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

M K TRIPATHI1, S MONDAL2, A MOR3 and I J REDDY4

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

Received: 14 March 2018; Accepted: 20 June 2018

ABSTRACT

The present study investigated the effect of various doses of oxytocin on in vitro PGF2α and PGE2 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, 10², 10³ and 10⁴ ng/ml) and cultured at 38.5°C, 5% CO₂ level for 24 h. The production of PGF2α and PGE2 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 PGF2α and PGE2 production in mid-luteal stage buffalo corpus luteum.

Key words: Corpus Luteum, Prostaglandin F2α, Prostaglandin E2, 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 2000). An integrated role of luteal PGF2α 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 PGF2α 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 PGF2α 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 PGF2α during the oestrous cycle (Schams and Berisha 2004). Oxytocin may be either luteotropic 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 oestrous cycle and the possible role of oxytocin directly on buffalo corpus luteum to produce PGF2α 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 luteal 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$_3\alpha$ were >0.01% for all. 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, 10x 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
(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 (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−ΔΔCT 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 PGF2α, PGE2 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, 10², 10³ and 10⁴ ng/ml) on in vitro PGF2α production in buffalo luteal cells is presented in Fig. 1a. The levels of PGF2α 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, 10², 10³ and 10⁴ng/ml oxytocin, respectively. The effect of oxytocin on PGF2α production was nonsignificant (P>0.05)

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers name and sequence (5’–3’)</th>
<th>Sequences used to design the primers</th>
<th>Amplification conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGFS</td>
<td>Forward: CCAGAGGAAACTGTTGGA</td>
<td>Cattle: AY135401 Denaturation: 95°C for 30 sec, Horse: AY304536 Annealing: 59°C for 30 sec and Pig: AY863054 Extension: 72°C for 30 sec</td>
<td></td>
</tr>
<tr>
<td>PGES</td>
<td>Forward: TGGTCATCAAATGTACGTGGT</td>
<td>Cattle: AY 032727 Denaturation: 95°C for 30 sec, Rat: BC088101 Annealing: 59°C for 30 sec and Horse: AY057096 Extension: 72°C for 30 sec</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Effect of oxytocin on PGF2α (a) and PGE2 (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 (Brozös 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.](image-url)
11 when luteal PGE2 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 PGE2 and PGF2α machineries necessary for autoregulation of its function and PGE2 and PGF2α 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 PGH2, the common metabolite of phospholipids. Cyclooxygenases (COX-1 and COX-2) catalyze the conversion of PGH2 to PGE2 and PGF2α respectively (Smith and Dewitt 2000).

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.

ACKNOWLEDGEMENTS

The authors express their gratitude to National Fund for Basic, Strategic and Frontier Application Research in Agriculture (Grant No. NASF AS2002), ICAR, New Delhi, for providing financial support to carry out this work. We also thank Director, NIANP, for providing the necessary facilities to carry out the research work. I am thankful to Director, CIRB who allowed me to conduct research work at NIANP. Special thanks are due to Dr P S P Gupta, Dr S Nandi, Dr A Mishra, Shiv Tripathi and all lab mates for assisting in our work. The help rendered by A Jagannath is duly acknowledged.

REFERENCES


