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ISOLATION AND CHARACTERIZATION OF LUTEAL CELLS IN BUFFALO (*BUBALUS BUBALIS*)

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Abstract : The objective of the study was to evaluate the morphological and functional characteristics of luteal cells isolated from buffalo ovary. Luteal cells exhibited columnar morphology and contact inhibition at the stage of confluence. Protein concentrations increased linearly from Day 3 to Day 7 of culture. DNA concentrations increased from Day 3 to Day 5 and then declined to Day 7 of culture. $PGF_{2\alpha}$ concentrations decreased progressively from Day 3 to Day 7 of culture. It was concluded that buffalo luteal cells could serve as an excellent model for studying the specific role of $PGF_{2\alpha}$ in maternal recognition of pregnancy and implantation.

Key words : reproductive efficiency prostaglandin luteal cell buffalo

INTRODUCTION

Early embryonic mortality is a major impediment hampering the reproductive efficiency of buffalo (23, 26, 34) and accounts for a main component of post fertilization losses. It has been observed that more than 40% of the total embryonic mortality occurs between days 8 and 17 of pregnancy in bovine (14). The early embryonic development including implantation of foetus and maintenance of pregnancy are critically influenced by embryo-maternal cross talk. The survivality of embryo during early embryonic life is mostly dependent on the efficiency with which the maternal recognition of pregnancy (MRP) is established. Maternal recognition of pregnancy results from signaling between the trophoblast of conceptus and the maternal system. These signals ensure maintenance of structural and functional integrity of corpus luteum (CL). The CL produces progesterone which is required to stimulate and maintain endometrial function that are permissive for early embryonic development, implantation, placentation and successful fetal and placental development.

Higher rates of early embryonic wastage during pre-implantation period may be

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associated with inadequate luteal function (25, 27) which may not only delay the embryogenic development but also impair the ability of embryo to continue its presence to the maternal system. Prostaglandins (PGs) are the major contributors to the regulation of reproductive processes viz., ovulation, luteolysis, implantaion, decidualization and parturition (22, 24, 28, 32). Prostaglandin E_2 (PGE_2) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are the major secretory products of uterine endometrium in ruminant. Both are known to induce opposite effects on uterus and corpus luteum. PGE_2 protects CL from the spontaneous regression (20) and helps in the maintenance of pregnancy. It enhances endometrial vascular permeability at the sites of blastocyst apposition and decidualization (16, 17). PGE_2 also plays a major role in blastocyst hatching (1) and implantation (12). In contrast, $PGF_{2\alpha}$ acts as the luteolytic agent (3) to control estrous cycle in ruminants. Although progesterone is the major luteal hormone, the CL also produces a number of other substances including PGs and oxytocin. The PGs are of particular interest, because of the involvement of $PGF_{2\alpha}$ in regression of the CL in many species. Production of $PGF_{2\alpha}$ directly by luteal cells has been reported in cows (21, 31), pigs (7, 9), rhesus monkeys (15), sheep (33, 35) and rats (30). Exploring the functions of luteal cell is important in order to understand the basic information of maternal recognition of pregnancy and implantation in buffalo. Nevertheless, there is no report which analyzes the functions of luteal cells in terms of prostaglandin production in vitro in buffalo. The aim of the present study is to isolate, culture and characterize luteal cells in buffalo.

MATERIALS AND METHODS

Source of materials

RPMI 1640, HBSS, L-glutamine, BSA, Calf Thymus DNA, type II collagenase were procured from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue, penicillin, gentamycin, streptomycin, amphotericin were from HiMedia, Mumbai; sodium carbonate, sodium hydroxide, copper sulphate, sodium potassium tartarate, folin ciocalteau reagent, diphenylamine, glacial acetic acid, sulphuric acid were from SRL, Mumbai. Prostaglandin ELISA kits were procured from Neogen, USA. Tissue culture plates and plastic strainer (70 µM pore size) were procured from BD Falcon, Bedford, MA, USA.

Collection of buffalo CL

Ovaries with CL from buffalo were collected at a local abattoir within 1 hr after exsanguination. The stages of the estrous cycle were determined according to previously described methods (8, 37) 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 developing corpus luteum (Stage I) initially appeared as red elevation protruding from ovarian surface, reaching its developmental stage with dark red projection between days 6 and 10 of the cycle. Between days 11 to 16 the matured corpus luteum changed its colour from dark red to orange, firm in consistency on palpation and demarcated from rest of the ovary. After day 16, corpus

luteum (Stage III) showed regressive changes like disappearance of blood vessels, decrease in size, change in colour from red to yellow and appeared as hard nodular structure. In the present study, luteal tissues of the midluteal phase (Days 6 to 15 of the estrous cycle) were used. The ovaries with CL were submerged in icecold 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 g/ml)] and transported to the laboratory.

Isolation of luteal cells

The luteal cells were separated by using a modification of procedures described by Hansel and Dowd (11) and Brunswig et al. (2). Corpus luteum was separated from surrounding tissues, chopped, rinsed a few time with Hanks Balanced Salt Solution (HBSS) medium supplemented with gentamycin (50 µg/µl, pH 7.4) and 0.1% bovine serum albumin (BSA) and incubated at 37°C for 1 hr in HBSS containing 0.1% collagenase (Type II collagenase from Clostridium histolyticum, pH 7.4). The cell suspension obtained from the digestion was filtered through plastic strainer (70 µM) to remove undissociated tissue fragments. The filtrate was washed 3 times by centrifugation at 600xg 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

After cell counting and viability determination, the epithelial cells were seeded at the rate of 1×10^5 viable cells in RPMI 1640 medium (Roswell Park Memorial Institute 1640 medium) at 38.5°C in presence of 5% CO_2 for 7 days. The medium was changed every 2-3 days until the confluency was reached. The spent medium was collected on day 3, 5 and 7 of culture to quantify the levels of prostaglandins.

Recovery of cells for protein and deoxy ribonucleic acid (DNA) quantifications

The cells were washed three times with Dulbecco Phosphate Buffer Saline (DPBS) and separated by centrifugation at 500×g for 15 min on Day 3, Day 5 and Day 7 of culture. Growth pattern was monitored by measuring the levels of protein by folin-phenol reagent (19) and of DNA by diphenylamine method (4) in the cell pellet. For quantitation of protein, 25 µl suspension of cell pellet was taken and the volume was made upto 500 µl with double distilled water. 5.5 ml of alkaline copper reagent (1 ml of 1% (w/v) $CuSO_4$ and 1 ml of 2% (w/v) Na Tartarate mixed with 98 ml of 2% (w/v) Na_2CO_3 in 0.1 M NaOH) was added and mixed well immediately and allowed to stand for 10 min at room temperature. 500 µl Folin Ciocalteau was added and incubated at room temperature in the dark for 30 min. The sample was read in 650 nm in spectrophotometer. A series of tubes containing 25, 50, 100, 150, 200 and 250 µg of BSA was prepared for standard. For estimation of DNA in cell pellet suspension, 25 µl suspension was taken and the volume was made upto 1 ml with double distilled water. Subsequently 2 ml of diphenylamine reagent (1 gm diphenylamine dissolved in 100 ml of glacial acetic acid and 2.75 ml concentrated H_2SO_4) was added and allowed to stand for 10 min at room temperature. After 10 min the reading was taken in 600 nm in spectrophotometer. A

series of tubes containing 10, 25, 50, 100, 150, 200 µg of calf thymus DNA was prepared for standard.

Determination of PGF_{2a}

The concentration of $PGF_{2\alpha}$ were determined in 50 µl aliquots of culture medium after 10 fold dilution with extraction buffer using Enzyme linked immunosorbant assay (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 18%.

Statistical analysis

Data were analyzed for descriptive statistics and significance has been obtained by using the Repeated Measures Analysis of Variance (SPSS 16.0).

RESULTS AND DISCUSSION

To the best of our knowledge and belief, this is the first study to report $PGF_{2\alpha}$ profiles in buffalo luteal cells during different days of culture. Methods for separation and culture of luteal cells have been reported for several species, but not in buffalo. In this study, we report the separation and culture of luteal cells from buffalo ovary using the modification of methods described for bovines by Hansel and Dowd (11) and Brunswig et al. (2). The ultrastructural characteristics of luteal cells are available for rat (29), cattle (5) and sheep (13) using transmission electron microscopy. Scanning electron microscopy revealed that the small and large luteal cells were spherical or slightly elongated in shape in bovines. We used the light microscopy for morphological characterization of buffalo luteal cells in culture. Functional characterization revealed that $PGF_{2\alpha}$ secretion decreased linearly with the increase in days in culture.

The microphotographs of Day 3, 5 and 7 luteal cells in culture are presented in Figures 1-3. The luteal cells began to attach to culture dishes within 24-36 hr after seeding and reached confluence after 6 to 7 day in culture. In primary culture, luteal cells exhibited columnar morphology and showed contact inhibition at the stage of confluence which agrees with earlier report by Chegini et al. (5) in bovines. The viability of luteal cells at the time of plating was greater than 90%. The luteal cell number increased linearly from Day 3 to Day 7 of culture. The pattern of cellular growth was evaluated by measurement of proteins and DNA content in cell pellet (Table I). Protein concentrations have been found to increase progressively with the time in culture. DNA concentrations increased progressively from 16±1.67 µg/25 µl on Day 3 to 26.80±0.80 µg/25 µl (P=0.004; n=5) on Day 5 and then

TABLE I : Growth pattern of luteal cells in culture.

Day of culture	*Protein (µg/25 µl)*	*DNA (µg/25 µl)*
Day 3	2.90±0.78[a]	10.63±1.02[a]
Day 5	15.7±1.34[ab]	13.87±0.55[b]
Day 7	23.10±1.80[bc]	16.28±1.46[c]
Significance	F=58.346; P<0.001	F=24.866; P<0.001

Non-identical superscripts are significant at 5% level of significance.

Fig. 1 : Luteal cells on Day 3 of culture, magnification X10.

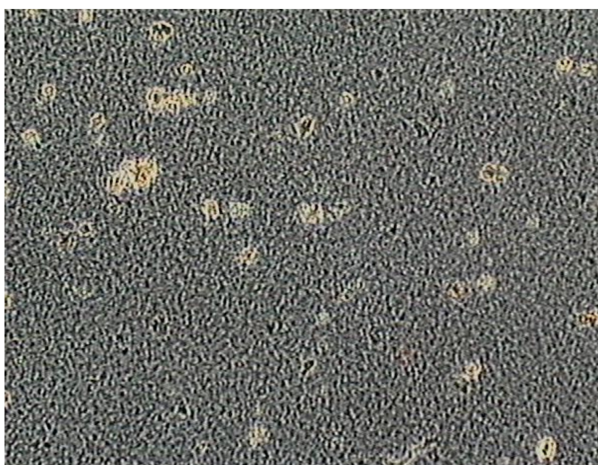

Fig. 2 : Luteal cells on Day 5 of culture, magnification X10.

declined to 18.4±0.68 μg/25 μl (P=0.002; n=5) on Day 7 of culture. The pattern of protein and DNA in buffalo luteal culture confirms the results of Mondal et al. (22, 24) in terms of increase in protein and DNA content with the time in endometrial cell culture. $PGF_{2\alpha}$ concentrations decreased from 16.85±6.64 pg/μg DNA (n=5) on Day 3 of culture to 10.46±2.43 pg/μg DNA (P= 0.0.227; n=5) on Day 5 of culture and thereafter to

Fig. 3 : Luteal cells on Day 7 of culture, magnification X10.

7.06±1.87 pg/μg DNA (P= 0.147; n=5) on Day 7 of culture (Fig. 4).

One of the most important aspects of luteal $PGF_{2\alpha}$ production is that there appears to be an auto-amplification loop such that treatment of luteal cells with $PGF_{2\alpha}$ induces production of $PGF_{2\alpha}$ by the luteal cells. Treatment in vivo with cloprostenol ($PGF_{2\alpha}$ analog) dramatically increased the in vitro production of $PGF_{2\alpha}$ in sheep (33) or pigs (10). Similarly, treatment of large luteal cells in vitro with $PGF_{2\alpha}$ or activation of $PGF_{2\alpha}$ second

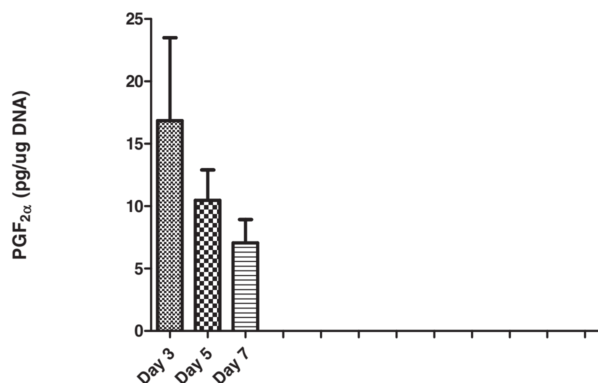

Fig. 4 : $PGF_{2\alpha}$ profiles during different days of luteal culture.

messenger pathways (free calcium, activation of PKC) increased $PGF_{2\alpha}$ production *in vitro* (7, 35). This increase in $PGF_{2\alpha}$ production only occurs in CL with luteolytic capacity (from pigs on day 17 of pseudopregnancy) but not in CL without luteolytic capacity (day 9 of the estrous cycle) (7).

Multiple intracellular mechanisms are involved with $PGF_{2\alpha}$ induced production of intraluteal $PGF_{2\alpha}$. Treatment with $PGF_{2\alpha}$ dramatically increased free intracellular calcium in large luteal cells (38) and free calcium will bind to cytosolic phospholipase 2 (cPLA2) causing translocation to the nuclear membranes. In addition, $PGF_{2\alpha}$ treatment activated mitogen-activated protein kinases (MAP kinases) (6) and activated protein kinase C (PKC, 38), both intracellular effectors that contribute to activation of cPLA2. There was no detectable increase in luteal cPLA2 mRNA after in vivo treatment with $PGF_{2\alpha}$ (36), suggesting that luteal changes in cPLA2 were probably mediated by protein activation rather than transcriptional regulation. Thus, treatment of CL with $PGF_{2\alpha}$ is likely to cause translocation and activation of cPLA2 through a number of key intracellular pathways allowing liberation of free arachidonic acid from membrane phospholipids. Treatment *in vivo* with $PGF_{2\alpha}$ also dramatically increased expression of COX-2 mRNA and protein (7, 18, 35). In vitro treatment of large luteal cells with $PGF_{2\alpha}$ or activators of the intracellular effector systems that are activated by $PGF_{2\alpha}$ also dramatically increased Cyclooxygenase-2 (COX-2) mRNA, protein, and $PGF_{2\alpha}$ production (7, 35). Thus, $PGF_{2\alpha}$ will activate both of the key rate-limiting steps in $PGF_{2\alpha}$ production by activating the cPLA2 protein and by inducing COX-2 activity.

The molecular mechanisms involved in $PGF_{2\alpha}$ induction of COX-2 have been analyzed. There was about a 40-fold induction of reporter gene expression following treatment of transfected large luteal cells with $PGF_{2\alpha}$ (39). This induction could be inhibited by a specific inhibitor of PKC, myristoylated pseudosubstrate for PKC. There are three key DNA response elements in the 5'flanking DNA region that act synergistically to regulate induction of COX-2 by $PGF_{2\alpha}$ in ovine large luteal cells (39). The most critical element is an E-box region that is about 50 bp upstream of the transcription initiation site. Thus, $PGF_{2\alpha}$ acting through PKC and an E-box DNA element, specifically increases COX-2 gene transcription. The physiological significance of this autoamplification pathway has not yet been clearly defined but it would allow small amounts of $PGF_{2\alpha}$ from the uterus to induce a dramatic increase in intraluteal $PGF_{2\alpha}$ production. Intraluteal $PGF_{2\alpha}$ production may be crucial for complete luteolysis. Induction of intraluteal $PGF_{2\alpha}$ production by $PGF_{2\alpha}$ only occurs in CL with luteolytic capacity. In cattle the lack of $PGF_{2\alpha}$ autoamplification in the early CL involves a lack of induction of COX-2. In contrast, in the pig CL there is induction of COX-2 mRNA and protein in either CL without luteolytic capacity (day 9) or with luteolytic capacity (day 17), but there is no increase in luteal $PGF_{2\alpha}$ production in day 9 CL.

In conclusion, we have developed *in vitro* system for isolation and culture of buffalo luteal cells that maintain their morphological characteristics and secrete $PGF_{2\alpha}$. This system can be used for studying the specific role of $PGF_{2\alpha}$ in maternal recognition of pregnancy and implantation in buffalo.

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