of both ligand (orexin) and its receptors in ovarian follicles cells indicated its autocrine and paracrine role in these investigations.

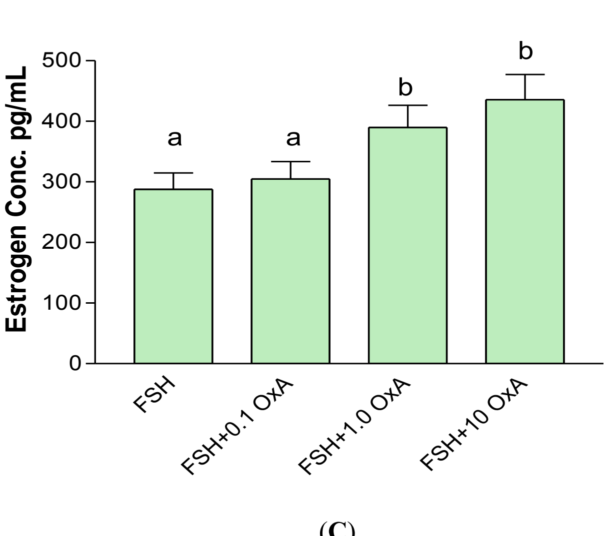
4.5 Effect of orexin on estrogen (E_2) secretion

GCs were collected from F4 follicles and were cultured for 48 h after attaining 75-80% confluency, without (control) or with dose of orexin (0.1, 1 and 10 ng/mL) alone or with follicle stimulating hormone (FSH) and insulin-like growth factor (IGF-I) (30ng/mL and 10ng/ml respectively). The Concentration of E2 in spent culture medium was 24.02±2.01 pg/ml. Treatment of orexin alone did not affect the E2 concentration, but orexin, with FSH and IGF-I increased E2 secretion in dose dependent manner shown in Fig. 4.8 A, B and C respectively. The results of present study are in agreement with finding of Ciccimarra *et al*., (2018) in which granulosa cell viability was significantly ($P<0.05$) increased E2 without differences in all the concentrations of OXA, whereas the highest concentration (10 ng/ml) showed stimulatory effect in swine. Porkka *et al*., (2004) reported orexin A and serum E2 concentration was highest on the day of pro-oestrous in young cyclic rats. The findings of present study are in contrast with observation of Nitkiewicz *et al*., (2014), where in *in vitro* conditions, orexin inhibited FSH-induced estradiol secretion in porcine granulosa cells. It could be possible due to dose concentration of orexin and due to different actions of orexin in different species.

(**A**)



(**B**)



(**C**)

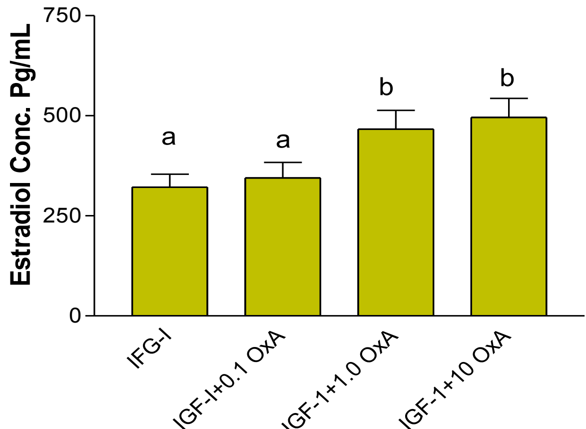


Fig. 4.8 : Concentration of estradiol (E2) in spent media of granulosa cells (GC) culture. GCs were cultured for 48 h in medium in presence or in the absence of orexin at a dose of 0.1,1.0 and 10ng/mL + FSH (30ng/mL) or orexin + IGF-I (10ng/mL). All values are shown as means±SE. Different superscripts denote statistically different value (P<0.05)

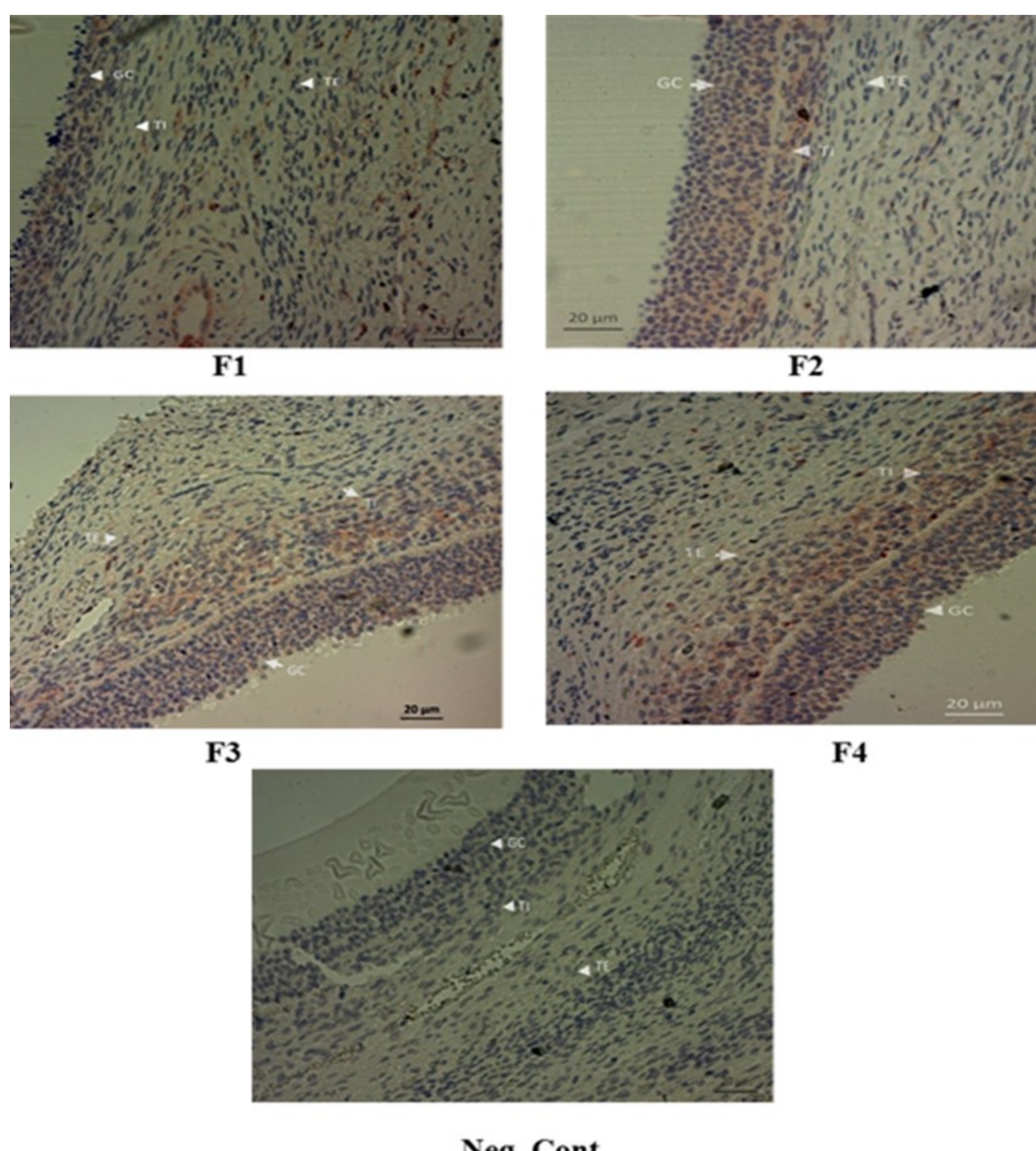


Plate 4.7: Distribution of orexin A in Immunoreactive cells in different stages of Follicular cell observed under 20X. The distribution of orexin positive cells in F1, F2, F3, F4 & Negative Control (arrow) GC (Granulosa Cell), TI (Theca Interna)

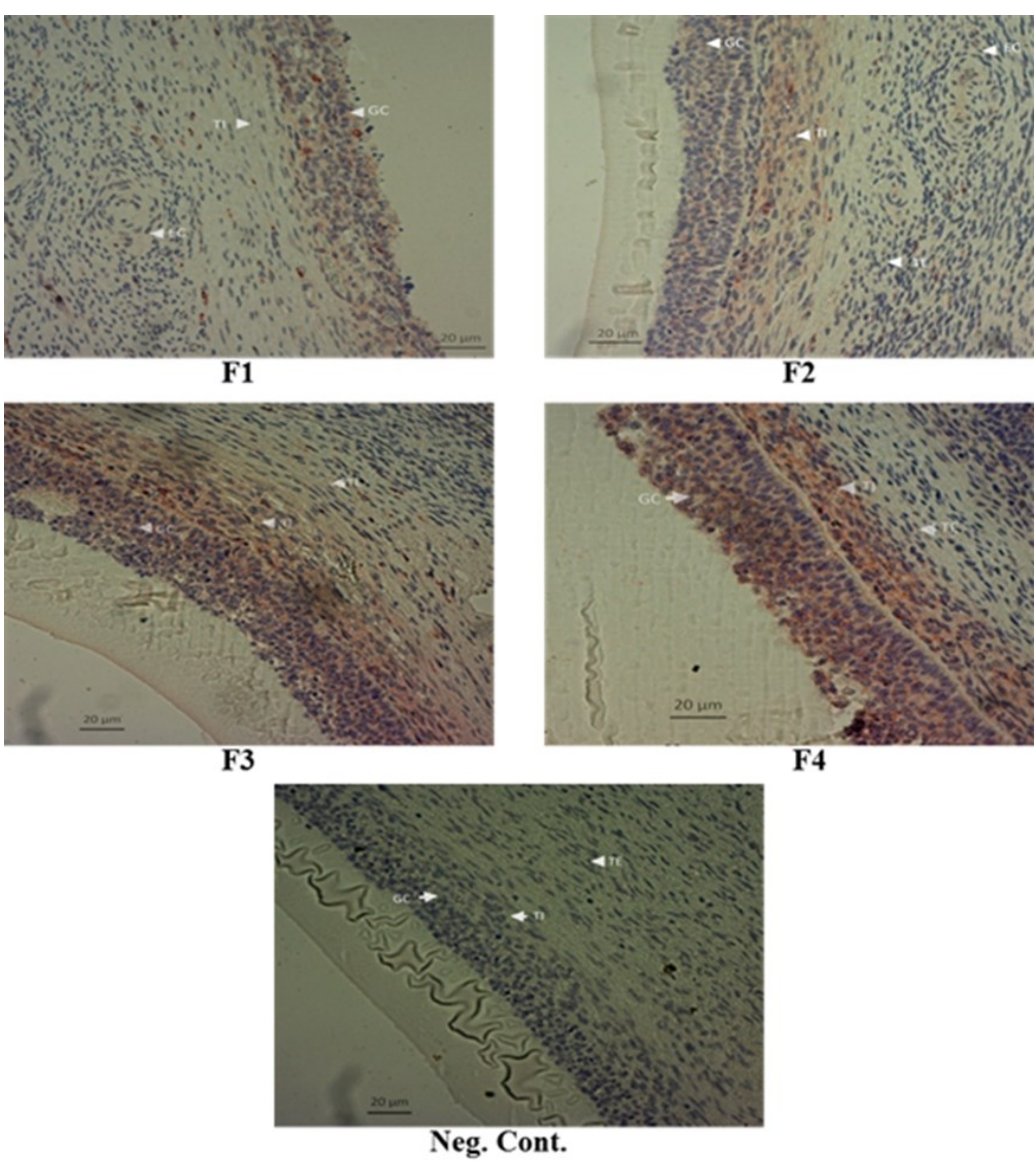


Plate 4.8: Distribution of orexin 1 receptor (OX1R) in immunoreactive cells in different stages of Follicular cell observed under 20X. The distribution of orexin positive cells in F1, F2, F3, F4 & Negative Control (arrow) GC (Granulosa Cell), TI (Theca Interna)

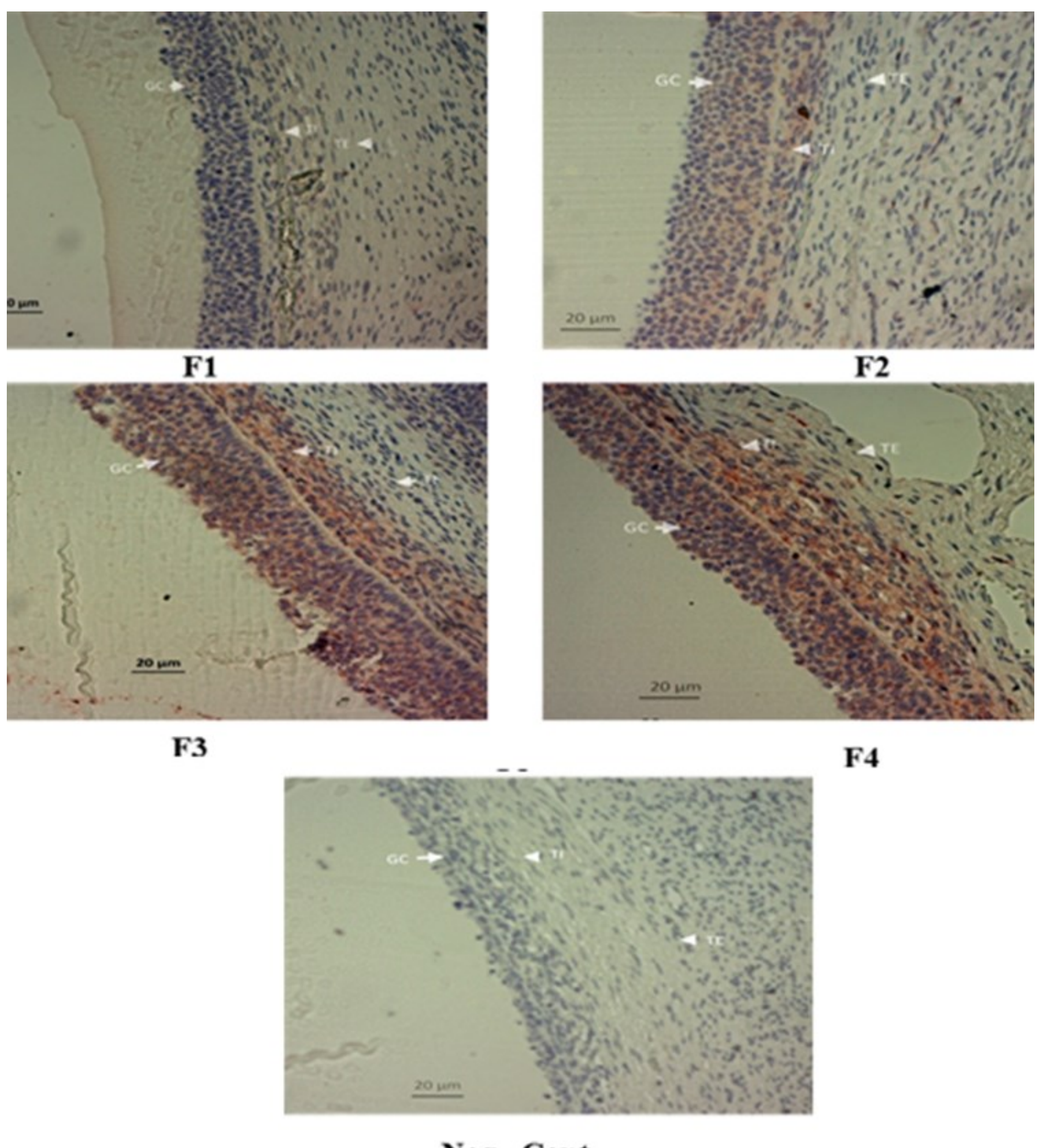


Plate 4.9: Distribution of orexin 2 receptor (OX2 R) in immunoreactive cells in different stages of Follicular cell observed under 20X. The distribution of orexin positive cells in F1, F2, F3, F4 & Negative Control (arrow) GC (Granulosa Cell), TI (Theca Interna)

4.5.1 Effect of orexin on CYP19A1 expression

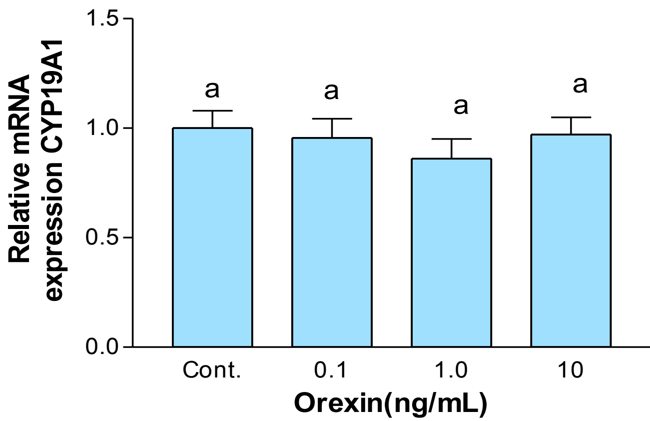
The expression of CYP19A1 from GCs treated with orexin (0.1, 1, & 10 ng/ml) either alone or in combination with FSH (30ng/ml) and IGF-I (10 ng/ml) is presented in Fig. 4.9 A, B and C. Basal orexin did not affect CYP19A1 expression, but in combination with FSH and IGF-I (OXA+FSH, OXA+IGF-I) increased E2 secretion significantly ($P<0.05$) with increase dose of orexin.

The result of present study are in agreement with earlier studies, in which orexin increases E2 secretion from cultured granulosa cell in rat (Cataldi *et al*., 2012) and in pig (Kiezun *et al*., 2017). However, the present finding does not agree with observation of Salim *et al*., (2016) in rat in which orexin treated hypothalamic mRNA CYP19A1 expression decreased with increase in dose. It might be possible due to different actions of orexin in different tissues and species. The decreased E2 secretion was probably due to inhibition of expression of CYP19A1 gene, the product of which is aromatase, an intermediate for E2 synthesis.

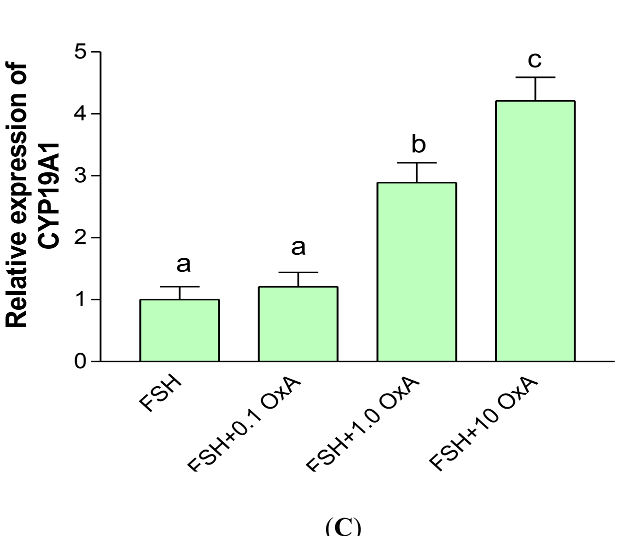
Orexin is not only the important regulator of neuroendocrine functions but also involved in reproductive functions through both at central and peripheral level. Present findings in buffalo also designate stimulatory effect of orexin at local level on steroidogenesis in corpus luteum and ovarian follicles. It offers the evidence of regulation of ovarian functions in buffaloes. However, the detail regulator interplay of orexin on ovarian functions required further more study.

The result of present study inferred the presence of orexin and its receptors (OX1R and OX2R) mainly on fully developed mature CL and ovarian follicles. Orexin has significant *in vitro* effect on estrogen and progesterone secretion in buffalo ovaries.

(**A**)



(**B**)



(**C**)

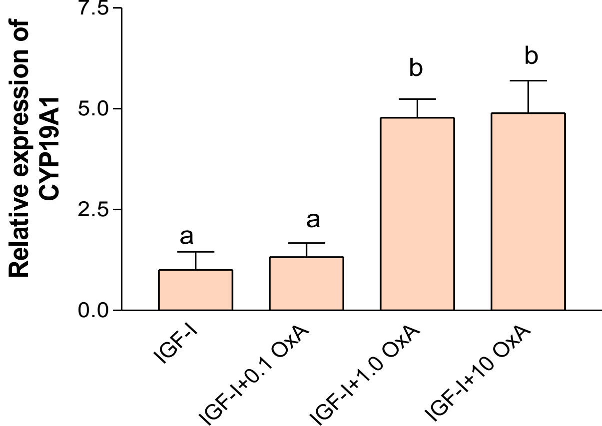


Fig. 4.9: A. Effect of orexin at the dose of 0.1,1.0 and 10 ng/mL on aromatase (CYP19A1) mRNA expression from cultured granulosa cells (GC) either alone or in presence of FSH (30ng/mL) and IGF-I (10ng/mL). Different superscripts denote statistically different values (P<0.05).

SUMMARY AND CONCLUSION

Buffalo (*Bubalus bubalis*) is a multi-purpose species and contributes significantly to rural economy and dairy industry in many developing countries across the world. The genetic development, mainly in reproductive performance stands high among agricultural research requirements of the developing countries. Nevertheless, a better understanding of the luteal and follicular dynamics and ovulation may be essential for increasing conception rate and for minimizing early embryonic losses. The failure in conception occurs may be due to defects in process of conversion of follicular structure into luteal structure. The follicle development involves the growth of unit of ovarian follicles, in which only one selected for pre-ovulation, maturation, while other follicles undergo atresia. In buffalo, the dominant follicle is classically developed up to 9mm in diameter and it will be the follicle to continue growing up to 14-20 mm size in diameter to be ovulate.

In domestic species ovarian cycle is categorized by repeated patterns of cellular proliferation, differentiation along with alteration in ovarian steroidogenic cells (granulosa, theca and luteal cells) which enhance follicular and luteal development. Recent studies showed involvement of orexin in ovarian functions. Orexin is a recently discovered peptide and the present study was carried out to explore the involvement of this peptide hormone in luteal and follicular growth and functions in the ovary of buffalo.

The first part of study, explored the expression and localization of orexin and its receptors in CL and confirmation of *in vitro* effect of orexins on steroid production. CL was classified in to the following stages; early (1-4), mid (5-10), late (11-16) and regressing (>17) phase of estrous cycle. Luteal tissue was frozen in liquid nitrogen and stored at -80^{0}C until RNA isolation. qPCR was applied to investigate mRNA, and localization were done by immunohistochemistry. Beta actin was used as a control for mRNA expression calculation. The result confirmed the hypothesis that orexin and its receptors were found in buffalo CL during estrous cycle. The relative

expression of mRNA of orexin varied according to the stage of estrous cycle. It was higher in early and regressing phase and lower in mid and late luteal phases in PPO and OX2R and low in late luteal phase and abundance in early, mid and regressing phases in OX1R. Results of mRNA expression were also supported by the localization of orexin and its receptors (OX1R and OX2R) by immunohistochemistry. Orexin A, a secretory protein synthesized from PPO and its receptors were observed in cytoplasm of small and large luteal cells, and the intensity of immunoreactivity is moderately positive. (Every image was score as per its intensity, for grading a 4-tire scoring system, that is strong positive (3+), moderately positive (+2), low positive (+1), and negative (0) was used) These observations indicated the autocrine and paracrine role of orexin in CL functions and development.

For further confirmation of orexins role in CL, an *in vitro* culture experiment was carried out. Luteal cells isolated form early(1st) stage CL were cultured *in vitro* and treated with orexin at 0.1, 1.0, and 10 ng/mL concentrations for 48 h after obtaining 75-80% confluence. Progesterone (P4) concentration in spent media of culture was 9.34 ng/mL. Orexin at a dose 0.1ng/mL progesterone (P4) concentration was comparable with control and at dose 1.0 and 10ng/mL there was significant ($P<0.05$) decline. In the present study inhibitory effect of orexin on progesterone secretion was observed. This may be attributed to species specific effect. mRNA expression of StAR, CYP11A1 and 3β-HSD in cultured cells varied significantly according to given treatment. mRNA expression of StAR gene was comparable between control and 0.1 ng/mL dose however significantly ($P<0.05$) decreased with increased doses of 1,0 and 10ng/mL. The expression pattern of CYP11A1 gene was similar to the StAR for 0.1 and 1.0 ng/mL dose significantly ($P<0.05$) decreased, while no significant difference between 1.0 and 10 ng/mL doses. The mRNA expression of 3β-HSD was significantly ($P<0.05$) declined in orexin treated cells at the dose 0.1, 1.0 and 10ng/mL and comparable at dose 1.0 and 10ng/mL. The decline in P4 secretion from luteal cells in response to orexin treatment is probably due to

decrease in expression of gene associated with P4 synthesis (i.e. StAR, CYP11A1and 3β-HSD).

The second part of study, investigated expression and localization of orexin and its receptor in ovarian follicles and effect of orexin on estradiol secretion and cell proliferation of granulosa cell culture in buffalo. Follicles were classified in four group according to size and the concentration of estradiol 19β (E2) in follicular fluid (FF): F1(small), 4-6mm diameter, E2 >0.5ng/ml of FF; F2 (medium), 7-9 mm, E2= 0.5-5 ng/ml; F3 (large) 10-13mm, E2=5-40ng/ml; F4 (pre-ovulatory), > 14mm, E2 > 180ng/ml). For RNA extraction, follicles were dissected out from ovarian stroma. The surround tissue (theca externa) was carefully removed with forceps under a stereo microscope. After aspiration of FF, each follicle was bisected and its inside wall was gently scraped and flushed with Ringer's solution to separate out the granulosa cells (GC) and the remaining follicle wall after GC separation formed the theca interna cells (TIC). GC and TIC isolated from each follicle were transferred in to separate tubes and labeled. The GC in the flushing solution was centrifuged at 3000g for 10 min at 4°C. The TIC and GC pellet were separately snap frozen in liquid nitrogen and stored at -80°C until RNA isolation. qPCR was applied to investigated mRNA, and localization were done by immunohistochemistry. Beta actin was used as a control for mRNA expression calculation.

It was observed that orexin and its receptor (OX1R and OX2R) expression significantly varied through the follicular phase in both GC and TIC. In GC, the mRNA expression of PPO and OX1R was significantly (P<0.05) higher in large and preovulatory follicles (F3 and F4). There was no significant variation in OX2R in GC among all stages of follicles. In TIC, mRNA expression of PPO was significantly (P<0.05) high in F4 and was comparable to F1, F2 and F3 follicles. The expression of OX1R was significantly (P<0.05) high in F4 followed by F3 and F2 and were comparable in F1 and F2. In TIC, the abundance of OX2R transcripts was significantly (P<0.05) highest in F4 follicles and comparable between F1, F2 and F3 with no significant difference. Results of mRNA expression were also supported by

the immunohistochemical localization of orexin and its receptors. Orexin and its receptors immunoreactivity was observed brown colour in cytoplasm of granulosa and theca cells. The intensity of immuno-staining observed in present study containing orexin and its receptor during follicle development and maturation with maximum intensity in F3 and F4 follicles. The localization of orexin and its receptors in somatic cells (GC and TIC) of ovarian follicle indicate autocrine and paracrine role of this peptide in buffalo follicle development.

In the *in vitro* study, granulosa cells isolated from pre-ovulatory or F4 follicles were cultured and treated with orexin (0.1, 1.0 and 10ng/mL) alone or with FSH and IGF-I (30ng/mL and 10ng/mL respectively) for two days after obtaining 75-80% confluence. E2 concentration was checked by ELISA from the spent media. The treatment of orexin alone did not affect the E2 concentration, but orexin with FSH and IGF-I increased E2 secretion in dose dependent manner (dose 0.1,1.0 and 10ng/mL). From the cells, relative mRNA expression for aromatase gene (CYP19A1) was demonstrated. The result showed that orexin enhance E2 secretion with FSH and IGF-I and mRNA expression ($P<0.05$) and localization of CYP19A1, in dose dependent manner in *in vitro* cultured GCs.

Salient observations:

- The relative mRNA expression of orexin and its receptor (OX1R and OX2R) as determined by RT-qPCR was found significantly ($P<0.05$) higher in early and regressing stage of CL and lower in mid and late luteal stage of CL.
- Immunoreactivity of orexin and its receptors was mostly restricted to small and large luteal cells. Immunoreactivity was observed highest during early and regressing luteal stage for both ligand and receptor.
- Orexin inhibited P4 production through decrease mRNA expression of StAR, CYP11A1 and 3βHSD in luteal cell culture.

- Relative mRNA expression of orexin and its receptor (OX1R and OX2R) increased with follicle development and found highest in large (F3) and pre-ovulatory (F4) or mature follicle.
- Orexin and its receptor immunoreactivity were observed in cytoplasm of granulosa and theca cells with increasing immunoreactivity in F3 and F4 follicle.
- Basal orexin did not affect aromatase gene CYP19A1 expression, however in combination with FSH and IGF-I, increased E2 production.

Conclusion

- The result of present study demonstrated the dynamics of expression and localization of orexin and its receptors in buffalo ovarian follicles and corpus luteum.
- Orexin exhibited steroidogenic effect on estrogen and progesterone secretion in granulosa and luteal cell culture indicating its role on follicle and luteal development and functions.

PROPOSED AREA OF FUTURE RESEARCH

1. To find exact role of orexin use of knockout or gene silencing technique.
2. To find systemic effect of orexin in hypothalamic-pituitary-ovarian axis.
3. To study role of orexin system in pregnancy / gestation
4. To study role of orexin system in male reproduction.

BIBLIOGRAPHY

Ali, I., V.S. Chouhan, S.S.Dangi, M. Gupta, U.Tandiya, I. Hyder and M. Sarkar (2014) Expression and localization of locally produced growth factors regulating lymphangiogenesis during different stages of the estrous cycle in corpus luteum of buffalo (*bubalusbubalis*). Theriogenology, 81(3), 428-436.

Baruselli, P.S., R.G. Mucciolo,J.A. Visintin, W.G. Voama, R.P. Arruda, E.H. Madureira, C.A. Oliveira and J.R. Molero (1997) Ovarian follicular dynamics during the estrous cycle in buffalo (*Bubalusbubalis*). Theriogenology 47, 1531-1547.

Basini, G.,R. Cicimarra,S. Bussolati, S. Grolli, L. Ragionieri, F. Ravanetti, M. Botti, F. Gazza A. Cacchioli, R. Di Lecce, A.M. Cantoni and F. Grasselli (2018) Orexin A swine corpus luteum. Domestic Animal Endocrinology, 64, 38-48.

Bonnavion, P. and de Lecea (2010) Hypocretins in the control of sleep and wakefulness. Current Nurology Neuroscience Reports, 10, 174-179.

Cataldi, N.I., V.A.R. Lux-Lantos and C.Libertun (2012) Effect of orexin A and B on expression of orexin receptors and progesterone release in luteal and granulosa ovarian cells. Regulatory Peptides. 178, 56-63.

Cataldi, N.I.,V.A.R. Lux-Lantos and C. Libertun (2014) Orexin A and B in vitro modify orexins receptors expression and Gonadotropins secretion of anterior pituitary cells of pro-estrous rates. Regulatory Peptides, 188, 25-30.

Chojnowska, K., A. Nitkiewicz, N. Smolinska, A. Maleszka, M. Kiezun, and T. Kaminski (2012) The effect of orexin A and B on the secretion of

progesterone and estradiol by porcine ovarian granulosa cells.Sekcia-Biotechnologie, 2.www.researchgate.net/pubication/282848349 April 2012.

Chouhan, V.S., R.P. Panda, V.P. Yadav, V. Babitha, F. A. Khan, G. K. Das, M. Gupta and M. Sarkar (2013) Expression and localization of vascular endothelial growth factor and its receptors in the corpus luteum during oestrous cycle in water buffaloes (*bubalusbubalis*). Reproduction in Domestic Animals, 48, 810-818.

Chouhan, V.S., S.S.Dangi, M. Gupta, V. Babitha, F.A. Khan, R.P. Panda, V.P. Yadav, G. Singh, M. Sarkar (2014) Stimulatory effect of vascular endothelial growth factor on progesterone production and survivability of cultured bubaline luteal cells. Animal Reproduction Science, 148, 251-259.

Ciccimarra, R.S., F.Bussolati,S. Grasselli, L. Grolli, F. Ragionieri, M. Ravanetti, F. Botti, A. Gazza, R. Cacchioli, A.M. DiLecce, A. Cantoni and G.Basini (2018) Orexin system in swine ovarian follicles. Domestic Animal Endocrinology, 62, 49-59.

Dall'Aglio, C., V. Pedini, P. Scocco, P. Ceccarelli (2010) Immunohistochemical evidence of orexin-A in the pancreatic beta cells of domestic animals. Research in Veterinary Science, 89,147-149.

Das G.K., B.L.Kumawat and F.A. Khan (2013) Ovarian follicular dynamics during estrous cycle and its aberrations during certain reproductive disorders in buffalo. Theriogenology Insight, 3(1), 37-46.

De Lecea, L.,T.S. Kilduff, C. Peyron,X. Gao,P.E. Foye and P.Danielson (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proceedings NationalAcadSciences, USA,95(1), 322-327.

Diep, C.H., A.R. Daniel,L.J.Mauro, T.P. Kunutson and C.A.Lange (2015) Progesterone action in breast, uterine, and ovarian cancers. Journal of Molecular Endocrinology, 54, R31-53.

Dobrzyn,K., N. Smolinska,L. Marta, K. Szeszko,E. Rytelewska, K. Kisielewska M. Gudelska and T.Kaminski (2018) The *in vitro* effect of progesterone on the orexin system in porcine uterine tissues during early pregnancy. Acta VeterinariaScandinavica, 60, 76.

Evans, A.C. and J.E. Fortune (1997) Selection of the dominant follicle in the cattle occurs in absence of differences in the expression of messenger ribonucleic acid for gonadotropin receptors. Endocrinology. 138, 2963- 2971.

FAOSTAT. (2019) Food and Agriculture organization of the United Nations. http://faostat.fao.org/site/573/DesktopDefault.aspx?PageID=339#ancor,2012.

Ginther, O.J., M.A.Beg, F.X. Donadeu and D.R. Bergfelt (2003) Mechanism of follicle deviation in monovular farm species. Animal Reproductive Science, 78, 239-257.

Gougeon, A. (1996) Regulation of Ovarian Follicular Development in Primates: Facts and Hypotheses.Endocrine Reviews, 17(2), 121-155.

Gupta, M., K.B. Bahiram,V.M. Sardar,J.P. Korde,S.P. Magar, S.W. Bonde and N.V. Kurkure (2019) Expression and localization of adiponectin and its receptors in ovarian follicles during different stages of development and the modulatory effect of adiponectin on steroid production in water buffalo. Reproductive Domestic Animal 54, 1291-1303.

Gupta, M., S, Dangi, V.S. Chaouhan,I.Hyder, V. Babith,V.P.Yadav,F.A. Khan,A. Sonwane, G. Shigh, G.K. Das,A. Mitra, S.Bag, and M. Sarkar (2014) Expression and localization of ghrelin and its functional receptor in corpus luteum during different stages of estrous cycle and the modulatory role of ghrelin on progesterone production in cultured luteal cells in buffalo. Domestic Animal Endocrinology, 48, 21-32.

Gupta, M., S.S. Dangi, G. Singh, M. and Sarkar (2015) Expression and localization of ghrelin receptor in ovarian follicles during different stages of development and modulatory effect of ghrelin on granulosa cells function in buffalo. General and comparative Endocrinology, 210, 87-95.

Inutsuka, A. and A. Yamanaka (2013) The physiological role of orexin /hypocretin neurons in the regulation of sleep / wakefulness and neuroendocrine functions. Frontiers in Endocrinology. 4 (18), 1-10.doi: 10.3389/fendo.2013.00018.

Jainudeen, M.R. and E.S.E. Hafez (1993) Cattle and buffalo: reproduction in farm animal (Ed. E.S.E. Hafez), 6th ed. Lea and fediger, Philadelphia, PA, USA. 315-329.

Johren, O., J. Steffi, J.Neidert, M. Kummer, A. Dendorfer and P. Dominiak (2001) Prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. Endocrinology, 142 (8), 3324-3331.

Joshi, D. and S.K. Singh (2017) Localization and expression of orexin A and its receptor in mouse testis during different stages of postnatal development. General and Comparative Endocrinology, 241, 50-56.

Kaminski, T., N. Smolinska, A. Nitkiewicz and J. Przala (2010b) Expression of orexin receptors 1(OX1R) and 2 (OX2R) in the porcine hypothalamus during the oestrous cycle. Journal of Physiology and Pharmacology, 61(3), 363-371.

Kaminski, T.,N. Smolinska, A. Nitkiewicz, J. Przala, A. Maleszka and M. Kiezun (2010a) Expression of orexin receptors 1(OX1R) and 2 (OX2R) in the porcine pituitary during the oestrous cycle. Animal Reproduction Science, Vol.117(1-2), 111-118.

Karteris, E., J. Chen, S. Harpal and Randeva (2004) Expression of human prepro-orexin and signaling characteristics of orexin receptors in the male reproductive system. Journal of clinical Endocrinology and Metabolism, 89(4), 1957-1962.

Kesielewska, K., E. Rytelewska, M. Gidesla, M. Kiezun, K. Dobrzyn, K. Szeszko, K. Bors, J. Wyrebek, T. Kaminski and N.Smolinska(2019) The effect of orexin B on steroidogenic acute regulatory protein, P450 side-chain cleavage enzyme, and 3βHSD gene expression, and progesterone and androstenedione secretion by the porcine uterus during early pregnancy and the estrous cycle. Journal Animal Science, 97, 851-864.

Kiezun, M., N. Smolinska, K. Dobrzyn, K. Szeszko, E. Rytelewska and T. Kaminski (2017) The effect of orexin A on CYP17A1 and CYP19A3 expression and on estradiol, estrone and testosterone secretion in the porcine uterus during early pregnancy and the estrous cycle. Theriogenology, 90, 129-140.

Lachance, Y., V.Luu-The, C. Labrie, J. Simard, M. Dumont,Y. de Launoit, S. Guerin, G. Leblanc and F. Labrie (1992) Characterization of human 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase gene

and its expression in mammalian cells. Journal of Biological Chemistry, 267, 3551.

Levanti, M., A, Germana and F. Abbate (2015) Orexin A expression in the ovary of dog and cat. Reproduction in Domestic Animals, 50, 247-250.

Li, M., N. Zu, C.S. Zhang, M.Y. Xie, Y.Z. Liu and X.J. Xu (2019) Orexin A promotes granulosa cell secretion of progesterone in sheep. Indian Journal Veterinary Research, 20 (2), 136-142.

Liguori, G., C. Squillacioti, L. Assisi, N. Mirabella, E. Lanagella, A. Costagliola and A. Vittoria (2017) Localization of orexin B and receptor 2 for orexins in testicular cytotypes of the camelid alpaca (*Vicugna pacos*). Reproduction in Domestic Animal, 52(3), 452-458. https://doi.org/10.1111/rda.12931

Luu-The, V. (2001) Analysis and characteristics of multiple types of human 17beta-hydroxysteroid dehydrogenase. Journal of Steroid Biochemical Molecular Biology, 76, 143-151.

Miller, W. L. and J. F. straus, (1999) Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein, StAR. Journal Steroid Biochemical Molecular Biology, 69, 131-141.

Mishra, S.R., N. Thakur,A. Somal, M.S. Parmar, R. Reshma,G. Rajesh and M. Sarkar (2016) Expression and localization of fibroblast growth factor (FGF) family in buffalo ovarian follicle during different stages of development and modulatory role of FGF2 on steroidogenesis and survival of cultured buffalo granulosa cells. Research in Veterinary Science, 108, 98-111.

Ning, H., Z. Lei, W. Zhang, J. Su, J. Song and X. Jia (2008) Expressions of prepro-orexin and OX1R mRNA in hypothalamus-pituitary-ovary axis of

Suzhong sows in oestrous cycle. Journal Nanjing Agriculture University China, 31(1), 87-91.

Nitkiewicz, A., N. Smolinska A. Maleszka, K. Chojnowska and T. Kaminski (2014) Expression of orexin and their precursor in the porcine ovary and the influence of orexins on ovarian steroidogenesis in pigs. Animal Reproductive Science, 148, 53-62.

Nitkiewicz, A., N. Smolinska, A. Maleszka, M. Kiezun and T. Kaminsrki (2012) Localization of orexin A and orexin B in the porcine uterus. Reproductive Biology, 12(2), 135-155.

Nitkiewicz, A., N. Smolinska, J. Przala and T. Kaminsrki (2010) Expression of orexin receptors 1 (OX1R) AND 2 (OX2R) in the porcine ovary during the oestrous cycle. Regulatory Peptides, 165, 186-190.

Peyron, C. and T. S. Kilduff (2017) Mapping the Hypocretin/Orexin Neuronal System: An Unexpectedly Productive Journey. Journal NeuroSciences. 37 (9), 2268-2272. Doi:10.1523/JNEUROSCI.1708-16.2016

Porkka-Heiskanen, T., A.Kalinchuk, L.Alanko, I. Huhtaniemi and D. Stenberg (2004) Orexin A and B levels in the hypothalamus of female rats: The effects of the estrous cycle and age. European Journal of Endocrinology, 150,737-42.

Ragionieri, L., F. Ravanetti, R. Di Lecce, M. Botti, R. Ciccimarra, S. Bussolati, G. Basini, F. Gazza and A.Cacchioli (2018) Immunolocalization of Orexin A and its receptors in the different structures of the porcine ovary. Annals of Anatomy, 218, 214–226.

Robinson, R.S., A.J. Hammond, G.E. Mann and M.G. Hunter (2008) A novel physiological culture system that mimics luteal angiogenesis. Reproduction, 135, 405-413.

Rosati, S. 2015. Histrelin for central precocious puberty- a single surgeon experience. Journal of surgical research, 198(2), 355-359.

Russell,S.H., C.J. Small, S.A. Kennedy, A. Stanley, A. Seth, K.G. Murphy, S. Taheri, M.A.Ghatei and S.R. Bloom (2001) Orexin A interactions in the hypothalamo- pituitary-gonadal axis. Endocrinology, 142, 5294-5302.

Sakurai, T., A. Ameniya, M. Ishii, I. Matsuzaki, R.M. Chemelli and H. Tanaka (1998) Orexins and orexin receptors: afamily of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell, 92 (4),573-58.

Salim, M., Z. Alishah, H. Khazali and F. Mahnoudi (2016) Orexin decrease aromatase gene expression in the hypothalamus of androgenized female rats. International Journal Fertility and Sterility, 10(2), 190-195.

Sarkar, M., S. S. Schilffarth, S.D. Schams, H.H. Meyer and B.B. Berisha (2010) The expression of leptin and its receptor during different physiological stages in the bovine ovary. MolecularReproduction Development,77, 174-181.

Sasson, R., R.K. Dearth, R.S. White, E.P. Chappell and P.L. Mellon (2006) Orexin A induces GnRH gene expression and secretion from GTI-7 hypothalamic GnRH neurons. Neuroendocrinology,84,353-363.

Schams, D. and B. Berisha (2004) Regulation of corpus luteum function in cattle- An overview. Reproduction in Domestic Animal, 39, 241-251.

Silveyra P., P.N. Catalano, V. Lux-Lantos and C. Libertun (2007b) Impact of pro-estrous milieu on expression of orexin receptors and prepro-orexin in rat hypothalamus: actions of Cetrorelix and Nembutal. Animal Journal of Physiology Endocrinology and Metabolism. 293, E820- 828.

Silveyra, P., V. Lux-Lantos and C. Libertun (2007a) Both orexin receptors are expressed in rat ovaries and fluctuate with the estrous cycle: effects of orexin receptor antagonists on gonadotropins and ovulation. Animal Journal of Physiology Endocrinology and Metabolism.293, E977- 985.

Singh C.V. and R.S. Barwal (2010) Buffalo breeding research and improvement stratergies in India. In the Buffalo in World, World Buffalo Congress, Buenos Aires, Argentina, April, 1024-1031.

Small, C.J., M.L. Goubillon, J.F. Murrau, A. Siddhqui, S. E. Grimshaw, H. Young, V. Sivanesan, T. Kalamatianos, A.R. Kennedy, C.W. Coen, S.R. Bloomand C.A.Wilson (2003) Central orexin A has site-specific effects on luteinizing hormone release in female rats. Endocrinology, 144, 3225-3236.

Smolinska, N., A. Nitkiewicz,A. Maleszka andM. Kiezun (2014) The effect of the estrous cycle on the expression of prepro-orexin gene and protein and the levels of orexin A and B in the porcine pituitary.Animal 8(2), 300-307. DOI: https://doi.org/10.1017/S1751731113002152

Smolinska, N., M. Kiezun, K. Dobrzyn, K. Szeszko, A. Maleszka and T. Kaminski (2015) Expression of the orexin system in the porcine uterus, conceptus and trophoblast during early pregnancy. Animal, 1 -12. Doi:10.1017/S175131115001020

Stocco, C., C. Telleria and G. Gibori (2007) The Molecular Control of Corpus Luteum Formation, Function, and Regression. Endocrine Review, 28(1), 117-149.

Storbeck K. H., P. Swart and A. C. Swart (2007) Cytochrome P450 side-chain cleavage: insights gained from homology modeling. Molecular Cell Endocrinology, 265, 65-70.

Tafuri, S., R. L. Muto, L.M. Pavone, S. Valiante, A. Costagliola, N. Stiano and A. Vittoria (2010) Novel localization of orexin A in the tubular cytotypes of the rat testis. Regulatory Peptides, 164, 53-57.

Taheri, S. and S. Bloom (2001) Orexins / hypocretins: waking up the scientific world. Clinical Endocrinology, 54, 421-429.

Taneja, M., A. Ali and G. Singh (1996) Ovarian follicular dynamics in water buffalo. Theriogenology, 46, 121-130.

Thakre, A., M. Gupta, S.P.Magar, K.B. Bahiram, V.M. Sardar, J.P. Korde, S.W. Bonde and I. Hyder (2021) Transcriptional and translational abundance of visfatin (NAMPT) in buffalo ovary during estrous cycle and its invitro effect on steroidogenesis. Domestic Animal Endocrinology, 75, 106583.

Warriach, H.M. and N. Ahmad (2007) Follicular waves during the oestrous cycle in Nili-Ravi buffaloes undergoing spontaneous and PGF2alpha-induced luteolysis. Animal Reproduction Science, 101 (3-4), 332-337.

APPENDIX

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.

3 Loading dye (6X)

Reagents used for Immunohistochemistry

1 PBS 0.01MM, 7.4 pH

Na_2HPO_4 : $2H_2O$	1.86 g/litter
KH_2PO_4	0.43 g/litter
NaCl	7.20 g/litter

2 1.5% BSA in PBS

3 Citrate Buffer (MW 294.1) 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.

4 3% H_2O_2 in Methanol — Dilute 10 times of 30%
(freshly prepare, protect from light)

VITA

The author Dr. Sardar Vikas Murlidhar was born on 10th March 1976 at Murtijapur, Dist. Akola in Maharashtra State. He passed his SSC examination from Shivaji High School, Akola, in the year 1991. He passed Higher Secondary School examination in the year 1995 from Radhakishan Laxminarayan Toshniwal (RLT) Sciences College, Ranpise-nagar, Akola. He completed his B.V. Sc & A. H. from the College of Veterinary & Animal Sciences, Parbhani the in year 2003 and M.V. Sc. in the year 2005 from Post Graduate Institute of Veterinary and Animal Sciences, Akola. He was engaged with Animal Help Foundation (AHF) as veterinary officer (VO) in project of Municipal Corporation, Ahemedabad, Gujrat from 2005 to 2007. He joined as Assistant professor, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Udgir in the year 2008.

He executed his further studies as in-service candidate during year 2016 to 2021 for the award of Ph.D. degree in Veterinary Physiology at Nagpur Veterinary College, MAFSU, Nagpur. He has rendered his service from 2008 to till date at constituent colleges of MAFSU i.e. COVAS, Udgir-2008-2010, PGIVAS, Akola 2010-2012, KNPVC, Shirval 2012-16, and NVC, Nagpur 2016 to till date, total he has 12 years' experience in teaching as Assistant Professor in MAFSU, Nagpur. He has published 12 research papers in National and International Journals. He attended 10 workshops and 05 conferences/symposiums.

He is registered member of Maharashtra State Veterinary Council (MSVC) and life member of Society of Animal Physiologist of India (SAPI), life member of Animal Physiologists Association (APA), life member of Association of Animal Scientists (AAS) and Life member of Indian Society for Sheep and Goat Production and Utilization (ISSGPU).

THESIS ABSTRACT

a)	Title of the thesis	**“EVALUATION OF OREXIN SYSTEM IN BUFFALO OVARY AND ITS *INVITRO* EFFECT OF STEROIDOGENESIS”**
b)	Full name of student	**SARDAR VIKAS MURLIDHAR**
c)	Name and address of Major Advisor	**Dr. J.P. KORDE** **Associate Professor and Head I/c** **Dept. of Veterinary Physiology,** **Nagpur Veterinary College, Nagpur**
d)	Degree to be awarded	Ph.D.
e)	Year of award of degree	2021
f)	Major subject	Veterinary Physiology
g)	Total number of pages in the thesis	51
h)	Number of words in the abstract	496
i)	Signature of Student	
j)	Signature, Name and Address of forwarding authority	**Associate Dean** **Nagpur Veterinary College,** **Nagpur**

ABSTRACT

The present research was conducted to investigate the expression and localization of orexin and its receptors (OX1R & OX2R) in corpus Luteum (CL) and follicles of buffalo ovary. The *in vitro* role of orexin on steroid production in these luteal and follicular cell was also evaluated. The qPCR, RT-PCR and immunohistochemistry techniques were applied to investigate mRNA expression and localization of orexin and its receptors at four stages early (CL1), mid (CL2), late

(CL3) and regressing (CL4) stage of estrous cycle. β-actin and RPL 15 was used as a control for mRNA expression calculation. It was observed that, orexin and its receptors expression of orexin varied according to developmental stages of CL. Expression of orexin was higher in CL1 and CL4 stage and lower in CL2 and CL3. Immunohistochemistry showed that orexin A and its receptors were localized in cytoplasm of large and small luteal cells with highest immunoreactivity observed in CL1 and CL4. For confirmation of stimulating role of orexin in CL luteal cells from 1^{st}stage, CL were cultured *in vitro* and treated with orexin at 0.1, 1 and 10 ng/mL after obtaining 75-80% confluence. Orexin at a dose 0.1ng/mL showed comparable concentration of P4 with control and at 1.0 and 10ng/mL there was significantly ($P<0.05$) decline in P4 secretion. The mRNA expression of StAR gene was comparable between control and 0.1ng/mL dose however, significantly ($P<0.05$) decreased with increased doses. The expression pattern of CYP11A1 was similar to that of StAR except non significant at dose 1.0 and 10 ng/mL. The mRNA expression of 3β-HSD was significantly ($P<0.05$) declined in orexin treated cells at the dose 0.1 and 1.0 ng/mL and at dose 1.0 and 10ng/mL are comparable. To demonstrate expression and localization of orexin and its receptor in ovarian follicles, follicles were classified into four groups according to size and the concentration of estradiol-19β (E2) in follicular fluid (FF): F1 (small), F2 (medium), F3 (large) and F4 (preovulatory). Theca interna cells (TIC) and granulosa cells (GC) were isolated from each follicle and total RNA were isolated. The level of gene expression of PPO, OX1R and OX2R varied through the follicular phase in both GC and TIC. In GC the mRNA expression of PPO and OX1R was significantly higher in F3 & F4 as compared to F1 & F2. There was no significant variation in OX2R in GC among all stage of follicles. In TIC mRNA expression of PPO was significantly ($P<0.05$) high in F4 and was comparable in F1, F2 & F3 follicles. The expression of OX1R was significantly ($P<0.05$) high in F4 followed by F3 and F2 while comparable in F1 and F2. In TIC the abundance of OX2R transcripts was significantly ($P<0.05$) higher in F4 follicles compared to F1, F2 & F3. Orexin A and its receptor immunoreactivity was observed highest in cytoplasm F3 & F4 follicles. The *in vitro* study showed that

the treatment of orexin alone did not affect the E2 secretion, but orexin with FSH and IGF-I increased E2 secretion in dose dependent manner (dose 0.1, 1.0 and 10ng/mL). The expression of aromatase CYP19A1 from GC treated with basal orexin did not affect but in combination with FSH and IGF-Iincreased E2 secretion significantly ($P<0.05$) with increase dose of orexin. This study provided evidences for role of orexin in luteal and follicular development and steroid production of buffalo.

प्रबंधसारांश

अ.	प्रबंधाचे शिर्षक	:	म्हशींच्या बिजकोषामधिल रचनाबध्द संस्थे मधे ओरेझीन संप्रेरकाचे मुल्यमापन आणि शरिराबाहेरील अंत:पात्र संवर्धनात स्टेरॉईड संप्रेरकाच्या जैवसंश्लेषणा वर होणारा परिणाम
ब.	विद्यार्थ्याचे पुर्ण नांव	:	सरदार विकास मुरलीधर
क.	मार्गदर्शकाचे नांव व पत्ता	:	डॉ. जे.पी. कोरडे सहयोगी प्राध्यापक व विभाग प्रमुख पशु शरीरक्रियाशास्त्र विभाग नागपूर पशुवैद्यक महाविद्यालय नागपूर
ड.	प्रदान करण्यात येणारी पदवी	:	पी. एच. डी.
इ.	पदवी प्रदान करण्याचे वर्ष	:	२०२१
फ.	मुख्य विषय	:	पशु शरीरक्रियाशास्त्र
ग.	प्रबंधातील एकूण पृष्ठे	:	५१
ह.	सारांशातील एकूण शब्द	:	४९८
ई.	विद्यार्थ्याची सही	:	
ज.	अग्रेषित करणाऱ्या अधिकाऱ्याची सही, नांव आणि पत्ता	:	सहयोगी अधिष्ठाता नागपूर पशुवैद्यक महाविद्यालय नागपूर

सारांश

सदर संशोधन म्हशीच्या विस्तारीत झालेल्या बिजकोषामध्ये ओरेझीन आणि त्यांचे घटक रिसेप्टार (OX1R,OX2R) यांचे अभिव्यक्ती आणि स्थान निश्चिती करण्यासाठी तसेच ओरेझीन चे मुल्यमापन ल्यूटीयल आणि बिजकोष पेशीतील स्टेरॉईड संप्रेरकाचा स्त्रावावरच्या प्रभावाचे अध्ययन शरीराबाहेरील संवर्धनात (अंत पात्र संर्वधनात) करण्यात आले आहे. ओरेझीन आणि त्याचे घटक रिसेप्टार (OX1R,OX2R) अभिव्यक्ती, स्थान निश्चिती व प्रमाण निर्धारीत करण्यासाठी कॉर्पस ल्युटीअम चे कार्यात्मक व रचनात्मक विकासानुसार चार टप्यात वर्गीकरण करण्यात आले, जसे

की प्रारंभीक (१–४ दिवस), मध्ये (५–१० दिवस), शेवट (११–१६ दिवस), आणि प्रतीगमीत कॉर्पस ल्यूटीयम (१७ दिवसानंतर) वर्गीकृत केलेल्या कार्पस ल्यूटीयम मधे ओरेझीन आणि त्यांच्या घटक रिसेप्टार अविर्भाव स्थान व प्रभावाचे विश्लेषण करण्यासाठी क्यु आरटी पि सीआर ,आर टी पी सी आर आणि इम्युनोहिस्टोकेमेस्ट्री तंत्रपध्दतीचा वापर करण्यात आला. ओरेझीन एम. आर. एनए. चा अविर्भाव बिटा एक्टीन व आर पि एल–१५ संप्रेरक ठेवून करण्यात आला. संशोधनाच्या परिणामातुन असे आठळले की, म्हैशीच्या कॉर्पस ल्युटीयम मध्ये ओरेझीन आणि त्यांचे रिसेप्टार अभिव्यक्ती अविर्भाव सर्वाधिक प्रारंभिक (१–४ दिवस), आणि प्रतिगमीक (१७ दिवसानंतर) टप्यात आणि सर्वात कमी मध्य (५–१०दिवस) आणि शेवटच्या टप्यात आढळली. ओरेझीन आणि त्याच्या घटक रिसेप्टार हे ल्युटियल पेशींच्या सायटोप्लाझम मध्ये अधिक प्रमाणात असल्याचे इम्युनोहिस्टोकेमेस्ट्री व्दारे निर्दशनास आले व हे प्रामुख्याने प्रारंभिक आणि प्रतिगमिक टप्यात सर्वाधिक आढळले. ओरेझीन संप्रेरकाची खात्री करून घेण्यासाठी अंत पात्र संर्वधनात (शरीरा बाहेरील पात्रात) ल्युटियल प्रारंभीक पेशीची ७५ ते ८० टक्के कृत्रीम वाढ पूर्ण झाल्यानंतर ४८ तासात ओरेझीन ची ०.१, १.० आणि १० नॅनोग्राम प्रती मिली मात्रा दिल्यावर प्रोजेस्ट्रॉनचे प्रमाण कंट्रोल गटाशी ०.१ नॅनोग्राम प्रती मिली मात्रेच्या सारखे आठळले. आणि १.० व १० नॅनोग्रॅम प्रति मिली या मात्रेत वृध्दी दिसुन आली. प्रोजेस्ट्रॉन संप्रेरकाचे प्रमाण सर्व ओरेझीन मात्रा सोबत व एम आर एन ए अभिव्यक्ती माध्यम जसे एस टि ए आर, सि वाय पी ११ ए १ आणि ३ बीटा एच एस डी मधे वेगळे आढळले.एस टि ए आर, जनूके हे एम आर एन ए अभिव्यक्ती मधे एस टि ए आर जनूके हे तूलनात्मक ०.१ नॅनोग्राम प्रती मिली मात्रे येवढा आठळला, शिवाय पी ११ ए १ जिन हा एस टि ए आर सारखा असुन ओरेझीन मात्रेच्या प्रमाणात कमी होतो. तसेच बीटा एच एस डी हा ०.१ व १.० नॅनोग्राम प्रतीमिली मधे कमी होत असून १.० व १० नॅनोग्रॅम प्रतिमिली मात्रे मधे तूलनात्मक आढळला. संशोधनाच्या दूसऱ्या टप्यात ओरेझीन आणि त्यांचे रिसेप्टार यांची अभिव्यक्ती व स्थान निश्चिती बिजकोशातील वर्गीकृत चार गटात विभागण्यात आली असून बिजकोशाचे वर्गीकरण एफ १ लहान, एफ २ मध्यम, एफ ३ मोठा आणि एफ ४ पूर्व अंडमोचन करण्यात आले. प्रत्येक बिजकोशातून थिका ईटरना व ग्रानूलोसा पेशीचे एकत्रीत आर एन ए काढण्यात आले. यांचे बिजकोशातील ग्रानूलोसा आणि थिकइटरना मधे ओरेझीन (पीपीओ) आणि त्यांचे रिसेप्टार (OX1R,OX2R)यांची पातळी बिजकोशाच्या टप्या नूसार आढळली. ग्रानूलोसा पेशीतील ओरेझीन (पीपीओ) आणि OX1R, यांची एम आर एन ए अभिव्यक्ती सर्वाधिक मोढया एफ ३ आणि एफ ४ पूर्व अंडमोचन बिजकोषात आढळते आणि OX2R मधे सर्व बिजकोशात सारखे पणा आढळतो. थिका इंटरना पेशीत ओरेझीन (पीपीओ) यांची एम आर एन ए अभिव्यक्ती सर्वाधीक एफ ४ मध्ये असून एफ १, एफ २, आणि एफ ३ हि तूलनात्मक आढळली. ओरेझीन रिसेप्टार १ ची एम आर एन ए अभिव्यक्ती सर्वाधीक एफ ४ मधे असुन एफ ३ आणि एफ २

खालोखाल आढळली. तसेच OX2R मध्ये सर्वाधीक एफ ४ बिजकोषात असून एफ १, एफ २, आणि एफ ३ मध्ये भिन्नता आढळून आली नाही. ओरॉझीन अ आणि त्यांचे रिसेप्टार हे ग्रानूलोसा आणि थिका पेशी ची स्थान नीश्चिती पेशीच्या जीव द्रव्यात (सायटोप्लाझम) दिसत असून ती सर्वाधिक (मोढया) एफ ३ आणि (पूर्व अंडमोचन) एफ ४ बिजकोषात आढळली. ग्रानूलोसा पेशीच्या अंत: पात्र संवर्धनात फक्त ओरेझीन असलेल्यात इस्ट्रोडिऑन च्या स्त्रावात वाढ सारखी दिसून येते. परंतू ओरेझीन सोबत एफ एस एच आणि आ जि एफ १ हया मध्ये इस्ट्रोडिऑल चे प्रमाण वाढत असून ते ओरेझीन च्या मात्रा च्या नूसार वाढतो. ग्रानूलोसा पेशी मध्ये अंत:पात्र संवर्धनात ओरेझीन इस्ट्रोडिऑल चा स्त्राव सि वाय पी १९ ए १ च्या अभिव्यक्तीत सूध्दा ओरेझीन च्या मात्रे च्या नूसार वाढतो. सदर संशोधन म्हशीच्या बिजकोषातील ल्यूटीयल आणि बिजांडयाची वाढ, त्यांचे कार्य आणि स्टेरॉईड संप्रेरके उत्पादन वाढवीण्यात ओरेझीन ची भुमीका असल्याचा पुरावा देते