RESEARCH ARTICLE Indian Journal of Animal Research, Volume 57 Issue 6: 702-707 (June 2023)

Effect of Tumor Necrosis Factor-α on *in vitro* Prostaglandin Production in Buffalo Corpus Luteum

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10.18805/IJAR.B-4346

ABSTRACT

Background: Corpus luteum plays key role in embryonic survival. Prostaglandins are the important regulator controlling the life span of corpus luteum. The present study investigated the effect of various doses of TNF_{α} on *in vitro* $PGF_{2\alpha}$ and PGE_2 production and expression profiling of PGFS and PGES mRNA in buffalo Corpus Luteum (CL).

Methods: Buffalo ovaries with mid-luteal phase CL were collected from the abattoir and CL were enucleated from surrounding tissues. Corpus luteum were finely chopped, rinsed with HBSS (Hanks Balanced Salt Solution) medium; supplemented with gentamycin and 0.1% BSA and incubated at 37°C for 1 hr in HBSS containing 0.1% collagenase. The cell suspension following filtration was washed by HBBS supplemented with gentamycin and 0.1% BSA (bovine serum albumin) and was treated with increasing doses of TNF_{α} (0.1, 0.5 and 1.0 nM) and cultured at 38.5°C, 5% CO_2 level for 24 hr.

Result: There was dose dependent increase in concentrations of $PGF_{2\alpha}$ and PGE_2 with increasing doses of TNF_{α}. The PGFS (prostaglandin F synthase) mRNA expression increased with increasing doses of TNF_{α}. However, there was decrease in PGES (prostaglandin E synthase) mRNA expression at 0.1 nM and 0.5 nM TNFα but PGES mRNA expression increased at 1.0 nM TNF_{α} as compared to control. It can be concluded that TNF_{α} may alter PGES and PGFS mRNA expression and prostaglandin secretion in buffalo CL.

Key words: Corpus luteum, Prostaglandin E synthase, Prostaglandin E_2, Prostaglandin F synthase, Prostaglandin $f_{2\alpha}$, Tumor necrosis factor-α.

INTRODUCTION

Premature luteal regression or inadequate luteal function is a major cause of early embryonic mortality in domestic ruminants including buffaloes. Early embryonic mortality is a major cause of reproductive failure in buffaloes during pre-implantation period (Mondal and Prakash, 2002; Mondal *et al*., 2009; Mondal *et al*., 2010) and accounts for 20% loss in buffaloes living closer to equator (Vale *et al.,* 1989). Prostaglandins namely prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) play important role during maternal recognition of pregnancy, implantation and establishment of pregnancy in livestock including buffalo (Nandi *et al*., 2012; Mondal, 2013). The life span of CL is controlled by complex interactions among various luteotrophic and luteolytic factors including prostaglandins. Prostaglandin E_2 protects the CL from spontaneous regression and play important role in maintenance of pregnancy (Akinlosotu *et al*., 1986; Christenson *et al*., 1994) whereas $PGF_{2\alpha}$ primarily secreted from the surface epithelium of the uterus cause regression of CL (Davis and Rueda, 2002). In spite of $PGF_{2\alpha}$ produced from endometrium, its secretion is also reported from CL in cows (Townson and Pate, 1994, 1996) and pigs (Diaz *et al.,* 2000). The small amounts of uterine $PGF_{2\alpha}$ stimulate the production of $PGF_{2\alpha}$ in the CL which indicates the presence of a positive feedback pathway in the ruminant CL (Diaz *et al*., 2002). $PGF_{2\alpha}$ can autoregulate its synthesis in CL and it has been proposed that $PGF_{2\alpha}$ produced locally in CL acts *via* a paracrine and/or autocrine mechanism to induce luteolysis (Wiltbank and Ottobre, 2003). Progesterone, oxytocin and various cytokines have been shown to affect luteal $PGF_{2\alpha}$ production in different species (Pate, 1988; Wiltbank and Ottobre, 2003; Tripathi *et al*., 2018; Grazul *et al.,* 1989).

Tumour Necrosis Factor-alpha (TNF_{α}), a cytokine possessing wide biological actions, is mainly produced by ovarian macrophages and increased during regression of the CL (Penny *et al*., 1999). TNF_{α} participates in the regression of the corpus luteum in bovine (Pate, 1995). Highest expression of its specific receptors (TNF-RI) is present in the bovine corpus luteum during luteolysis (Friedman *et al.,* 2000; Korzekwa *et al.,* 2008). The luteolytic action of TNF_{α} may be mediated by the intraluteal production of $PGF_{2\alpha}$ as TNF_{α} dramatically stimulated $PGF_{2\alpha}$ production by cultured luteal cells of cow (Benyo and Pate, 1992). There is an integrated role of luteal $PGF_{2\alpha}$ and PGE_2 in auto-regulation of CL function (Arosh *et al.,* 2004). Intrauterine

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How to cite this article: Tripathi, M.K., Mondal, S., Reddy, I.J. and Mor, A. (2023). Effect of Tumor Necrosis Factor-α on *in vitro* Prostaglandin Production in Buffalo Corpus Luteum. Indian Journal of Animal Research. 57(6): 702-707. DOI: 10.18805/IJAR.B-4346.

Submitted: 28-10-2020 **Accepted:** 04-03-2021 **Online:** 07-04-2021

administration of PGE_2 had been found to protect the CL from spontaneous and/or induced luteolysis in ruminants (Pratt *et al*., 1977). The production of endometrial prostaglandins is governed by the enzymes prostaglandin E and F synthase (PGES and PGFS) which convert PGH_2 to PGE_2 and $PGF_{2\alpha}$, respectively. There is paucity of information regarding the role of TNF_α in regulation of $PGF_{2\alpha}$ and PGE_2 secretion and expression of PGES and PGFS mRNA in buffalo CL. Keeping this in view the present work was undertaken to investigate the effect of TNF_α on *in vitro* prostaglandin production and expression of PGES and PGFS mRNA in buffalo CL.

MATERIALS AND METHODS

Collection of corpus luteum and identification of stage of estrous cycle

Buffalo ovaries with CL were collected from a local abattoir within 1 hr after exsanguinations. The ovaries were then submerged in ice-cold physiological saline (0.9% NaCl; pH 7.4) containing antibiotics [penicillin (10 IU/ml), streptomycin (100 g/ml), amphotericin (2 g/ml) and L-glutamine (100 µg/ml)] and transported to the laboratory. The stages of the estrous cycle were determined according to previously described methods (Mondal *et al*., 2004; Ghosh and Mondal, 2006; Chouhan *et al.,* 2013) and were categorized to Stage I (days 3 to 5 of the cycle), Stage II (days 6 to 15 of the cycle) and Stage III (days 16 to 21 of the cycle). The Stage I is the developing stage, Stage II is mature one while Stage III is in the regressed stage of corpus luteum. The mid-luteal phase of estrous cycle was determined by macroscopic examination of the CL by observing the change of colour from dark red to orange, firm in consistency on palpation and demarcated from rest of the ovary (Mondal *et al*., 2013).

Luteal cell Isolation

Mid stage corpus luteum were immediately separated from the surrounding tissues of the ovaries, chopped, rinsed a few times with Hanks Balanced Salt Solution (HBSS, H9394 Sigma) medium supplemented with gentamycin (50 µg/µl, pH 7.4, TC026, Hiimedia) and 0.1% bovine serum albumin (BSA, RM3158, Himedia) and incubated at 37°C for 1 hr in HBSS containing 0.1% collagenase (Type II collagenase from Clostridium histolyticum, pH 7.4, C6885, Sigma). The cell suspension obtained from the digestion was filtered through plastic strainer (70 µM) to remove undissociated tissue fragments. There after the filtrate was washed three times by centrifugation at 600 × g for 10 min with HBBS supplemented with gentamycin and 0.1% BSA. The number of viable cells that excluded Trypan blue was counted using a haemocytometer.

Culture of luteal cells and treatment with TNF_α

After cell counting and viability determination the luteal cells were seeded at the rate of 1×10^5 viable cells in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 100 µg glutamine, 1 µl gentamicin (50 µg/µl; TC026-1G, Himedia) and 10% FBS (Fetal Bovine Serum; F2442, Sigma) and were treated with increasing doses of TNF_α (0.1, 0.5 and 1.0 nM) followed by incubation at 38.5°C and 5% CO_2 level for 24 hours. Cells along with spent media were harvested and centrifuged at 8000 rpm for 6 minutes to isolate cell pellet. The spent media was collected for quantification of prostaglandins. The cell pellet was kept in 100 µl RLT-lysis buffer (Qiagen, Germany) containing β-mercaptoethanol and stored at -80°C for expression of PGEs and PGFS mRNA.

Quantification of prostaglandins

$PGF_{2\alpha}$ concentrations were estimated in 50 ml aliquots of culture medium after 10fold dilution with extraction buffer using ELISA kit (404710, 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%. Concentrations of PGE_2 were estimated 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, 13,14 dihydro-15 keto-prostaglandin $F_{2\alpha}$, prostaglandin D_2 and prostaglandin $F_{2\alpha}$ were >0.01% for all. Intra and inter-assay coefficients of variation were less than 13%.

RNA isolation

Cultured cell pellets of both the treatment and control groups were used for the extraction of total RNA 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. RNA integrity 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 synthesis kit (Biorad iScript™ cDNA synthesis kit, 1708890, Biorad, USA) was used to synthesize cDNA following the protocol - I script reverse transcriptase (1 µl), 5X iScript reaction mix (4 µl), Nuclease free water (14 µl) and (100 ng) RNA (1 µl) to make total volume 20 µl. The reactants were 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 were 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 25 µl total volume. Reaction mixture contained of 2 µl first strand of cDNA, 10X Buffer (2.5 µl), 50 mM dNTP Mix (0.5 µl), 25 mM $MgCl_2$ (2.5µl), forward primer (1 µl), reverse primer (1 µl), Mili Q water (15.3 µl) and 0.2 µl Taq DNA Polymerase (5U/µl). Following initial denaturation at 95°C for 5 min, amplification (30 cycles) was carried out under the conditions mentioned in Table 1, followed by final extension at 72°C for 10 min.

The generated cDNA fragments were resolved by agarose gel (1%) electrophoresis.

Real time PCR

PCR analyses were performed with three replicates per sample of each gene using the STEP ONE PLUS real time PCR system (Applied Bio systems, USA), Fast SYBR Green Master Mix (KK4604, Applied Bio systems, USA) and gene specific primers for both housekeeping and target genes (Table 1). Primer 3 program (Rozen and Skaletsky, 2000) was used for designing the primers with an annealing temperature of 59°C and amplification size of less than 250 bp. Primer efficiencies were checked by 10 fold serial dilution of cDNAs (Svec *et al.,* 2015) and ranged between 95 and 105%. β-actin was used as endogenous control. Thermal cycling was carried out 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). The specificity of each PCR product was determined by melt curve analysis and amplicon size determination was carried out by agarose gel electrophoresis (2%). Negative control consisted of all the 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 controls serves as calibrator.

Statistical analysis

The results are shown as the Mean±S.E.M. of values obtained from 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 TNF_α (0.1 nM, 0.5 nM, 1.0 nM) on in vitro $PGF_{2\alpha}$ production in buffalo luteal cells is presented in Fig 1(a). The concentrations of $PGF_{2\alpha}$ were 110.2±11.53, 122.6±19.04, 124.6±22.68, 130.4±15.96 pg/ml in control, 0.1 nM, 0.5 nM and 1.0 nM TNF_α treatment group, respectively. The effect of various doses of TNF_α (0.1 nM, 0.5 nM and 1.0 nM) on in vitro PGE_2 production in buffalo luteal cells is presented in Fig 1(b). The levels of PGE_2 were 2.27±0.36, 2.84±0.37, 2.99±0.42 and 3.17±0.57 pg/ml in control, 0.1 nM, 0.5 nM and 1.0 nM TNF_α, respectively.

The effect of different doses (0.1 nM, 0.5 nM, 1.0 nM) of TNF_α on PGES and PGFS mRNA expression is presented in Fig 2(a) and 2(b). TNF_α causes dose dependent increase in PGFS mRNA expression. However, there was no significant difference in PGFS expression among different groups. There was non-significant ($P>0.05$) decrease in PGES mRNA expression at 0.1 nM and 0.5 nM but increase at 1.0 nM concentrations of TNF_α in comparison to control group. However, there was no significant differences ($P>0.05$) in PGES and PGFS mRNA expression among various groups.

To the best of our knowledge and belief, this is the first study to report the effect of TNF_α on *in vitro* prostaglandin production and expression of PGFS and PGES mRNA in buffalo Corpus luteum. The present study showed that TNF_α stimulated $PGF_{2\alpha}$ and PGE_2 production by cultured buffalo luteal cells. There was dose dependent increase in the concentrations of both $PGF_{2\alpha}$ and PGE_2 with the increasing doses of TNF_α. However, no significant difference in $PGF_{2\alpha}$ and PGE_2 concentrations were found among the different treatment groups. Since early corpus luteum is relatively resistant to $PGF_{2\alpha}$, mid stage CL was chosen in the present study to see the effect of TNF_α on luteal cell $PGF_{2\alpha}$ production. Our results are in alignment with the earlier report of Benyo and Pate (1992) wherein TNF_α stimulated $PGF_{2\alpha}$ secretion by the cells in a dose-dependent fashion in mid-stage bovine CL. Similarly, Sakumoto *et al.* (2000a) reported that TNF_α significantly stimulated both $PGF_{2\alpha}$ and PGE_2 secretion in the bovine luteal cells in a dose-dependent fashion. In porcine species also TNF_α had been shown to stimulate both $PGF_{2\alpha}$ and PGE_2 secretion in a dose-dependent manner in cultured mid luteal cells (Miyamoto *et al*., 2002). The production of both PGE_2 and $PGF_{2\alpha}$ was dose-dependently increased by recombinant human TNF_α ($rTNF_\alpha$) in cultured human granulosa-lutein cells (Wang *et al*., 1992).

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

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

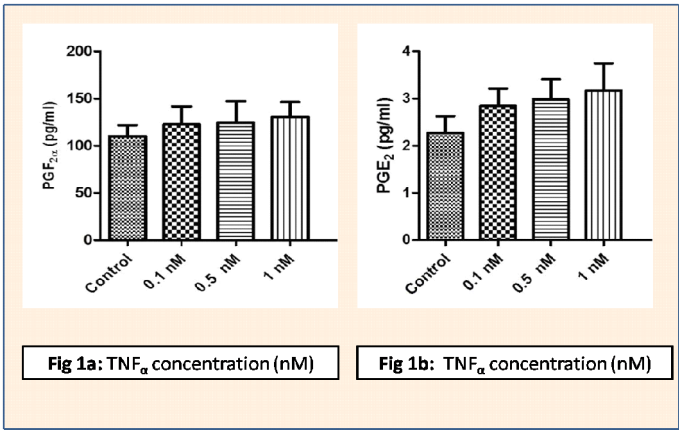

Fig 1: Effect of TNF_{α} on $PGF_{2\alpha}$ and PGE_2 production by luteal cells in buffalo.

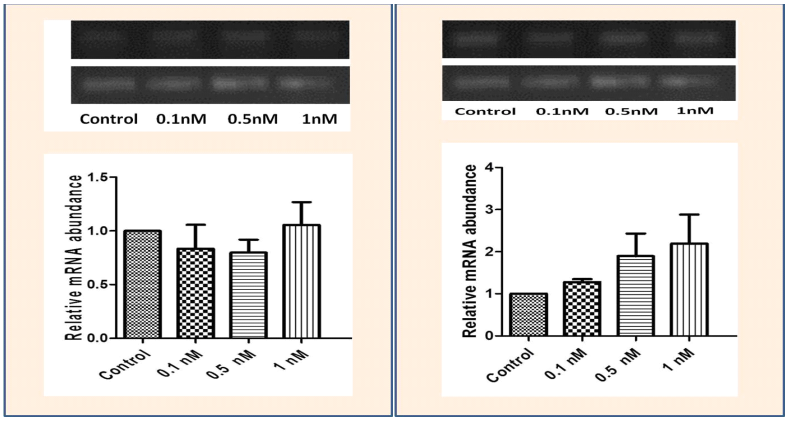

Fig 2: Effect of increasing concentrations of TNF_{α} on PGES (a) and PGFS mRNA expression in buffalo luteal cell.

However, our results are not in agreement with the findings of Skarzynski *et al.* (2003) and Korzekwa *et al.* (2008) who reported that infusion of lower concentration of TNF_{α} caused the increase in plasma $PGF_{2\alpha}$ level and luteolysis whereas its higher concentration stimulated PGE_2 level and prolongs the estrous cycle in cattle. (Skarzynski *et al.*, 2003; Korzekwa *et al.,* 2008) which may be due to species difference. The stimulatory effect of TNF_{α} on $PGF_{2\alpha}$/PGE_2 had also been shown in various tissues of different species including bovine endometrium (Miyamoto *et al.*, 2000; Okuda *et al.*, 2002; Skarzynski *et al.*, 2003), equine endometrium (Szóstek *et al.*, 2014), porcine endometrium (Blitek and Ziecik, 2006; Waclawik *et al.*, 2010), porcine maternal placenta (Jana *et al.*, 2008), human luteal phase endometrial cells (Chen *et al.,* 1995) and human fetal membranes (Kent *et al.*, 1993).

Most prostaglandins are produced from arachidonic acid (AA) which is released from the membrane phospholipids via the action of Cytosolic phospholipase A2 (cPLA2) (Wiltbank and Ottobre, 2003). Cyclooxygenases (COX-1 and COX-2) convert AA 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 formation of PGE_2 and $PGF_{2\alpha}$ respectively from PGH2 (Smith and Dewitt, 1996). 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). The present study showed that TNF_{α} increased PGFS mRNA expression in dose dependent manner. Our findings gain support from the finding of Korzekwa *et al.* (2011) who reported that TNF_{α} increased mRNA expression of PGFS in immortalized bovine luteal endothelial cell line. In our results the stimulatory effect of TNF_{α} on both $PGF_{2\alpha}$ and PGE_2 production indicated that the regulation of synthesis of both PGs by TNF_{α} may be at some common sites like substrate availability, phospholipase A_2 or cyclooxygenase enzyme activity as well at PGFS level in case of $PGF_{2\alpha}$ synthesis but it may not be at PGES level in case of PGE_2 synthesis as Parent *et al.* (2002) reported that TNF_{α} stimulated PGE_2 output but not COX-2 expression. Therefore, it is possible that TNF_{α} may stimulate the production of PGE_2 by increasing the availability of arachidonic acid which is the common substrate for PGs synthesis. Another source of $PGF_{2\alpha}$ is PGE_2, which could be converted to $PGF_{2\alpha}$ by PGE2 9-keto reductase (Duffy, 2015). TNF_{α} stimulated $PGF_{2\alpha}$ secretion through stimulation of phospholipase A_2 (PLA2) and MAPK pathways in bovine luteal cells (Sakumoto *et al.,* 2000b). TNF_{α} is potent

stimulators of luteal prostaglandin production and appears to act primarily by stimulating phospholipase A_2, (Pate, 1995). TNF_α stimulated $PGF_{2\alpha}$ production by luteal cells is dependent upon the stimulation of phospholipase A_2 through mechanisms requiring synthesis of RNA and protein (Townson and Pate, 1996). The mechanism of action of TNF_α on PGE_2 is not well documented in luteal cell. However, Waclawik *et al*. (2010) reported that TNF_α significantly stimulated PGE_2 synthesis and release through up-regulation of prostaglandin-endoperoxide synthase 2 mRNA, as well as PGE_2 synthase (mPGES1) mRNA and protein expression in endometrial luminal epithelial cells of porcine collected from days 11-12 of the estrous cycle and pregnancy. Similarly, in another experiment in equine endometrium, Szóstek *et al*. (2014) reported that TNF_α stimulated PGE_2 production to a greater extent and $PGF_{2\alpha}$ secretion to a lower level through up-regulation of PG synthases mRNA transcription.

CONCLUSION

The overall result indicates that TNF_α may serve as an important modulator of prostaglandins production in the developing stage of buffalo corpus luteum; however, the possible physiological role of TNF_α for simultaneous increase of intra-luteal $PGF_{2\alpha}$ and PGE_2 production seems counterintuitive and needs further detailed studies to clarify its precise role.

ACKNOWLEDGEMENT

We thank Director, NIANP for providing the necessary facilities for conducting the research work. Thanks are due to Director, CIRB for granting permission to conduct research work at NIANP Bangalore, India.

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