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Effect of oxytocin on *in vitro* prostaglandin production and expression of PGFS and PGES mRNAs in buffalo corpus luteum

M K TRIPATHI[1], S MONDAL[2], A MOR[3] and I J REDDY[4]

ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka 560 030 India

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ABSTRACT

The present study investigated the effect of various doses of oxytocin on *in vitro* $PGF_2\alpha$ and PGE_2 production and expression profiling of PGFS and PGES mRNA in buffalo CL. Buffalo ovaries with mid-luteal phase CL were collected from the abattoir and CL was separated from surrounding tissues, chopped, rinsed with HBSS medium supplemented with gentamicin and BSA and incubated at 37°C for 1 h in HBSS containing collagenase. The cell suspension following filtration was treated with increasing doses of oxytocin (1, 10, 10^2, 10^3 and 10^4 ng/ml) and cultured at 38.5ºC, 5% CO_2 level for 24 h. The production of $PGF_2\alpha$ and PGE_2 were not significantly different among different treatment groups as compared to control. The expression of PGES and PGFS mRNAs were not significantly different among different treatment groups as compared to control. It can be concluded that oxytocin may not directly stimulate $PGF_2\alpha$ and PGE_2 production in mid-luteal stage buffalo corpus luteum.

Key words: Corpus Luteum, Prostaglandin $F_2\alpha$, Prostaglandin E_2, Oxytocin

Early embryonic mortality is a major problem in the improvement of reproductive performance of buffaloes and causes reproductive failure during pre-implantation period (Mondal and Prakash 2002, Mondal *et al*. 2009, Mondal *et al.* 2010). The majority of embryonic mortality occurs during early embryonic period with rates ranging from 20–44% reported in beef cattle (Humbolt 2001) and 20% in buffaloes living closer to equator (Vale *et al*. 1989). Corpus luteum (CL) is the major source of progesterone hormone which is essential for the maintenance of pregnancy. The survivability of the corpus luteum depends upon the intrigued interactions among various luteolytic and luteotropic factors. Prostaglandins (PGs) are the important regulators of the luteal life cycle. $PGF_2\alpha$ secreted from uterine endometrium is involved in the regression of the CL in many species (Davis and Rueda 2002), whereas PGE_2 has been reported to exhibit luteoprotective functions (Akinlosotu *et al*. 1986, Christenson *et al*. 1994). In addition to the endometrium, luteal tissues also possess an inherent capacity to produce prostaglandins in many mammalian species (Townson and Pate 1994, Townson and Pate 1996, Diaz *et al*. 2000). An integrated role of luteal $PGF_2\alpha$ and PGE_2 in autoregulation of CL function has been reported (Arosh *et al*. 2004).

Present address: [1]Scientist (tripathimanoj123@rediffmail.com), Division of Livestock and Fish Management, ICAR-Research Complex for Eastern Region, Patna, Bihar, India. [2,4]Principal Scientist (sukanta781@gmail.com, reddyij123@gmail.com), [3]PhD Scholar (dravants81@gmail.com), Animal Physiology Division.

Oxytocin has long been used in field conditions for milk let down by the farmers. Oxytocin leads to luteal regression indirectly by causing the release of $PGF_2\alpha$ from the endometrium leading to shortened luteal phases in cows (Fuchs *et al*. 1996, Balaguer 1999). Cows administered oxytocin on days 4 to 7 after inseminations are at a higher risk of pregnancy loss (Yildiz and Erisir 2006)**.** In addition to the indirect effect of oxytocin on CL life cycle regulation via endometrial $PGF_2\alpha$ secretion, delay in luteal development caused by oxytocin infusion occurs via a direct influence on the developing corpus luteum (Wathes *et al*. 1992). There may be local interactions between oxytocin and $PGF_2\alpha$ that play a role in the regulation of the CL (Bennegård-Edén *et al.* 1995). Ovarian oxytocin seems to be the one among several physiological regulators of the synthesis and secretion of luteal $PGF_2\alpha$ during the oestrous cycle (Schams and Berisha 2004). Oxytocin may be either luteotrophic or luteolytic depending on its concentration by altering progesterone production in early pregnant cattle and can influence the corpus luteum of early pregnancy directly, thus providing another site of oxytocin action in the control of luteal function besides the uterus (Tan *et al*. 1982). It appears from the previous reports that oxytocin administration has diversified consequences on embryonic survivability depending on the species and stage of the estrous cycle and the possible role of oxytocin directly on buffalo corpus luteum to produce $PGF_2\alpha$ seems reasonable. Thus, it is hypothesised that exogenous administration of oxytocin may be directly involved in the luteal prostaglandin production and affects the embryonic survivability in

buffaloes. Information on role of oxytocin in the regulation of luteal $PGF_2\alpha$ and PGE_2 secretion and expression of PGFS and PGES mRNA involved in synthesis of $PGF_2\alpha$ and PGE_2, respectively, in buffalo CL is completely lacking. A proper understanding of the physiological effect of oxytocin on the buffalo corpus luteum might pave a way towards the manipulation of the luteal life cycle. Keeping this in view the present work was proposed with the objectives to investigate the effect of oxytocin on *in vitro* prostaglandin production and expression of PGES and PGFS mRNAs in buffalo corpus luteum.

MATERIALS AND METHODS

Collection of buffalo corpus luteum: Buffalo ovaries with corpus luteum were collected immediately after slaughter from a local abattoir and were brought to the laboratory within 30 min while keeping in ice-cold physiological saline (0.9% NaCl; pH 7.4) containing antibiotics [streptomycin (100 µg/ml), penicillin (10 IU/ml), amphotericin (0.2 µg/ml) and L-glutamine (100 µg/ml)].

Identification of mid stage corpus luteum: Corpus luteum was removed from the ovaries. The stages of the estrous cycle were determined based on morphological characteristics of corpus luteum according to the previously described methods (Mondal *et al.* 2004, Ghosh and Mondal 2006). The mid-luteal phase corpus luteum were identified by observing the change of its colour from dark red to orange, firmness in consistency on palpation and demarcated from rest of the ovary (Mondal *et al.* 2013).

Isolation of luteal cells: After determining the stages, the mid stage corpus luteum were separated and rinsed with Hanks balanced salt solution (HBSS) medium supplemented with gentamicin (50 µg/µl, pH 7.4) and 0.1% bovine serum albumin (BSA). The CL were finely chopped mixed in HBSS containing 0.1% collagenase (Type II collagenase from *Clostridium histolyticum*, pH 7.4) followed by incubation at 37°C for 1 h. Following incubation, the cell suspension obtained was filtered through plastic strainer (70 µM) to remove undigested tissues. The filtrate was washed 3 to 4 times by centrifugation at 600×g for 10 min with HBBS supplemented with gentamycin and 0.1% BSA. The viable cell number that excluded Trypan blue was counted using a haemocytometer.

Treatment of luteal cells with oxytocin: The luteral cells were seeded @ 1×10^5 viable cells in RPMI 1640 medium supplemented with 1 µl gentamicin (50 µg/µl), 100 µg glutamine and 10% FBS (fetal bovine serum) in 6 well plate and were treated with increasing doses of oxytocin (1, 10, 10^2, 10^3 and 10^4 ng/ml) followed by incubation at 38.5°C and 5% CO_2 level for 24 h. Cells and the spent media were harvested from the plates and centrifuged at 8,000 rpm for 6 min. The spent media was collected for quantification of prostaglandins. The cell pellet obtained was kept in 100 µl RLT-lysis buffer containing beta-mercaptoethanol (10 µl for each ml RLT buffer) and stored at –80°C for expression studies of PGF synthase and PGE synthase mRNA. The concentrations of $PGF_2\alpha$ were determined in 50 ml aliquots of culture medium after 10-fold dilution with extraction buffer using ELISA kits supplied by Neogen, USA. The sensitivity of the assay was 0.002 ng/ml. The cross reactivity of the antisera against 6-keto prostaglandin $F_{1\alpha}$, 13, 14 dihydro-15 keto-prostaglandin $F_{2\alpha}$, prostaglandin D_2 and prostaglandin E_2 were 3.05%, 0.05%, 0.05% and <0.01%, respectively. The intra- and inter-assay coefficients of variation were less than 15%. The concentrations of PGE_2 were determined in 50 ml aliquots of culture medium after 5-fold dilution with extraction buffer. The sensitivity of the assay was 0.002 ng/ml. The cross reactivity of the antisera against 6-keto prostaglandin $F_{1\alpha}$, 13,14 dihydro-15 keto-prostaglandin $F_{2\alpha}$, prostaglandin D_2 and prostaglandin $F_2\alpha$ were >0.01% for all. The intra- and inter-assay coefficients of variation were less than 13%.

Quantification of prostaglandins by ELISA: The concentrations of $PGF_2\alpha$ were determined by ELISA in 50 ml aliquots of culture medium after 10-fold dilution with extraction buffer using ELISA kits supplied by Neogen, USA. The cross reactivity of the antisera against 6-keto prostaglandin $F_{1\alpha}$, 13, 14 dihydro-15 keto-prostaglandin $F_{2\alpha}$, prostaglandin D_2 and prostaglandin E_2 were 3.05%, 0.05%, 0.05% and <0.01%, respectively. The sensitivity of the assay was 0.002 ng/ml. The intra- and inter-assay coefficients of variation were less than 15%. The concentrations of PGE_2 were determined in 50 ml aliquots of culture medium after 5 fold dilution with extraction buffer. The cross reactivity of the antisera against 6-keto prostaglandin F_1, 13, 14 dihydro-15 keto-prostaglandin $F_{2\alpha}$, prostaglandin D_2 and prostaglandin $F_2\alpha$ were >0.01% for all. The sensitivity of the assay was 0.002 ng/ml. The intra- and inter-assay coefficients of variation were less than 13%.

Isolation of RNA: Total RNA was isolated from the cultured cell pellets using RNeasy Minikit (M/s Qiagen, Germany) as per manufacturer's protocol and quantified by nanodrop spectrophotometer (Eppendorf, Germany) at 260 nm and 280 nm. The integrity of RNA was checked by formaldehyde-agarose gel (1%) electrophoresis and visualized under UV light after staining with ethidium bromide.

Reverse transcription polymerase chain reaction (*RT-PCR*): cDNA was synthesized by using cDNA synthesis kit (BIORAD iScriptTM cDNA synthesis kit) following the protocol—Iscript reverse transcriptase (1 µl), 5× iScript reaction mix (4 µl), Nuclease free water (14 µl) and (100 ng) RNA (1 µl) to make total volume 20 µl. The reaction was incubated at 25°C for 5 min, 42°C for 30 min followed by 85°C for 5 min. Following first strand synthesis, PCR amplification of PGES and PGFS was carried out using gene specific primers designed on the basis of aligned nucleotide sequences available in GenBank (Table 1). PCR amplification was carried out in a total volume of 25 µl. Reaction mixture contained of 2 µl first strand of cDNA, 10× Buffer (2.5 µl), 50 mM dNTP Mix (0.5 µl), 25 mM $MgCl_2$ (2.5 µl), forward primer (1 µl), reverse primer (1 µl), Mili Q water (16.3 µl) and 0.2 µl Taq DNA Polymerase

Table 1. Gene specific primers used for amplification of PGFS and PGES cDNA

Gene	Primers name and sequence (5'–3')	Sequences used to design the primers	Amplification conditions
PGFS	Forward: CCAGAGGAAACTGTTGGATTTC Reverse: AACCCCACGTTGTATCTGGTAG	Cattle: AY135401 Horse: AY304536 Pig: AY863054 Dog: AY875970	Denaturation: 95°C for 30 sec, Annealing: 59°C for 30 sec and Extension: 72°C for 30 sec
PGES	Forward: TGGTCATCAAAATGTACGTGGT Reverse: AACAGGAAGGGGTAGATGGTCT	Cattle: AY 032727 Rat: BC088101 Horse: AY057096 Pig: AY857634	Denaturation: 95°C for 30 sec, Annealing: 59°C for 30 sec and Extension: 72°C for 30 sec
β-actin	Forward: AATTCCATCATGAAGTGTGACG Reverse: GATCTTGATCTTCATCGTGCTG	Cattle: AY141970 Buffalo: DQ661647 Sheep: U39357 Goat: JX046106	Denaturation: 95°C for 30 sec, Annealing: 59°C for 30 sec and Extension: 72°C for 30 sec

(5 U/µl). After initial denaturation at 95°C for 5 min, 30 cycles of amplification were carried out under the conditions mentioned in Table 1, followed by a final extension at 72°C for 10 min. The generated cDNA fragments were resolved by agarose gel (1%) electrophoresis.

Real time PCR: Fast SYBR Green Master Mix (Applied Bio systems, USA) and gene specific primers for both housekeeping and target genes with 3 replicates per sample of each gene were used for PCR analyses using STEP ONE PLUS real time PCR system (Applied Bio systems, USA). The primers were designed with the Primer 3 program (Rozen and Skaletsky 2000) with an annealing temperature of 59°C and amplification size of less than 250bp. The efficiencies of the primer were checked by 10 fold serial dilution of cDNAs (Svec *et al*. 2015) and ranged between 95 and 105%. β-actin was utilised as endogenous control. Thermal cycling was performed as per manufacturer's protocol (95°C, 20 sec followed by 40 cycles of 95°C, 3 sec and 60°C, 25 sec and a melt curve of 95°C (15 sec), 60°C (1 min) and 95°C (15 sec). Melt curve analysis was used for determining the specificity of each PCR product and agarose gel electrophoresis (2%) was used for determining the amplicon size. Negative control consisted of all components of qRT-PCR mix except cDNA was used for all primers. The relative quantification of gene expression changes were recorded after normalizing for β-actin expression computed by using $2^{-\Delta\Delta CT}$ method (Svec *et al.* 2015) in which CT value from the controls served as calibrator.

Statistical analysis: The results are shown as the mean±SEM of values obtained from 4 separate experiments, each performed in triplicate. The differences in concentrations of $PGF_2\alpha$, PGE_2 and transcript abundance were analyzed by ANOVA followed by Tukey's multiple comparison tests using the statistical package of Graph Pad Prism 5, San Diego, USA. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The effect of various doses of oxytocin (1, 10, 10^2, 10^3 and 10^4 ng/ml) on *in vitro* $PGF_2\alpha$ production in buffalo luteal cells is presented in Fig. 1a. The levels of $PGF_2\alpha$ were 114.5±25.66, 100.3±33.5, 92±33.25, 114.5±32.2, 115.5±31.78 and 119.8±42.03 pg/ml in control, 1, 10, 10^2, 10^3 and 10^4ng/ml oxytocin, respectively. The effect of oxytocin on $PGF_2\alpha$ production was nonsignificant ($P>0.05$)

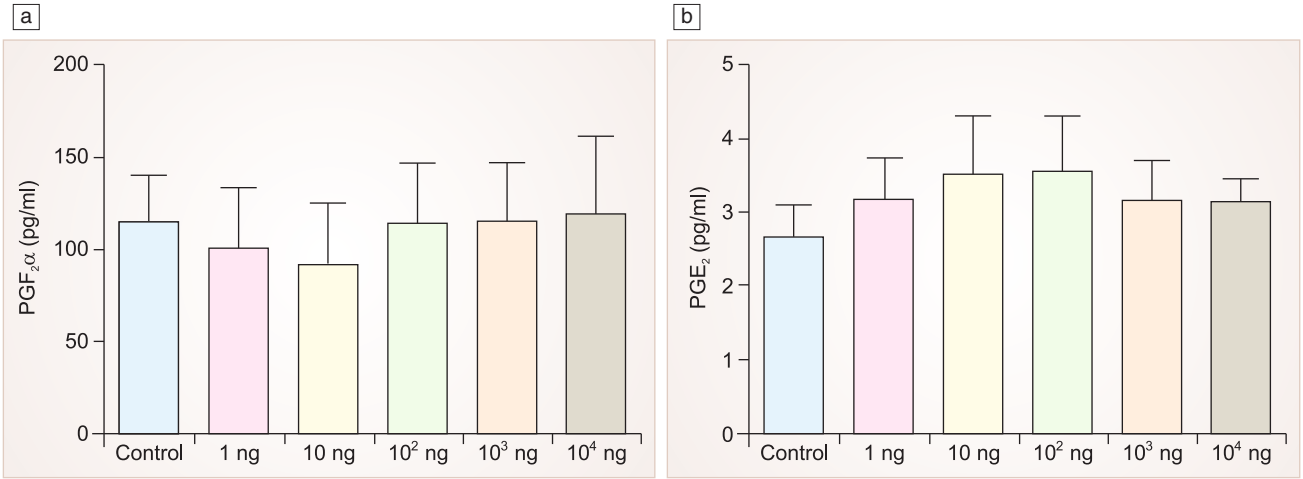

Fig. 1. Effect of oxytocin on $PGF_2\alpha$ (**a**) and PGE_2 (**b**) production by luteal cells in buffalo.

however 10 ng/ml was noted as the most effective dose to decrease $PGF_2\alpha$ level. The effect of various doses of oxytocin (1, 10, 10^2, 10^3 and 10^4 ng/ml) on *in vitro* PGE_2 production in buffalo luteal cells is presented in Fig. 1b. The levels of PGE_2 were 2.66±0.44, 3.17±0.57, 3.51±0.8, 3.56±0.76, 3.15±0.56, 3.15±0.32 pg/ml in control 1, 10, 10^2, 10^3 and 10^4 ng/ml oxytocin treatments, respectively. There was non-significant (P>0.05) increase in PGE_2 level with all treatment group as compared to control though 100 ng/ml was noted as the most effective dose to induce PGE_2 production.

The effect of different doses of oxytocin (1, 10, 10^2, 10^3 and 10^4 ng/ml) on PGFS and PGES mRNA expression is presented in Figs 2a,b, respectively. The expression of PGFS mRNA increased in all treatment groups as compared to control except 1 ng/ml treatment group. There were no significant (P>0.05) differences in PGFS mRNA expression among the treatment groups. The expression of PGES mRNA decreased as compared to control among all treatment groups except 10^3 ng/ml and lowest expression was observed with 10 ng/ml oxytocin. No significant differences (P>0.05) were observed among the various groups.

Oxytocin is well known to be involved in the process of luteolysis by stimulating the endometrial $PGF_2\alpha$ production in domestic ruminants. The endometrial $PGF_2\alpha$ further stimulates the production of luteal $PGF_2\alpha$ which acts via a paracrine and/or autocrine mechanism to induce luteolysis that indicates the presence of a positive feedback pathway in the ruminants CL (Auletta and Flinta 1988, Diaz *et al.* 2002). Oxytocin receptors are localized in both large and small luteal cells of bovine species and high expression of oxytocin (OT) in CL of ruminants is well-established (Schams and Berisha 2004). The newly formed corpus luteum is relatively resistant to $PGF_2\alpha$ (Levy *et al.* 2006) and so mid stage CL was chosen in the present study to see the effect of oxytocin on luteal cell $PGF_2\alpha$ production. The present study showed that the effect of oxytocin on $PGF_2\alpha$ and PGE_2 production from buffalo corpus luteum was non-significant, which indicates that oxytocin may not be involved in the process of luteolysis through a mechanism of direct stimulation of $PGF_2\alpha$ production from corpus luteum nor it may have any luteotropic effect through PGE_2 secretion in the mid stage buffalo corpus luteum. Our results are in agreement with the finding of Olofsson *et al.* (1992) who reported that oxytocin was found to be without effect on prostaglandin secretion on all days tested in isolated corpora lutea of adult pseudopregnant rats throughout the luteal life-span. Oxytocin treatment had no significant effect on luteal $PGF_2\alpha$ concentrations in pseudopregnant rats (Cao and Chan 1993). Similarly, oxytocin infusion in cattle had no remarkable effects on luteal size, progesterone and PGFM ($PGF_2\alpha$ metabolite) plasma levels (Brozos *et al.* 2012). Although in the present study we have not studied the effect of oxytocin on luteal progesterone production but some previous literature seems to support our finding in one way that oxytocin stimulates progesterone release from bovine corpus luteum (Miyamoto and Schams 1991) which indicates that oxytocin may be involved in functional luteal maintenance by increasing steroidogenesis and not luteolysis. On the other hand the findings of Grazul *et al.* (1989) is in contradiction with ours who reported that oxytocin increases $PGF_2\alpha$ release by bovine luteal cells as compared to control irrespective of day of estrous cycle. Our results also do not conform with the findings of Bennegrad-Eden *et al.* (1995) who reported that a single injection of oxytocin into corpus luteum of human female caused an increase in serum $PGF_2\alpha$ metabolite level, fall in serum progesterone level and shortened the luteal phase and fall in progesterone level could be prevented by giving PG synthase inhibitor. Systemic infusions of OT-antagonist between days 12 and 20 of the estrous cycle reduce the amplitude of PGFM pulses, but do not prevent luteolysis (Kotwica *et al.*1999). Oxytocin seems to have no regulatory action on PGE_2 production level in buffalo corpus luteum although there was nonsignificant increase in PGE_2 level in all the treatment groups as compared to control. Oxytocin treatment had no effect on PGE_2 synthesis, except on day

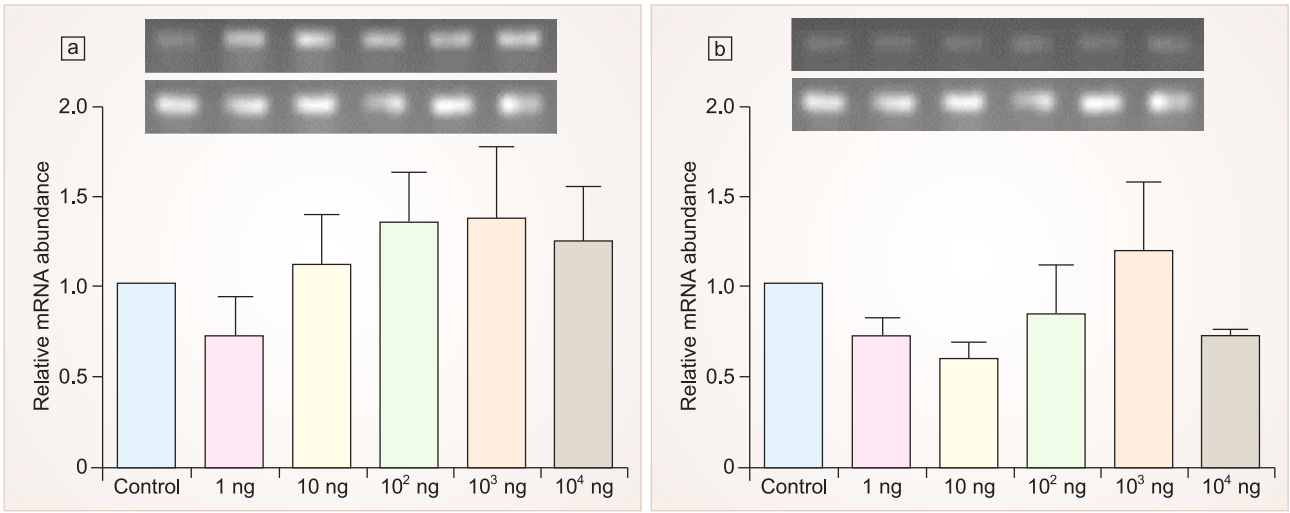

Fig. 2. Effect of increasing concentrations of oxytocin on PGFS (**a**) and PGES (**b**) mRNA expression in buffalo luteal cell.

11 when luteal PGE_2 concentrations of the oxytocin-treated rats were significantly higher than the corresponding control values (Cao and Chan 1993).

The production of prostaglandins depends on the level of expression of different enzymes involved in the process of PG synthesis. The bovine CL possesses all of the PGE_2 and $PGF_2\alpha$ machineries necessary for autoregulation of its function and PGE_2 and $PGF_2\alpha$ machineries are selectively and specifically expressed in CL (Arosh *et al*. 2004). Arachidonic acid is the precursor of most of the prostaglandins and is released from the membrane phospholipids via the action of cytosolic phospholipaseA2 (cPLA2). The first control point in the prostaglandin biosynthetic pathway is the release of the fatty acid from the phospholipids. Cyclooxygenases (COX-1 and COX-2) convert arachidonic acid into PGH_2, the common metabolite for various PGs including PGE_2 and $PGF_2\alpha$. PGE synthase (PGES) and PGF synthase (PGFS), catalyze the conversion of PGH2 to PGE_2 and $PGF_2\alpha$ respectively (Smith and Dewitt 1996). Our investigation reveals that there is no significant effect of oxytocin on PGFS and PGES mRNA expression in cultured luteal, which further supports our finding that oxytocin does not cause luteal prostaglandin ($PGF_2\alpha$ and PGE_2) production. There is no report available in any domestic species regarding the expression of PGFS and PGES mRNA as a result of oxytocin treatment in the corpus luteum, however, Waclawik *et al*. (2010) reported that in porcine endometrial luminal epithelial cells oxytocin did not have the effect on PGFS mRNA and protein expression and on $PGF_2\alpha$ release from LECs on days 11–12 of the estrous cycle or pregnancy. Similar results were reported where oxytocin did not stimulate PGFS and PGES expression in bovine myometrial cell *in vitro* (Slonia *et al*. 2009).

From this study, it can be concluded that oxytocin may not significantly alter prostaglandin production as well as prostaglandin F synthase and prostaglandin E synthase mRNA expression in cultured buffalo luteal cells.

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