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Freezing of endometrial epithelial cells of buffalo cultured *in-vitro*

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Prostaglandins are important regulators of various reproductive events, viz. luteolysis, ovulation, implantation, parturition and pregnancy recognition in farm animals. $PGF_{2\alpha}$ plays a major role in luteolysis and regulation of estrous cycle whereas PGE_2 exert action opposite to $PGF_{2\alpha}$ and favour establishment of pregnancy by its luteoprotective action. The endometrium is a complex tissue containing mainly epithelial and stromal cells. Both types of cells produce isoform of PG, but have different morphological and physiological properties (Fortier *et al.* 1988, Asselin *et al.* 1997, Rajkumar *et al*. 2010). Endometrial epithelial cells mainly secrete $PGF_{2\alpha}$ whereas stromal cells are the principal source of PGE_2. An *in-vitro* system for epithelial cell culture, therefore, constitutes a good model to study regulation of prostaglandin synthesis (Parent *et al.* 2003, Guzeloglu *et al.* 2004), which avoids animal handling, ethics problem and variability of results too. Recently, epithelial cell culture in buffalo has also been established in our laboratory (Rajkumar *et al.* 2010), as a model to study the regulation and modulation of prostaglandin production.

Cryopreservation has most commonly been used method for the long term storage of biological cells. Freezing and storage of endometrial cells allow accessibility of cells to conduct experiment throughout the year, reduce repeated procurement of genital tracts for isolation of cells, as well as reduce variability of results too. In order to study the regulatory mechanism involved in PG synthesis related to either luteolysis or establishment of pregnancy, there is a need to cryopreserve uterine endometrial cells to conduct experiment *in-vitro* at any point of time. Cryopreservation of cells derived from reproductive tracts e.g. bovine endometrial epithelial and stromal cells (Murakami *et al.* 2003), bovine endothelial cells from the corpus luteum (Acosta *et al.* 2007), porcine endometrial epithelial cells (Kim *et al.* 2010) and equine endometrial epithelial and stromal cells (Szostek *et al.* 2012) was established. Till date, no report on cryopreservation of buffalo endometrial epithelial cells has been reported so far. The objective of the present experiment was to study the effect of freezing on morphological and functional characteristic of endometrial epithelial cell of buffalo cultured *in-vitro*.

Isolation of endometrial epithelial cells: Buffalo uteri (3) along with ovaries bearing corpus haemmorrhagicum (day 1–5) were procured from the local abattoir. White side test and uterine cytology was done to rule out infection. Buffalo endometrial epithelial cells were isolated by enzymatic digestion as per Rajkumar *et al*. (2010) with some modifications. Briefly, the myometrial layers were dissected and placed in a beaker containing 0.3% trypsin III in HBSS and digested for 2 h at 5% CO_2, 95% humidified air at 37°C. Cell pellet was finally suspended in 20 ml RPMI-1640 medium with 10% FCS, 50µg/ml gentamicin and 0.25µg/ml amphotericin-B, plated onto 70 cm^2 culture flask and incubated for 3 h to allow attachment of stromal cells. The unattached epithelial cells in the supernatant were collected and counted using haemocytometer and viability was determined by trypan blue dye exclusion test. The supernatant containing epithelial cells was diluted to adjust the cell concentration 2×10^5 cells per ml in RPMI-1640 media and plated on 6 well plate precoated with matrigel diluted in 1: 3 ratio and cultured at 37°C. Media changed every 2 days until monolayer. Further, epithelial cells were subjected to indirect immuno-fluorescent staining for specific marker i.e. cytokeratin (epithelial cells).

After monolayer formation, cells were harvested using 0.25% trypsin and 0.02% EDTA for 5–10 min at 37°C. Final cell pellet was suspended in chilled freezing media (70% culture media + 10% DMSO + 20% serum) to adjust cell concentration at density of 1×10^6 cells/ml after counting and viability assessment. One ml of cell suspension was dispensed into 2 ml cryovial and subjected to slow freezing in cryo 1°C freezing container (isopropanol bath) and kept at – 80°C in deep freezer. Cells were thawed by plunging cryovials into water bath maintained at 37°C. Cells were counted and viability was tested again. Growth characteristics of the frozen thawed epithelial cells were evaluated by re-culturing the cells.

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Supernatant was collected on day 7 for $PGF_{2\alpha}$ estimation using ELISA kit, and cells were harvested for protein concentration determination by Bradford method. Total $PGF_{2\alpha}$ concentration per well was expressed as picograms per microgram of total cellular protein. The data are mean±SEM of values obtained in 3 separate experiments, each performed in triplicate. Independent 't' test was used to show the statistical significance among basal production of $PGF_{2\alpha}$ in frozen and unfrozen cells using SPSS software version 16.

Frozen stored buffalo endometrial epithelial cells after thawing appeared as polygonal/spherical with smaller clumps retaining its normal morphological characteristics when compared to unfrozen epithelial cells indicating no major morphological differences between frozen and unfrozen endometrial epithelial cells. Frozen cells when recultured reached confluency or monolayer by day 7–8 culture in a similar fashion as unfrozen epithelial cells. The viability of frozen thawed epithelial cells at the time of seeding estimated by Trypan blue dye exclusion was lower than unfrozen cells (60 vs 90%). The loss of viability of cells may be due to stress of freezing-thawing process causing injury to the plasma membrane, a primary target of cryo-damage (Gao and Crister 2000). The results are in comparable with the findings of Murakami *et al.* (2003) and Szostek *et al.* (2012) as they also reported no morphological difference between frozen thawed and unfrozen endometrial epithelial cells in cattle and equines, respectively.

Production of $PGF_{2\alpha}$ is a useful parameter for determining the functional status of endometrial epithelial cells. The mean $PGF_{2\alpha}$ production in frozen epithelial cells after monolayer (1.727±0.133 pg/µg proteins) was lower but statistically non-significant (P>0.05) than the unfrozen cells (2.793±0.020 pg/µg protein) indicating susceptibility of these cells to freezing and thawing process. The freezing process may cause the epithelial cells to loose arachidonic acid which might have reduced production of $PGF_{2\alpha}$ (Murakami *et al.* 2003). Results of the present study demonstrated successful freezing of buffalo endometrial epithelial cells without any major impairment in morphological characteristics but with certain loss of its functionality and viability.

SUMMARY

The aim of the present study was to freeze buffalo endometrial epithelial cells cultured *in-vitro* with its morphological and functional characteristics. Epithelial cells isolated by enzymatic digestion from buffalo uterus, were cultured and frozen at –80°C using DMSO as cryoprotectant. Frozen cells were thawed at 37°C and recultured. The culture media was collected at confluence for $PGF_{2\alpha}$ estimation using ELISA. Frozen stored buffalo endometrial epithelial cells immediately after thawing appeared as small clumps and spherical/polygonal in shape retaining its normal morphological characteristics. The viability of frozen thawed epithelial cells at seeding was 60%. Freezing and thawing did not affect morphology of buffalo endometrial epithelial cells, however, basal production of $PGF_{2\alpha}$ in frozen cells were lower than unfrozen cells (1.727±0.133 vs 2.793±0.020 pg/µg protein). The results demonstrated that buffalo endometrial epithelial cells can be frozen without any major impairment in their morphological characteristics but with certain loss of its functionality and viability.

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REFERENCES

Acosta T J, Yoshioka S, Komiyama J, Lee S H, Grazul-Bilska A T and Skarzynski D J. 2007. Effects of storage and passage of bovine luteal endothelial cells on endothelin-1 and prostaglandin $F_{2\alpha}$ production. *Journal of Reproduction and Development* **53**: 473–80.

Asselin E, Drolet P and Fortier M A.1997. Cellular mechanisms involved during oxytocin induced prostaglandin $F_{2\alpha}$ production in endometrial epithelial cells *in-vitro:* Role of cyclooxygenase–2. *Endocinology* **138**: 4798–4805.

Fortier M A, Guibault L A and Grasso F. 1988. Specific properties of epithelial and stromal cells from the endometrium of cows. *Journal of Reproduction and Fertility* **83**: 239–48.

Gao D and Crister J K. 2000. Mechanism of cryoinjury in living cells. *Institute for Laboratory Animal Research Journal* **41**: 187–96.

Guzeloglu A, Michel F and Thatcher W W. 2004. Differential effects of interferon-ô on the prostaglandin synthetic pathway in bovine endometrial cells treated with phorbol ester. *Journal of Dairy Science* **87**: 2032–41.

Kim T S, Kwon E H, Kim S U, Choi H S, Lim H J and Park S B. 2010. Activation and expression of urokinase-type plasminogen activator are modulated by freezing/thawing process through activation of redox signal pathway in primary porcine endometrial cells. *Cryobiology* **60**: 211–16.

Murakami S, Shibaya M, Takeuchi K, Skarzynski D J and Okudu K. 2003. A passage and storage system for isolated bovine endometrial epithelial and stromal cells. *Journal of Reproduction and Development* **49(6)**: 531–38.

Parent J, Villenenve C, Alexenko A P, Ealy A D and Fortier M A. 2003. Influence of different isoforms of recombinant trophoblastic interferons on prostaglandin production in cultured bovine endometrial cells. *Biology of Reproduction* **68**: 1035–104.

Rajkumar R, Singh S K, Kumar A, Shankar U and Agarwal S K. 2010. Morphological and functional integrity of buffalo endometrial epithelial cells cultured *in- vitro. International conference on Physiological Capacity Building in- Livestock under Changing Climate Scenario.* SAPI (Society of Animal Physiologist of India). pp. 18. 11–13 Nov 2010 at IVRI, Izatnagar.

Szostek A Z, Siemieniuch M J, Galvao A M, Lukasik K, Zieba D, Ferreira-Dias G M and Skarzynski. 2012. Effects of cell storage and passage on basal and oxytocin-regulated prostaglandin secretion by equine endometrial epithelial and stromal cells. *Theriogenology* **77**: 1698–1708.