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Abundance of adiponectin mRNA transcript in the buffalo corpus luteum during the estrous cycle and effects on progesterone secretion *in vitro*

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ABSTRACT

Adiponectin is an adipocyte derived cytokine implicated in energy homeostasis, insulin resistance and is involved in the regulation of reproduction both centrally and peripherally in animals. The present study was conducted to investigate *adiponectin* (ADIPOQ) and its receptors ADIPOR1 and ADIPOR2 abundance of mRNA transcript and protein in different stages of corpora lutea (CL) development during the estrous cycle of water buffalo and to determine the effect of adiponectin on cultured luteal cells of water buffalo (*Bubalus bubalis*). The results indicate adiponectin, ADIPOR1, and ADIPOR2 were present in buffalo corpora lutea (CL) throughout the estrous cycle. The abundance of *adiponectin* and its receptors was greater in the early and regressing and was less in mid- and late-stages of CL functionality. Adiponectin and its receptors were localized in the cytoplasm of small and large luteal cells. Furthermore, luteal cells were cultured in the *in-vitro* culture system and were treated with 1 and 10 μg/mL dose of adiponectin for 48 h. Adiponectin at both doses decreased ($P < 0.05$) progesterone (P_4) secretion from cultured luteal cells and also suppressed the abundance of factors involved in P_4productionv [Steroidogenic Acute Regulatory Protein (*STAR*), cytochrome P45011A1 (*CYP11A1*) and 3β-hydroxysteroid dehydrogenase (*HSD3B1*) at the 10 μg/mL dose as compared to adiponectin non-supplemented cells]. In conclusion, results of the present study indicate adiponectin and its receptors are present in bubaline CL and adiponectin inhibits P_4 production in cultured luteal cells. The findings indicate adiponectin affects luteal dynamics and reproductive functions in water buffalo.

1. Introduction

Adipose tissue functions as an endocrine organ with the production of various factors including adipokines that regulate adipocyte differentiation, energy metabolism, insulin resistance, inflammation, immunity, cancer, angiogenesis and also control fertility and reproduction (Badman and Flier, 2007; Dupont et al., 2012; Reverchon et al., 2014). Adiponectin is an adipokine that is primarily produced by mature adipocytes and is abundantly present in blood (2 to 20 μg/mL) of mammals (Maeda et al., 1996; Arita et al.,

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2012). Adiponectin is a 30 kDa 244 amino acid glycoprotein present in plasma, alsoknown as ACRP30, ADIPOQ, and GBP28 (Kadowaki and Yamauchi, 2005). The secretion of adiponectin is inversely related to amount of adipose tissue and the circulating concentration of adiponectin is two to three times greater in females as compared to males (Campos et al., 2008; Oliveira et al., 2017). The function of adiponectin is mediated by binding to two specific receptors, Adiponectin receptors 1 and 2 (ADIPOR1 and ADIPOR2) (Yamauchi et al., 2003). These receptors belong to a separate family of receptors, the progestin and adipoQ receptors (PAQR) (Tang et al., 2005). The receptors contain seven transmembrane domains, however, are structurally and functionally different from G-protein coupled receptors (Kadowaki and Yamauchi, 2005).

A growing body of evidence in recent years indicates several hormones such as leptin, ghrelin, orexin, insulin which regulate body metabolism also modulate reproductive functions (Sarkar et al., 2010; Kumar et al., 2012; Gupta et al., 2014, 2015; Ciccimarra, et al., 2018). It is believed that adiponectin is also an important regulator of metabolism and, therefore, it also regulates reproductive functions. Adiponectin modulates reproductive processes at both the central hypothalamus-pituitary axis (Wena et al., 2008; Rodriguez-Pacheco et al., 2008) and directly in peripheral tissues such as the ovary, uterus, oocyte/embryo (for review see Dobrzyn et al., 2018). Adiponectin and its receptors have been identified in different organs of the female reproductive system in various species. Gene transcript and protein of adiponectin, ADIPOR1 and ADIPOR2 have been reported to be present in ovarian cells of rats (Chabrolle et al., 2007a), chickens (Chabrolle et al., 2007b), humans (Chabrolle et al., 2009), cattle (Tabandeh et al., 2010), pigs (Maleszka et al., 2014a; 2014b) and goats (Oliveira et al., 2017). Adiponectin and its receptors are also present in the oviduct (Archanco et al., 2007), uterus (Smolinska et al., 2014) and placenta (Caminos et al., 2005). All these findings indicate adiponectin has a direct action on the reproductive axis in different species.

Results of studies indicate adiponectin modulates the regulation of steroidogenesis in ovarian cells (Lagaly et al., 2008; Maillard et al., 2010). Results of some more recent studies indicate adiponectin has a contrasting effect on ovarian steroidogenesis. In the ovary of pigs, adiponectin increases LH and insulin-induced estradiol (E_2) secretion from theca interna cells whereas it decreases P_4 production in luteal cells and LH and insulin-induced P_4 production in theca interna cells (Maleszka et al., 2014a). Similarly, adiponectin decreased insulin-induced P_4 and androstenedione production in theca cells of cattle (Lagaly et al., 2008). Inconsistent with these findings, adiponectin increases IGF-I and insulin-induced E_2 and P_4 production in granulosa cells of cattle (Mailard et al., 2010). In poultry, adiponectin increases IGF-1-induced P_4 secretion in second and third/fourth largest ovarian yellow follicles and decreases P_4 production in response to gonadotropins (LH and FSH) in the third/fourth largest ovarian follicles (Chabrolle et al., 2007b). In rat granulosa cells, treatment with adiponectin resulted in increased P_4 secretion and E_2 production when there was treatment with insulin-like growth factor-I (IGF-I; Chabrolle et al., 2007a).

In the bubaline species, there are few studies of the adiponectin system and its effect on ovarian steroidogenesis. In the present study, therefore, it was hypothesized, based on previous research with other species, that adiponectin and its receptor modulated luteal dynamics in water buffalo. The present study, therefore, was conducted to investigate mRNA and protein adiponectin abundances in buffalo corpora lutea and to study the *in-vitro* effect of recombinant adiponectin on P_4 secretion in luteal cells of water buffalo.

2. Materials and methods

2.1. Reagents

All reagents used in this study were obtained from Sigma-Aldrich (USA) unless otherwise specified.

2.2. Ethical clearance

As the samples were obtained from the slaughterhouse, the permission of the local ethical committee was not required. The study did not involve the handling of animals.

2.3. Collection of CL during estrous cycle

The reproductive tracts of water buffalo (*Bubalus bubalis*) were collected from a registered and licensed government local abattoir 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 (Chouhan et al., 2013). Ovaries ($n = 40$), each with the CL, were used to extract 10 CL per group for RNA studies. The CL 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.

2.4. RNA isolation and RT-PCR

2.4.1. RNA isolation

Total RNA was extracted from CL of buffalo by using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instruction. The RNA was treated with DNase 1 (Sigma-Aldrich, USA) to remove any possible DNA contamination. The RT-PCR was

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

Gene	Sequences of nucleotide (5′–3′)	Amplicon length (bp)	Efficiency (%)	Annealing temperature (°C)	EMBL accession no. or reference
Adiponectin (ADIPOQ)	For: TCTTCACCTACGACCAGTATCAG Rev: ACATTATCTGCATAGACCCCATTG	139	96.4	58	MG868932
ADIPOR1	For: TGCTCGGTTTCGTGCTGTT Rev: CGATCCCTGAATAGTCCAGCTTGG	213	102.4	60	MG868933
ADIPOR2	For: ATACACACCGAGACAGGCAA Rev: GACTGTGTGGAACAGCCATGAG	189	95.7	60	MG969349
STAR	For: CTGCGTGGATTAACCAGGTTCG Rev: CCAGCTCTTGGTCGCTGTAGAG	84	97.3	60	Gupta et al., 2014
CYP11A1	For: AGTTCGAGGGATCCTACCCAGA Rev: AGCCATCACCTCCGTGTTCAG	146	104.4	60	Gupta et al., 2014
HSD3B1	For: GATCATCTGCCTGTTGGTGGA Rev: GTGGATGACCACTGAGGTGC	191	98.6	60	Gupta et al., 2014
ACTB	For: TCTCACGGAGCGTGGCTACAG Rev: CTGCTCGAAGTCCAGGGCCACGTA	100	96.8	62	Gupta et al., 2014
RPL15	For: TGGGCTACAAGGCCAAACAA Rev: GCTTCGAGCAAACTTGAGCTGG	140	105.6	60	MG969348

Abbreviations: ADIPOQ, adiponectin; ADIPOR1, adiponectin receptor 1; ADIPOR2, adiponectin receptor 2; HSD3B1, 3beta hydroxysteroid dehydrogenase; CYP11A1, cytochrome P450 family 11 subfamily A member 1; STAR, steroidogenic acute regulatory protein ACTB, beta actin; RPL15, ribosomal protein 15; EMBL, European molecular biology laboratory.

used to detect *adiponectin (ADIPOQ)*, *ADIPOR1* and *ADIPOR2* in CL of buffalo and factors involved in P_4 production (Steroidogenic Acute Regulatory Protein (*STAR*), cytochrome P450 11A1 (*CYP11A1*) and 3β-hydroxysteroid dehydrogenase (*HSD3B1*). The integrity of total RNA was assessed using a 1.5% agarose gel using 1 × TAE as electrophoresis buffer and gels were stained with ethidium bromide. There was an expected yield of total RNA in all the samples. The purity and concentration of total RNA were assessed using nanodrop procedures (Eppendorf, Germany). The isolated RNA samples were free from protein contamination because the OD 260: OD 280 values were greater than 1.8.Total RNA (1 μg) was reverse transcribed in a 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 s at 42 °C and with storage at 4 °C.The cDNA was stored at −20 °C for long term use.

2.4.2. Primers

The RT-qPCR procedures were used to detect *adiponectin* (*ADIPOQ), ADIPOR1* and *ADIPOR2* in the CL of buffalo and factors involved in P_4 synthesis [Steroidogenic Acute Regulatory Protein (*STAR*), cytochrome P450 11A1 (*CYP11A1*) and 3β-hydroxysteroid dehydrogenase (HSD3B1)]. Primers of *adiponectin (ADIPOQ)*, *ADIPOR1*, *ADIPOR2,* β-actin (*ACTB*) and ribosomal protein 15 (*RPL15*) were designed using the Fast PCR (Version: 6.2.73) software. Published primers were used for, STAR, *HSD3B1* and CYP11A1 (Gupta et al., 2014). Details of the primers used, primer efficiencies and annealing temperature are provided in Table 1.

2.4.3. Quantitative RT-PCR (RT-qPCR) analysis

Targeted cDNAs were quantified using real-time GoTaq® qPCR master mix procedures (Promega, USA) and specific primers in a total volume 15 μL reaction containing 7.5 μLGoTaq® 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. The PCR was performed with specific primer pairs of *adiponectin (ADIPOQ)*, *ADIPOR1*, *ADIPOR2*, *STAR*, *CYP11A1*, *HSD3B1*, *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 s, annealing at 58 °C for adiponectin, 60 °C for*ADIPOR1*, *ADIPOR2*, *STAR*, *HSD3B1*, *CYP11A1* and *RPL15,* 62 °Cfor ACTB for 25 s, extension at 72 °C for30 s, with a final extension at 72 °C for 15 s and final hold at 4 °C. The PCR products were assessed using a 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 $2^{-\Delta\Delta CT}$ 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.

2.5. Western blot

Total protein was obtained by triturating luteal tissues of diff ;erent stages (n = 10/group) in liquid nitrogen and the triturated tissue was suspended in RIPA lysis buff ;er (Himedia laboratories, India) and protease inhibitor cocktail (Sigma-Aldrich, USA), homogenized and centrifuged at 12,000xg. Protein samples from10 CL per group were pooled and the experiment was replicated thrice for each protein. The protein concentration in the supernatant which contains mostly the total soluble protein was estimated using the Bradford protein assay. Furthermore, the supernatant was diluted in sodium dodecyl sulfate (SDS) sample buff ;er (2X laemmli buffer), followed by boiling for 5 min. The protein samples (80 μg from each group of CL) were subjected to 12% PAGE,

electro-transferred onto polyvinylidene difluoride (PVDF) membrane (0.45μ pore size) and blocked with 3% bovine serum albumin (BSA) before incubation with primary antibodies [rabbit monoclonal anti-adiponectin (Cell Signaling Technology, USA, Cat. No. C45B10), rabbit polyclonal anti-ADIPOR1 (Sigma-Aldrich, USA, Cat. No. SAB1307251) and rabbit polyclonal anti-ADIPOR2 (Sigma-Aldrich, USA, Cat. No. SAB1411435) at a 1:800 dilution, and rabbit monoclonal anti-β-actin (Cell Signaling Technology, Cat. No. 4970) at a 1:1000 dilution] overnight at 4 °C. After incubation, the membrane was washed thrice with TBS-Tplus 0.01% Tween 20 for 5 min each then anti-rabbit secondary antibody conjugated with horseradish peroxidase (Sigma-Aldrich, USA, Cat. No. A0545) was added at a 1:10,000 dilution and incubated at 37 °C for 1 h. After washing three to four times in TBS-Tween 20 solution, the positive signals were detected by incubating the membrane in 3,3′-diaminobenzidine tetrahydrochloride (DAB, Roche, Sigma-Aldrich, USA) diluted in peroxide buffer for 10–15 min. The bands were visualized using white light and recorded using a gel documentation system. Densitometric analysis was conducted using image-J software.

2.6. Immunohistochemistry

The CL at different stages of functionality were separated from ovary and were fixed with 10% neutral buffer formalin (NBF), dehydrated through a series of graded alcohols, paraffin-embedded, serial sectioned (5 μm), mounted on 2% APES (Sigma-Aldrich, USA, Cat. No. A3648) coated slides and dried at 37 °C overnight. Five representative CL for each group were used for immunohistochemistry studies. Deparaffinization was conducted in xylene, followed by rehydration in a series of graded alcohols at room temperature, subjected to antigen retrieval in sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% Tween-20) in a microwave at 160 to 320 W for 20 min and then allowed to cool at room temperature. After washing twice in 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, the nonspecific background was eliminated by blocking using 3% BSA (dissolved in 1xPBS) for 2 h at 37 °C. Subsequently, sections were probed with rabbit anti adiponectin (Sigma-Aldrich, USA, Cat. No. SAB2108566,), anti-ADIPOR1 (Sigma-Aldrich, USA, Cat. No. SAB1307251) and rabbit anti-ADIPOR2 (Cloud-Clone Corp. USA, Cat No. PAA132Hu01) antibodies at 1:100 dilutions. Sections were washed twice for 5 min in PBS and were incubated for 1 to 1.5 h at 37 °C using Goat HRP conjugated secondary antibody (Sigma-Aldrich, Cat. No. A0545). The slides were washed twice in PBS and staining was assessed using an AEC staining kit according to the manufacturer's instructions (Sigma-Aldrich, Cat. No. AEC 101). The control slides were processed under similar conditions except for the omission of the primary antibody. Furthermore, sections were washed with distilled water and observed using a microscope (Axio Lab A1 from Zeiss). Images were obtained using the installed camera of Axio Cam ERC 5C.

2.7. Cell culture and estimation of P4 concentration

2.7.1. Luteal cell culture

To evaluate the effect of the adiponectin on luteal function, a luteal cell culture model was developed using cells isolated from fresh CL. Luteal cells were cultured as described previously (Chouhan et al., 2014). Ovaries were collected from a local abattoir and transported back to the laboratory in 1XPBS at 37 °C. In all experiments, stage IICL (days 5–10) were used and these were selected based on criteria previously described (Gupta et al., 2014). The CL were removed from the ovary with all connective tissue and blood (whenever present) trimmed away and then minced using BP blades. The minced luteal tissue was washed with culture medium by centrifuging three times for 5 min at 12,000 × *g*. After centrifugation, the pellet containing various cells (including luteal, endothelial, pericytes and fibroblasts) was dispersed by incubating the luteal tissue in DMEM/F12 medium (Himedia, India) containing 2 mg/mL collagenase I type 1A, 25 μg/mL DNase I (Sigma-Aldrich, USA) and 0.5% BSA Fraction V for 2 × 45 min in an incubator at 37 °C and shaking manually for10 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 using centrifugation for 5 min at 250 × *g* with DMEM/F12 media. The supernatant was discarded. Later, erythrocyte lysis was accomplished by washing the pellet with RBC lysis buffer. The pellet was again washed with DMEM/F12 media. Cells were re-suspended in DMEM/F12 medium containing 10% Fetal Bovine Serum (FBS; Gibco life technologies, Thermo Fisher Scientific, USA) and antibiotic/antimycotic solution (10,000 units penicillin, 10 mg streptomycin, 25 μg amphotericin B per mL). Cell viability was determined using trypan blue exclusion dye and was greater than 90%. The cells were then plated at 1.5×10^5 viable cells per well in a 24-well plate in a humidified CO_2 (5%) incubator at 37.5 °C. The cells were allowed to attach and grow until there was 75% to 80% confluence with replacing the media every 48 h. Then cells were maintained in DMEM/F12 media without FBS for 24 h and then cells were treated with fresh media (without FBS) containing different concentrations (0, 1 and 10 μg/mL) of recombinant adiponectin (Biovender, Czech Republic, Cat. No. RD172029100) and were maintained for 48 h. The doses of the adiponectin were selected based on the earlier reports (Lagaly et al., 2008; Maillard et al., 2010). Control cells were grown in media without adiponectin. Four replicates were evaluated for each experimental condition. After 48 h, the spent media was collected and stored for P_4 assay and RNA analyses after isolation from cells using TRIZOL reagent as previously described in this manuscript.

2.7.2. P_4 ELISA assay

Concentrations of P_4 were quantified in serum-free spent media from primary bubaline luteal cells after 48 h of culture for each experimental condition using an ELISA kit (Neogen Life Sciences, USA, Cat. No. 402310) to confirm steroidogenic properties. The results were expressed as the concentration of the P_4 (ng/mL).The intra- and inter-assay coefficients of variation were less than 10%. The range of detection of P_4concentration was 0.4 to 40 ng/mL. The sensitivity of detection of P_4 was 2.9 ng/mL at 50% binding and

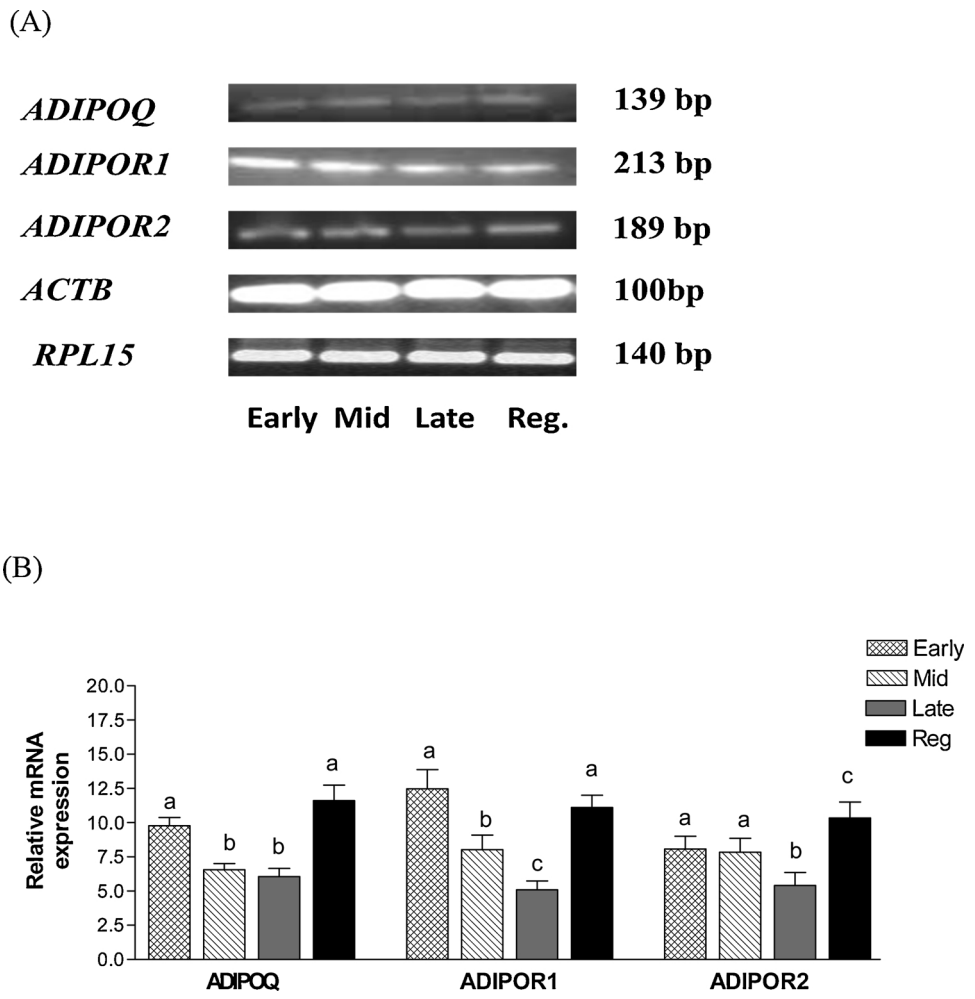

Fig. 1. (A) Representative images of amplification of *adiponectin (ADIPOQ)* (139 bp), *ADIPOR1*(213 bp), *ADIPOR2*(189 bp), *ACTB* (100 bp) and *RPL15* (140 bp) mRNA transcript by qPCR at different stages of CL development in the water buffalo. (B) Relative abundance mRNA profile of *adiponectin (ADIPOQ)*, *ADIPOR1* and *ADIPOR2* genes 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); Total RNA extraction using trizol method and cDNA preparation were conducted using commercial kit; qPCR was done using SYBR green chemistry; The tissue with least abundance (greatest Cq) served as calibrator to calculate the fold change; RPL15 and β-actin (ACTB) were used as reference gene to calculate ΔCq; Oneway ANOVA was conducted to assess whether there were between group differences and Tukey's *post hoc* honestly significant difference test was conducted to detect pair-wise mean differences; Minimum level of significance was set at 95%; Values are expressed as means ± SEM; Different superscripts indicates difference in values ($P < 0.05$).

0.35 ng/mL at 80% binding. Results are reported as mean ± SEM data and were obtained from three to four independent cultures, and each treatment had four replicates.

2.8. Statistical analysis

All experimental data are reported as the mean ± SEM. The differences in relative abundances of mRNA transcripts for adiponectin (*ADIPOQ*), *ADIPOR1*, *ADIPOR2* in CL during different stages of the estrous cycle and the abundances of protein 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 statistical significance of the difference in relative abundances of mRNA for *STAR*, *CYP11A1*, *HSD3B1* in cultured luteal cell and P_4concentration in spent culture media was assessed using a one-way ANOVA followed by the Duncan's multiple comparison test. The differences were considered to have occurred if $P < 0.05$.

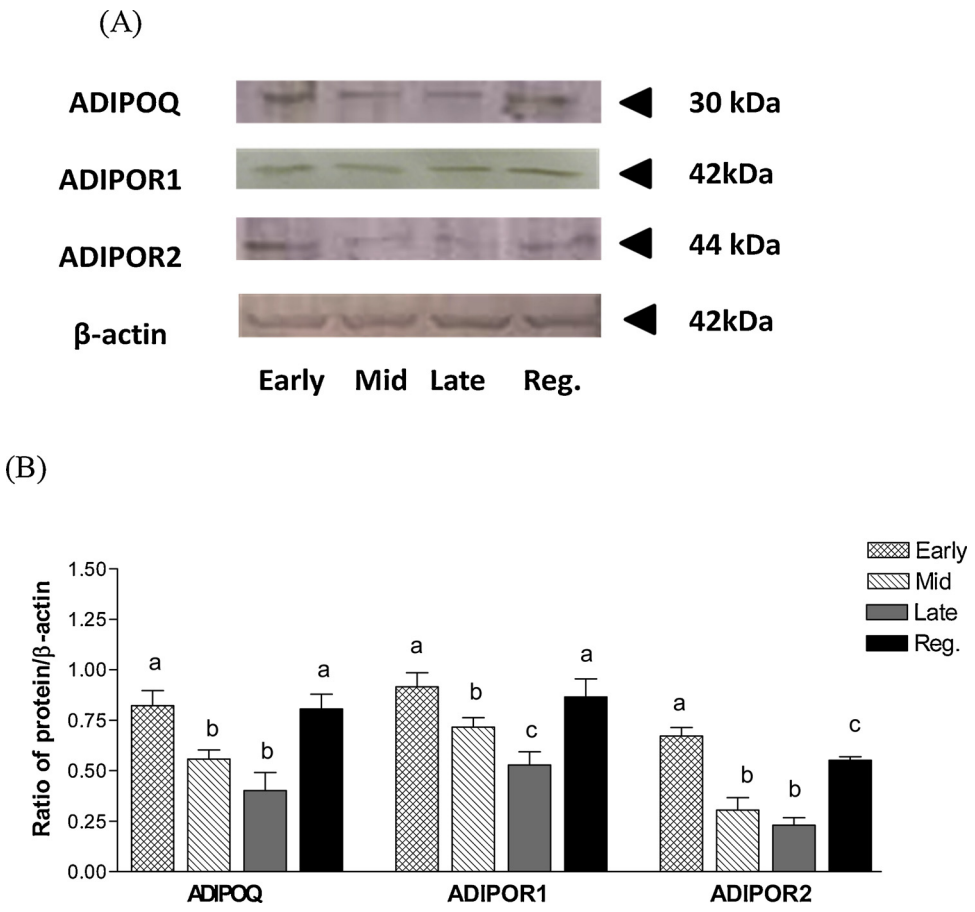

Fig. 2. Adiponectin (ADIPOQ), ADIPOR1 and ADIPOR2protein abundance determined by Western blotting analysis in different stages of CL during estrous cycle in water buffalo; Luteal protein was loaded at 80 µg/ well, resolved in 12% SDS-PAGE and electro-transferred to the PVDF membrane; Primary antibody was used at 1:800 for adiponectin, ADIPOR1, ADIPOR2 and 1;1000 for β-actin. while secondary antibody was used at 1:10,000(A) representative immunoblots of adiponectin (ADIPOQ), ADIPOR1 and ADIPOR2; β-actin was used as reference protein; The relative molecular weight of each protein is indicated on the right end of each blot;(B) Relative abundance of adiponectin (ADIPOQ) and its receptors (ADIPOR1 and ADIPOR2); densitometric analysis of adiponectin, ADIPOR1 and ADIPOR2 protein relative to β-actin protein was conducted using Image-J software; protein samples of 10 CL per group was pooled together and for each protein, the experiment was replicated thrice for each protein; One-way ANOVA was conducted to assess whether there were between group differences and Tukey's *post hoc* honestly significant difference test was done to find the pair-wise mean difference; Values are expressed as means ± SEM; Different superscripts indicate differences between values ($P < 0.05$).

3. Results

3.1. Relative abundances of mRNA for adiponectin (ADIPOQ) and its receptors (ADIPOR1 and ADIPOR2) in CL

Relative abundances of qPCR product were determined using the $2^{-\Delta\Delta CT}$ method. The tissue with the least abundance (greatest Cq) was used as calibrator. The geometric mean of Cq of ACTB and RPL15 was considered to be the reference/internal control. The values for amplification of adiponectin, *ADIPOR1*, *ADIPOR2*, *ACTB* and *RPL15*using qPCR are depicted in Fig. 1A and the relative mRNA abundances of adiponectin, ADIPOR1, and *ADIPOR2*are depicted in Fig. 1B. The values obtained with analysis of the adiponectin, ADIPOR1, and *ADIPOR2* (Fig. 1B) using qRT-PCR indicated that both ligand (*adiponectin*) and receptors (*ADIPOR1* and *ADIPOR2*) are present in the CL at each stage of functionality. The relative abundance of transcripts of *adiponectin* and its receptors (*ADIPOR1* and *ADIPOR2*) varied with stage of the luteal phase. The relative abundance of mRNA for *adiponectin* and *ADIPOR1* was greater ($P < 0.05$) during the early luteal stage (days 1–4) and regressing stage (days > 17) whereas the abundance was less ($P < 0.05$) in mid (days 5–10) and late-luteal (days 11–16) stages. The relative abundance of *ADIPOR2* was greater ($P < 0.05$) in the early- and mid-luteal stages, decreased in the late-luteal stage and subsequently increased during the regressing-stage of the CL.

3.2. Protein abundance of adiponectin and its receptors in CL

The presence of adiponectin and its receptor proteins ADIPOR1 and ADIPOR2 during diff ;erent stages of buff ;alo CL functionality

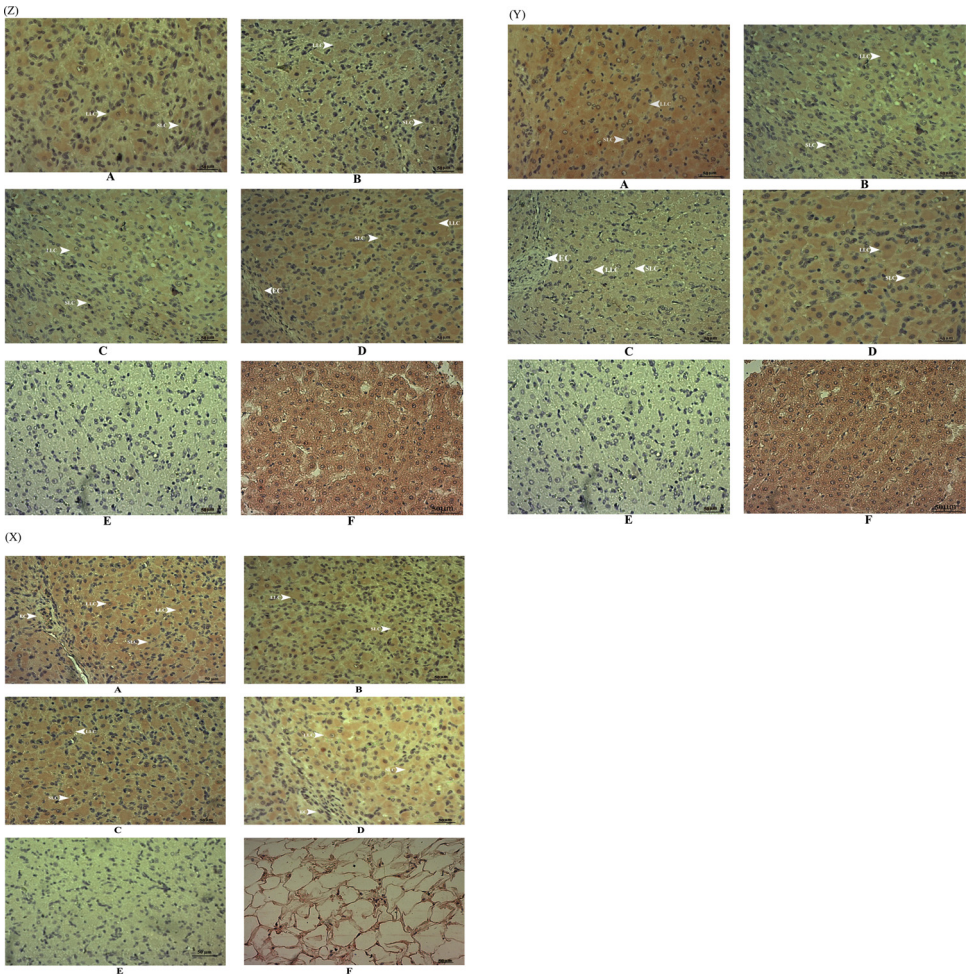

Fig. 3. Immunohistochemical localization of adiponectin (X), ADIPOR1 (Y) and ADIPOR2 (Z) in buffaloCL; antigen retrieval was conducted using sodium citrate buffer method; BSA 5% was used to minimize the non-specific binding; AEC-immunoperoxidase staining was performed on 5 μm thick paraffin-embedded buffalo CL using antibodies against adiponectin, ADIPOR1 and ADIPOR2 in dilution of 1:100; Immuno-specific staining is red-brown;Sections were counterstained with hematoxylin; Representative pictures show immunoreactivity against adiponectin, ADIPOR1 and ADIPOR2 (A, earlyluteal phase; B, mid-luteal phase; C, late luteal phase, D, regressing CL; E, negative control; F, positive control); Negeative control sections were stained by omitting primary antibody; Adipose tissue was taken as positive control for adiponectin and liver was taken as positive control for ADIPOR1 and ADIPOR2;Adiponectin and receptors were localized predominantly in the cytoplasm of large luteal cells and to some extent in small luteal; Small luteal cell (SLC); Large luteal cell (LLC); Endothelial cell (EC); Scale bar = 50 μm.

was verified by western blot procedures. Proteins of adiponectin, ADIPOR1, ADIPOR2, and β-actin were present in the CL. The major bands and protein for adiponectin/ADIPOQ (˜ 28 kD), ADIPOR1 (˜44 kD), ADIPOR2 (˜46 kD) and β-actin (˜44 kD) are evident inFigs. 2 A and 2B. Protein abundances (ratio of adiponectin/AdipoR1/AdipoR2 protein optical density/β-actin protein optical density) of adiponectin (ADIPOQ), ADIPOR1, and ADIPOR2) were greater ($P < 0.05$) in the early (days 1–4) and regressing (days > 17) luteal stages and was less in the mid- (days 5–10) and late-(days 11–16) luteal stages of functionality.

3.3. Immunohistochemical localization of adiponectin and its receptors in CL

The morphology of the CL sections was first evaluated using hematoxylin and eosin staining. The immunoreactivity of adiponectin, ADIPOR1, and ADIPOR2 was observed using immunohistochemistry procedures with the stage-specific difference in reactivity. The localization of both ligand and the receptor proteins was easily detected in various cell types at different stages of CL functionality classification (Fig. 3 X, Y and Z). The intensity of immunostaining varied from phase to phase and the number of positive cells was affected by stages of the estrous cycle. The immunoreactivity was present in the cytoplasm of luteal cells and was greater during early and regressing phases of CL functionality for adiponectin and receptors. The negative controls, with isotype IgG and without primary antibodies, had only weak background staining. Adipose tissue was used for a positive control for adiponectin and liver was used for positive control for ADIPOR1 and ADIPOR2. Positive control had strong/intense immunoreactivity of adiponectin and its receptors.

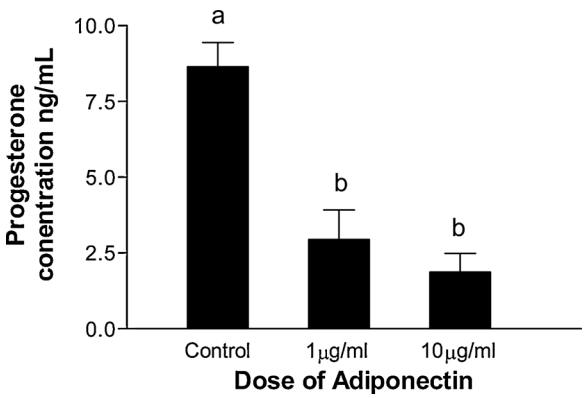

Fig. 4. Concentration of P_4 in spent media of bubaline luteal cell culture; Luteal cells were cultured for 48 h in a DMEM/F12 medium supplemented with 10% FBS; When the culture reached 75%–80% confluency, cells were treated with adiponectin at 0, 1 μg/mL and 10 μg/mL for 48 h in culture medium without FBS (n = 4_per dose); Production of P_4 in the supernatant (spent media) was assayed by ELISA; Results are expressed as means ± SEM of four independent experiments; All values are reported as mean ± SEM; Different superscripts indicate differences between values ($P < 0.05$).

3.4. Effect of adiponectin on P_4 secretion and mRNA abundance of STAR, CYP11A1, and HSD3B1

The concentration of P_4 in the spent media of control culture of luteal cells was 8.64 ng/mL. Addition of adiponectin to the media at both the doses (1 and 10 μg/mL) resulted in a decrease ($P < 0.05$) in P_4 concentration (Fig. 4).

The increase in abundance of enzymes/factors affecting P_4 production (*STAR*, *CYP11A1*, and *HSD3B1*) is depicted in Fig. 5A.Abundances of *STAR*, *CYP11A1,* and *HSD3B1* mRNA transcripts (Fig. 5B) in cultured luteal cells varied markedly with dose. The relative abundances of adiponectin mRNA transcripts in treated samples was compared with that of control samples in which there was no treatment imposed. The relative abundance of *STAR* mRNA transcript was similar to that of the control and 1 μg/mL adiponectin dose groups, however, was less ($P < 0.05$) than that of the control with the 10 μg/mL dose. The relative abundance of *CYP11A1* was less ($P < 0.05$) in adiponectin-treated samples at both doses. The relative abundance of HSD3B1mRNA transcript was less ($P < 0.05$) in adiponectin-treated cells in a dose-dependent manner.

4. Discussion

In the present study, for the first time, the presence of the adiponectin and related modulatory compounds were assessed in buffalo corpora lutea during the estrous cycle. Although abundances and immunolocalization of the adiponectin system and *in-vitro* effects of adiponectin in ovarian cells has already been studied in other species, the abundance of mRNA transcript and protein of adiponectin and its receptors and the effects of adiponectin in ovarian cells have not been studied in water buffalo.

The results of the present study indicate adiponectin and its receptor are present in the buffalo CL throughout the estrous cycle and the abundance varies with different stages of luteal function. In earlier studies the adiponectin system has been detected to be functional in ovarian cells of rats (Chabrolle et al., 2007a), chickens (Chabrolle et al., 2007b); humans (Chabrolle et al., 2009), cattle (Tabandeh et al., 2010; Maillard et al., 2010), pigs (Maleszka et al., 2014a; 2014b) and goats (Oliveira et al., 2017). In the present study, the mRNA and protein of adiponectin and its receptors (ADIPOR1 and ADIPOR2) were in greatest abundance in the early and regressing stages of CL functionality and was least in the mid- and late-luteal phases. The results of present study are consistent with observations of Tabandeh et al. (2010) in cattle. In this previous study, quantity of transcripts was greater for adiponectin, ADIPOR1 and ADIPOR2 during the initial stage of CL development and at the time of regression of CL as compared to the fully functional CL. Similarly there were greater abundances of adiponectin in early and regressing phase of CL in pigs (Maleszka et al., 2014a), however, the abundance of ADIPOR1 and ADIPOR2were reported to be greater in fully functional corpora lutea and less in corpora hemorrhagica (early CL) and regressing corpora lutea (Maleszka et al., 2014b). These differences in abundances of receptors are probably due to species variations. Adiponectin and its receptors have also been detected in granulosa cells, theca cells, cumulus cells in cattle, pigs and goats and quantity of mRNA in CL is greater than other ovarian cells (Maillard et al., 2010; Maleszka et al., 2014a; 2014b; Oliveira et al., 2017).

The results for presence of mRNA and protein in luteal tissues in the present study were further substantiated by immunohistochemical localization of adiponectin, ADIPOR1, and ADIPOR2during different stages of CL functionality. Immunoreactivity of adiponectin, ADIPOR1, and ADIPOR2wasobserved in small and large luteal cells and the intensity of immunoreactivity was greater in the early stage CL functionality and regressing CL. In previous studies adiponectin and the receptors have been immunolocalized in different cells of ovarian follicles and CL in the ovary of rats, cattle and goats (Chabrolle et al., 2007a; Maillard et al., 2010; Oliveira et al., 2017). The presence of the ligand adiponectin and the receptors ADIPOR1 and ADIPOR2in luteal cells indicated there were autocrine and paracrine functions of adiponectin in luteal dynamics in water buffalo (*Bubalus bubalis*).

The detection of mRNA and protein and localization of adiponectin provided indirect evidence of the involvement of adiponectin

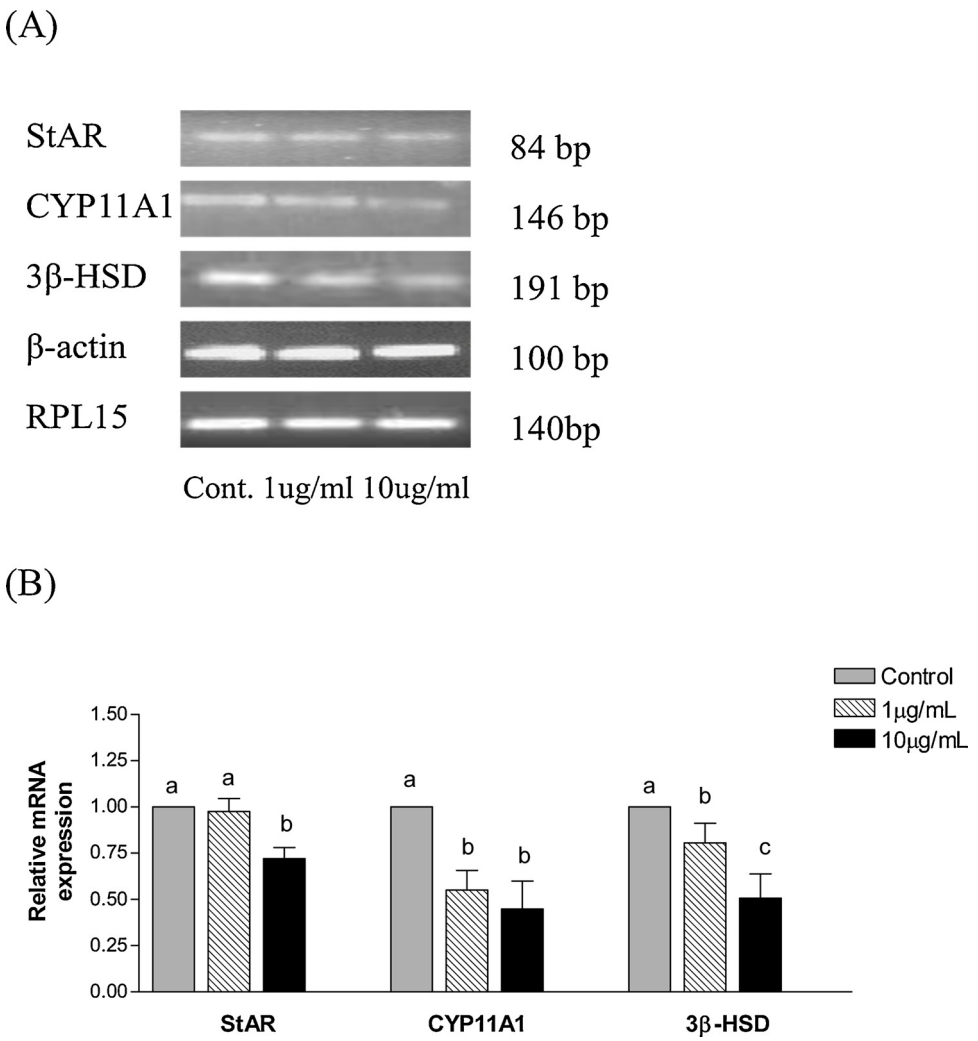

Fig. 5. (A) Representative pictures of amplification of *STAR* (84 bp), *CYP11A1* (156 bp), *HSD3B1* (191 bp), *ACTB* (100 bp) and *RPL15* (140 bp) genes in cultured luteal cells using qPCR;(B) Relative mRNA abundances of enzymes/factors involved in P_4 production(*STAR*, *CYP11A1,* and *HSD3B1*) from cultured luteal cells treated with adiponectin for 48 h at 0, 1 and 10 μg/mL ($n=$ four CL); Luteal cells were cultured for 48 h in a DMEM/F12 medium supplemented with 10% FBS; When the culture reached 75% to 80% confluency, cells were treated with adiponectin at0, 1 μg/mL and 10 μg/mL for 48 h in culture medium without FBS (n = 4_per dose);Cell lysate was used for the extraction of total RNA and cDNA preparation; Relative abundance was calculated using the $2^{-\Delta\Delta CT}$ method;RPL15 and β-actin (ACTB) were used as reference gene to calculate ΔCt; Results are reported as mean ± SEM; Different superscripts indicate differences between values ($P < 0.05$).

in luteal functions and dynamics. This finding, however, is not sufficient to draw a conclusive inference about the functions of adiponectin in the luteal tissues. To evaluate the effect of adiponectin on P_4secretion and functions of enzymes/factors affecting in P_4 production, the luteal cells were cultured and treated with different doses (0, 1 and 10 μg/mL) of adiponectin as previously described in this manuscript for the present study. The results of the *in-vitro* study indicate that adiponectin at both doses (1 and 10 μg/mL) decreased P_4 secretion from luteal cells. Adiponectin treatment at the 10 μg/mL dose suppresses the abundance of *STAR*, *CYP11A1*, and *HSD3B1*mRNA transcripts. There were similar results in a study of Maleszka et al. (2014a) in which treatment with adiponectin decreased basal P_4 secretion by cultured luteal cells. Similarly, Maillard et al. (2010) reported that recombinant adiponectin supplementation to media decreased insulin but induced $P_4$and E_2secretionsby granulosa cells of cattle. In another study with cattle, adiponectin decreased insulin-induced P_4 and androstenedione production as well as attenuated IGF-I-induced, modulation of *CYP11A1*, and *CYP17A1*mRNA transcript abundances in theca cells (Lagaly et al., 2008). A similar mechanism is not observed in some species because treatment with adiponectin increased P_4 and E_2 production in response to insulin-like growth factor-I (IGF-I) in granulosa cells of rats (Chabrolle et al., 2007a). In the chicken ovary, basal adiponectin treatment had no effect on P_4 production in the follicle, however, it increased the IGF-1-induced P_4secretion from F2 and F3/4 follicles although it markedly reduced by about half the P_4 production in response to gonadotropins (LH and FSH) in F3/4 follicles(Chabrolle et al., 2007b). In geese ovarian granulosa cells, adiponectin stimulated P4 production ($P < 0.01$) and weakly inhibited E_2 production (Meng et al., 2019). The effect

of adiponectin on steroidogenesis varied in different species, different ovarian cells and in the presence or absence of other growth factors. There was no effect of adiponectin on cellular proliferation or apoptosis of luteal cells of buffalo in a previous study (Gupta et al., 2019).

In the current study, the greater abundances of adiponectin and its receptors during the early stage of CL, during regression of CL and the lesser abundances during the mid- and late-luteal stages indicate that CL growth is associated with a decrease of adiponectin and associated regulatory or secreted compounds. The regression of CL is correlated with an increase of adiponectin and the receptor genes. There are important functions of $PGF_{2\alpha}$ in luteolysis and concentrations of $PGF_{2\alpha}$ and leukotrienes increase during regression of CL (Fields et al., 1992; Tsai and Wiltbank, 1998). Adiponectin functions to modulate the expression of genes associated with peri-ovulatory remodeling of ovarian follicles such as cyclooxygenase-2 (COX-2), prostaglandin E synthase (PGES), and vascular endothelial growth factor (VEGF) (Ledoux et al., 2006). The P_4 concentration is less in the early stage of the estrous cycle when CL are small and increases with increased size and weight during the estrous cycle (Mann, 2009), furthermore, it decreases during CL regression (Ginther and Beg, 2012). Accordingly, in the current study, adiponectin was greatest when P4 concentration was less and vice versa. Hence, apparently adiponectin and related modulatory compounds function in luteolysis because there are greater circulating concentrations of P_4 secreted when fully functional CL are present, the decrease in adiponectin during mid and late luteal phase possibly attenuates the inhibitory effect of adiponectin on P_4production by these cells in water buffalo. The inhibitory actions on P_4secretion by adiponectin could be mediated by inhibition of actions of enzymes/factors involved in P_4 production (STAR, CYP11A1, and HSD3B1).

5. Conclusion

Results of the present study indicate there are changes in the adiponectin modulatory system in buffalo CL during the estrous cycle. The results indicated that adiponectin and its receptors are present in buffalo CL throughout the estrous cycle and the relative abundances of the mRNA are greater during the early and regressing phases of CL functionality. Furthermore, in the current study adiponectin had an inhibitory effect on P_4 production and abundances of factors involved in P_4 production in the buffalo CL. From the study it can be concluded that adiponectin has a possible regulatory function in luteal dynamics and reproductive functions in water buffalo (*Bubalus bubalis*).

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