

Original Research Article

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Expression Profile of Orexin System in Corpus Luteum of Buffalo during Estrous Cycle

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ABSTRACT

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Orexin A and B are neuropeptides synthesized mainly in the lateral hypothalamus and are associated with a variety of physiological functions such as energy homeostasis, sleep and wakefulness, feeding behavior, as well as the reproductive system. The biological actions of the hormones are mediated via two distinct G protein-coupled receptors, termed orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R). The aim of the current study was to investigate the gene expression of orexin and its receptors OX1R and OX2R in different stages of corpus luteum (CL) during estrous cycle in (*Bubalus bubalis*). The result showed that transcript of pre-pro-orexin (PPO), OX1R and OX2R was present in buffalo CL throughout the estrous cycle however the level of transcript varied through different stages of CL. The abundance of transcript of orexin and its receptor was high higher $P < 0.05$ in early (CL1, days 1-4) and regressing stage (CL4, days >17) of CL whereas the expression was significantly lower ($P < 0.05$) in mid (CL2, days 5-10) and late luteal (CL3, days 11-16) stage during estrous cycle. Presence of both ligand (orexin) and receptors (OX1R) and (OX2R) in CL of buffalo ovary suggest the autocrine and paracrine role of orexin in corpus luteum functions and indicate the role of orexin in regulation of reproductive functions in water buffalo.

Introduction

Reproductive cycle in mammals is characterized by sequential proliferation, differentiation and conversion of ovarian follicular cells followed by development and decay of the CL in a cyclic manner. The corpus luteum (CL) is a transitorily functioning organ and plays a key role in the regulation of the estrous cycle and in the safeguarding of pregnancy which is chiefly

performed by progesterone, the main steroid hormone synthesized by CL (Stocco *et al.*, 2007) The development, maintenances, lysis, of CL and steroid production are important events in mammalian reproduction. The CL undergoes regression after maturation and disappeared from the ovary and allowing the initiation of a new cycle. The role of pituitary gonadotropins in the process of luteal function is well recognized. However, recent studies also showed evidences of modulatory

role by locally produced factors such as steroid hormones, peptides and growth factors in the CL development, function and regression by autocrine and paracrine manner (Berisha and Schams, 2005).

Orexins are neuropeptides with pleiotropic functions, involved multiple physiological processes, particularly related to food intake and several aspects of the reproductive cycle (Ragionieri *et al.*, 2018). Orexins are also called hypocretins and they are synthesized by neurons located mainly in the lateral hypothalamus (Taheri and Bloom 2001). Orexin A and orexin B are two forms of orexins produced by cleavage of a single precursor protein named pre-pro-orexin (PPO) and discovered simultaneously by two independent laboratories (Sakurai *et al.*, 1998; de Lecea *et al.*, 1998). Orexin A contains 33 amino acid residuals however, orexin B contains 28 amino acid residuals peptide.

Orexins exerts the biological action by binding to two closely related G protein coupled receptors: orexin receptor type 1 (OX1R) and orexin receptor type 2 (OX2R). OX1R is having high affinity to orexin A and OX2R binds both orexins with same affinity (Sakurai *et al.*, 1998; de Lecea *et al.*, 1998). Orexin A and orexin B are widely distributed and associated with functions like regulation of blood pressure (Shirasaka *et al.*, 1999), sleep wake cycle, regulation of hypothalamo-pituitary adrenal axis (Spinazzi *et al.*, 2006) and hypothalamo-pituitary-gonadal axis (Porkka-Heiskanen *et al.*, 2004).

Apart from the other organs orexin, OX1R and OX2R has been detected in hypothalamus and ovary in porcine (Ning *et al.*, 2010; Ciccimarra *et al.*, 2017; Kaminski *et al.*, 2018), ovary of canine and feline (Levanti *et al.*, 2015) and rat ovary (Dobrzyn *et al.*, 2018). Expression of orexin has also been documented in placenta and uterus

(Smolinska *et al.*, 2015). Evidences are also existing regarding the role of orexin on steroid secretion from granulosa cells, theca interna cells and luteal cells (Nitkiewicz *et al.*, 2014). Above works suggested the role of orexin in regulation of reproduction at central and peripheral level; however the reports regarding bovine and bubaline species are lacking. Hence the Hence, the present study was carried out to explore the expression of orexin and its receptors in corpus luteum (CL) of buffalo ovary during estrous cycle.

Materials and Methods

Collection of ovaries and separation of CL

The reproductive tracts of water buffalo (*Bubalus bubalis*) were collected from a local slaughterhouse where ethical issues related to slaughter are governmentally regulated and the samples were transported on ice within 10 to 20 min after slaughter to the laboratory. The stage of the estrous cycle was classified based on macroscopic observations of the ovaries (color, consistency, CL stage, number, and size of follicles) and the uterus (color, consistency, and mucus) as described previously (Gupta *et al.*, 2014). Ovaries (n = 40), each with the CL, were used to extract 10 CL per group for RNA studies. The CLs from each ovary were classified as being in the following stages of functionality: CL1, early luteal phase (days 1–4) CL2, mid-luteal phase (days 5–10); CL3, late luteal phase (days 11–16) and CL4, regressing CL (days >17) of the estrous cycle. Luteal tissue (n=10 per classification stage/group) was frozen in liquid nitrogen and stored at -80°C until RNA isolation.

RNA isolation

Isolation of total RNA was carried out CL of buffalo using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's

instruction. DNase I treatment was given to isolated RNA to get rid of any apparent DNA contamination. RT-PCR was used to detect pre-pro-orexin (PPO), orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R) in CL of buffalo RNA integrity was checked by running it on 1.5% agarose gel using $1\times$ TAE as electrophoresis buffer and gels were stained with ethidium bromide. The yield of RNA in all the samples was as per expectation. The purity and concentration of total RNA was assessed using nanodrop (Eppendorf, Germany). The isolated RNA samples were free from protein contamination as the OD 260: OD 280 values were greater than 1.8. One μg of total RNA was reverse transcribed in 20 μL of final volume of reaction containing 4 μL 5X reaction buffer, 3 μL MgCl_2 , 1 μL PCR nucleotide mix, 1 μL RNase inhibitor, 0.5 μL reverse transcriptase, 1.5 μL oligo- (dt 15) primer, 9 μL RNA template (1 μg) + nuclease-free water followed by incubation for 15 min at 50°C and 2 min 30 sec at 42°C and with storage at 4°C. The cDNA was stored at -20°C for long term use.

Primers

The RT-qPCR procedures were used to detect PPO, OX1R and OX2R in the CL of buffalo. Primers of PPO, OX1R and OX2R, were designed using the Fast PCR (Version:6.2.73) software. Published primers were used for beta-actin (ACTB) and ribosomal protein 15 (RPL15) (Gupta *et al.*, 2019). Details of the primers used, primer efficiencies and annealing temperature are provided in Table 1.

Quantitative RT-PCR (RT-qPCR) analysis

The quantification of targeted cDNAs was done by real-time PCR using GoTaq® qPCR master mix (Promega, USA) and specific primers in a total volume 15 μL reaction

containing 7.5 μL GoTaq® qPCR master mix, 0.5 μL forward primer (0.5 mM), 0.5 μL reverse primer (0.5 mM), 1.0 μL cDNA template at a 50 ng/ μL concentration, 5.5 μL nuclease-free water. PCR was performed with specific primer pairs of PPO, OX1R, OX2R, ACTB, and RPL15. The samples were initially denatured at 95°C for 2 min, then 40 PCR cycles were processed denaturation at 95°C for 20 sec, annealing at 60°C for PPO, OXR1, OXR2, and RPL15, 62°C for ACTB for 25 sec, extension at 72°C for 30 sec, with a final extension at 72°C for 15 sec and final hold at 4°C. The PCR products were run on 1.5% agarose gel stained with ethidium bromide. Optical data were collected at the end of each extension step and relative abundance of the PCR product was determined using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001). The tissue with the least abundance (greatest Cq) was utilized as calibrator. The geometric mean of Cq of ACTB and RPL15 was utilized as a reference/internal control.

Statistical analysis

All experimental data are shown as the mean \pm SEM. The differences in relative expression of mRNA transcripts for PPO, OX1R and OX2R in CL during different stages of the estrous cycle were assessed using the software SPSS.17 by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test. The differences were considered to have occurred if $P < 0.05$.

Results and Discussion

The Relative expression of qPCR product was determined by the $2^{-\Delta\Delta\text{CT}}$ method. The tissue with lowest expression (highest Ct) was taken as calibrator. The geometric mean of Cq of ACTB and RPL15 was taken as reference/internal control. The specific amplified PCR

products for PPO, OX1R and OX2R is presented in figures 1A, 2A and 3A respectively. The PCR product was 179 bp for PPO, 144 bp for OX1R, 77 bp for OX2R, 100 bp for ACTB and 140 bp for RPL15. The relative mRNA expression of PPO, OX1R and OX2R is presented in figures 1B, 2B and 3B respectively. The expression of PPO and OX2R was significantly higher ($P < 0.05$)

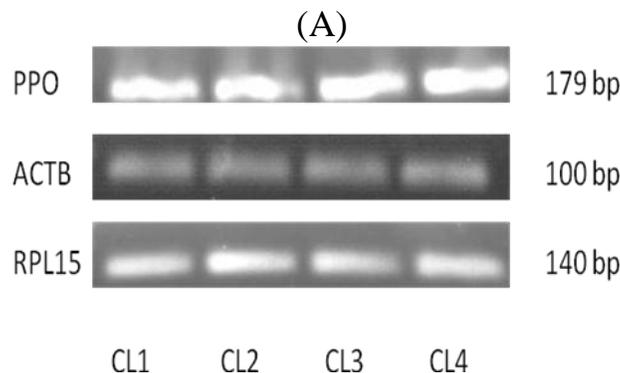
in early (CL1) and regressing (CL4) stage as compared to mid (CL2) and late (CL3) luteal stage. The expression between CL1 and CL4 as well as CL2 and CL3 was comparable. The expression of OX1R was lower in late luteal phase as compared to early and regressing CL. The mRNA abundance of OX1R was comparable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

Table.1 Gene transcript, primer sequence (5'-3') and resulting fragment size

Target Gene	Primer sequence 5'-3'	Amplicon length (bp)	Efficiency (%)	Accession No. / Reference
PPO	For: ATC TCT CCC CCT TTC CCG TC Rev: AGGAGACCTTTGTAGAGG AAG GAT	179	98.2	XM_006055754.1
OX1R	For: TCC CAG AGC CAA CAG AAG GT Rev: CAC TAA GGC TGA CGG GCA T	144	103.4	XM_006054498.1
OX2R	For:CCGTGGCCGCTGAAATAAAG Rev:AAACACCAAGAGCACCACCA	77	96.4	XM_019985477.1
ACTB	For: TCT CAC GGA GCG TGG CTA CAG Rev:CTGCTCGAA GTCCAG GGC CACGTA	100	96.8	Gupta <i>et al.</i> , 2019
RPL 15	For: TGGGCTACA AGGCCA AACAA Rev: GCT TCGAGCAAACCTTGAGCTGG	140	105.6	Gupta <i>et al.</i> , 2019

Abbreviations: PPO, Prepro-orexin; OX1R, Orexin receptor 1; OX2R, Orexin receptor 2; ACTB, β -Actin and RPL 15, Ribosomal Protein L 15

Fig.1 (A) Representative images of amplification of *pre-pro-orexin (PPO)* (179 bp), *ACTB* (100 bp) and *RPL15* (140 bp) genes by qPCR at different stages of CL development in the water buffalo. (B) Relative mRNA expression profile of *pre-pro-orexin (PPO)* in different stages of CL development during estrous cycle in water buffalo (n=10/group); (early CL, days 1–4; mid CL, days 5–10; late CL, days 11–16; regressing CL, days >17 of estrous cycle); Values are expressed as means \pm SEM; Different superscripts denote statistically different values ($P < 0.05$)



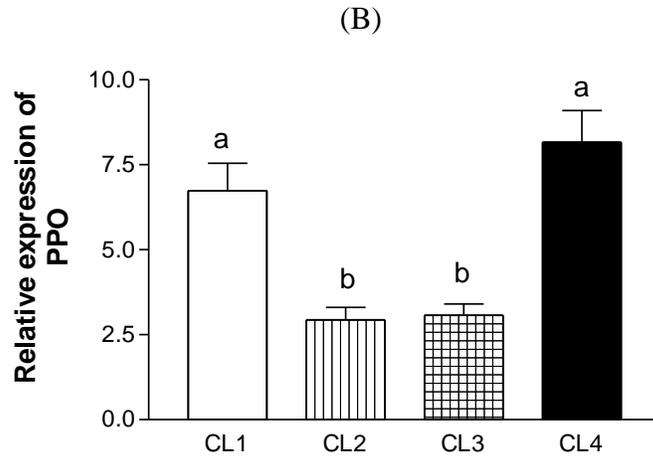


Fig.2 (A) Representative images of amplification of *orexin receptor 1* (*OX1R*) (144 bp), *ACTB* (100 bp) and *RPL15* (140 bp) genes by qPCR at different stages of CL development in the water buffalo. (B) Relative mRNA expression profile of *OX1R* in different stages of CL development during estrous cycle in water buffalo (n=10/group); (early CL, days 1–4; mid CL, days 5–10; late CL, days 11–16; regressing CL, days >17 of estrous cycle); Values are expressed as means \pm SEM; Different superscripts denote statistically different values ($P < 0.05$)

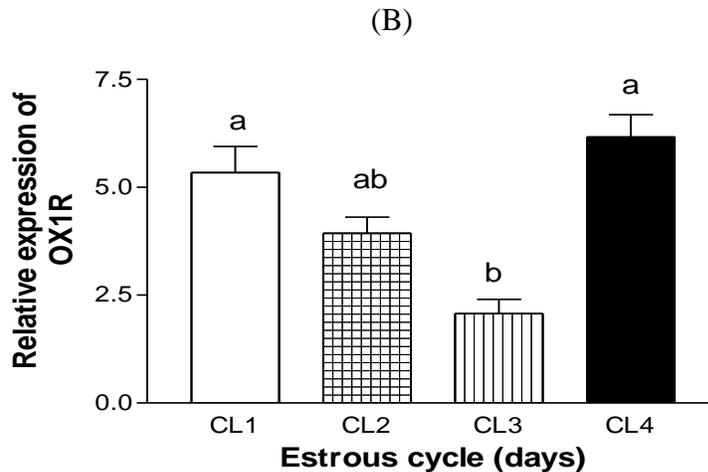
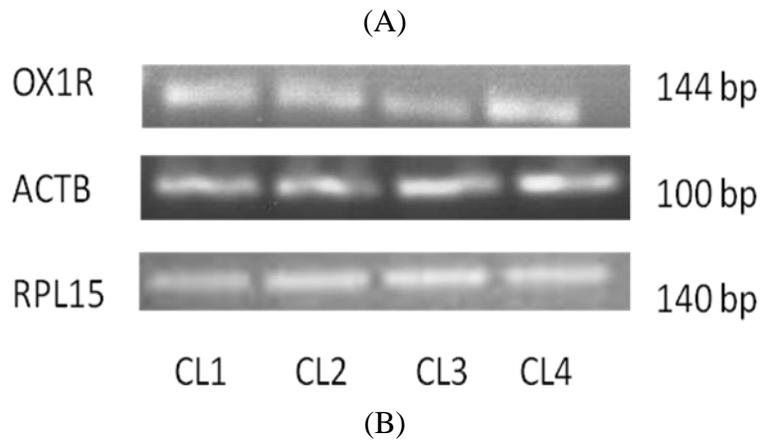
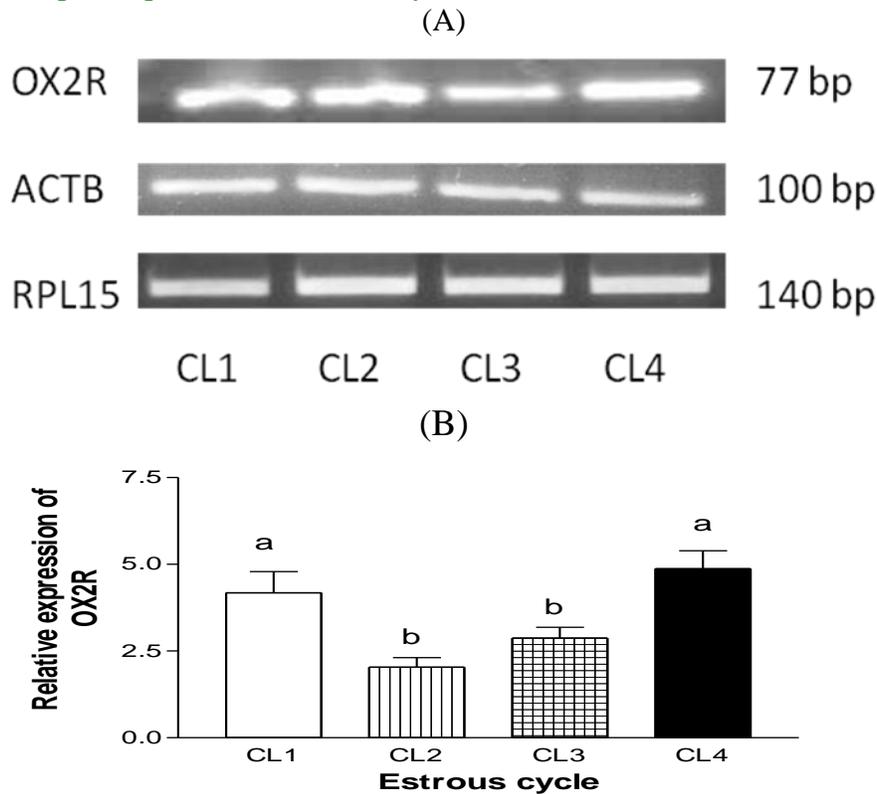


Fig.3 (A) Representative images of amplification of *orexin receptor 2 (OX2R)* (77 bp), *ACTB* (100 bp) and *RPL15* (140 bp) genes by qPCR at different stages of CL development in the water buffalo. (B) Relative mRNA expression profile of *OX2R* in different stages of CL development during estrous cycle in water buffalo (n=10/group); (early CL, days 1–4; mid CL, days 5–10; late CL, days 11–16; regressing CL, days >17 of estrous cycle); Values are expressed as means \pm SEM; Different superscripts denote statistically different values ($P < 0.05$)



The present study, for the first time established the presence of orexin and its receptors in buffalo corpora lutea during estrous cycle. Although expression and immunolocalization of the orexin system and in-vitro effects of orexin in ovarian cells been studied in other species however the reports regarding expression of orexin system in ovarian cells of buffalo was lacking. The present study revealed orexin and its receptor are expressed in buffalo corpus luteum throughout the estrous cycle and the mRNA expression was consistent throughout the estrous cycle. The abundance of transcript of orexin and its receptors was high in early and regressing stage of corpora lutea of buffalo ovary.

The results of the current study are backed by earlier studies in which orexin system has been detected in ovarian cells of pig (Nitkiewicz *et al.*, 2010, Kaminski *et al.*, 2010, Nitkiewicz *et al.*, 2014; Basini *et al.*, 2018), rat (Silveyra *et al.*, 2007, Cataldi *et al.*, 2012) dogs and cat (Levanti *et al.*, 2015). In present study higher expression of orexin and its receptor is observed in corpora hemorrhagica and regressing corpora lutea. The result of present are in agreement with observations of Nitkiewicz *et al.*, (2014) in pig in which it was found that the quantity of transcripts of orexin, OX1R and OX2R was highest in early and regressing stage of CL. However the expression of OXR1 was reported higher early (days 2-3) and mid

(days 10-12) luteal stage (Nitkiewicz *et al.*, 2010) which is in contrast to our findings. These differences are probably due to species variations. Similarly, Silveyra *et al.*, (2007) observed that the abundance of transcript of both orexin receptors in rat ovaries changes during the cycle with an increase in the proestrous phase and suggested that gonadotrophins are involved in the control of orexin system expression. It has also been documented that orexin at both central and peripheral level modulate the steroid synthesis and secretion. Orexin increased estradiol production from granulosa cells of pigs however no effect on progesterone production was observed (Ciccimarra *et al.*, 2017). Orexin at 10 nM dose suppressed FSH-induced oestradiol secretion by granulosa cells in pigs (Nitkiewicz *et al.*, 2014). Orexin expression has also been detected at higher level i.e. in hypothalamus (Silveyra *et al.*, 2007) and pituitary (Smolinska *et al.*, 2014). Studies have also confirmed the regulation of Hypothalamic-pituitary-gonadal axis by regulating GnRH, FSH and LH by orexin (Small *et al.*, 2003; Barb and Matteri, 2005). All these earlier observation and the results of the current study indicate the regulatory role of orexin in ovarian functions and reproductive cycled in buffalo.

In conclusion, results of the present study indicate that the dynamics of orexin abundance is coexistent with the stage-specific functional variations in buffalo CL during the estrous cycle. The results showed the presence of orexin and its receptors CL during the estrous cycle. Still, for precise conclusions to be drawn, particularly concerning the intracellular mechanism of visfatin influence on gonadal steroidogenesis and its possible effects on ovaries, further *in-vitro* and *in-vivo* studies are required to elucidate the role of orexin in ovarian physiology.

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