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Isolation and characterization of endometrial luminal epithelial and stromal cells *in vitro*

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Abstract
In this study, we have described a simple method of isolating endometrial luminal epithelial (LE) and stromal (ST) cells using ovine uteri samples obtained from the abattoir. Endometrial lining were digested in trypsin, collagenase and bovine serum albumin dissolved in Hanks balanced solution. The cells were seeded into a 24-well plate at a concentration of 5×10^5 per well, while some were snap frozen and kept at -80^0C for RNA extraction at a later date to determine the presence of leucocytes in the cell culture system. Some cells were also cultured into a 4-well chamber slide for standard immunocytochemistry. At 100% confluence, the media were removed and cells were processed for RNA extraction. The extracted RNA was used in a conventional PCR to detect the presence of progesterone and oestrogen receptors. The cell population was determined by cell morphology and immunocytochemical staining with cell specific staining of cytokeratin and vimentin. The mean yield was 49×10^6 cells per 50 gm of digested endometrial tissue. Separated stromal cells were of higher purity percentage (ST; 90 ± 4%; against LE; 10 ± 4%) than epithelial cells which tended to have more stroma (LE; 83 ± 10%, against ST; 17 ± 10%). At 100% confluence (usually day 7-8), the mean percentages LE and ST in the mixed culture were 59% and 41% respectively. The LE expressed cytokeratin protein while, ST expressed vimentin markers. The absence of CD45 marker confirmed the absence of leucocyte in the cell culture system. Progesterone and oestrogen receptor were present in the cell culture. Finally, this study offers a simple method using a single digestion process to isolate mix population of endometrial LE and ST, while comparing favourable with other methods with double digestion system in term of cells yield and characteristic features of the isolated cells.

Keywords: Cytokeratin, Luminal epithelia, Oestrogen, Progesterone, Stroma, Vimentin
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Introduction

The primary function of uterus is to support a pregnancy. Implantation requires a reciprocal interaction between the implantation-competent blastocyst and receptive maternal endometrium. It has been a growing concern of reproductive biologists to understand this complex process in order to institute concepts towards attaining optimal reproductive capacity and health in animal and human subjects. *In vivo* study presents the most physiological conditions under which cell differentiation and implantation occurs. However, the extraordinary complexity of living organisms is a great barrier to the identification of individual cells components and the exploration of their basic biological functions. The information from *in vivo* studies on implantation in human is limited due to obvious ethical issues (Aplin *et al*., 2008). Indeed, implantation related studies in domestic species particularly in sheep have contributed immensely to the ever growing body of knowledge on the molecular mechanism underlying implantation in mammalian species (Lee & DeMayo, 2004; Spencer

et al., 2004). Animal model is not, however, without its own limitations such as (i) poor reproducibility of results (ii) limitation in manipulating experimental condition and (iii) legal issues with animal welfare as well as economic and time constraints. Thus, in vitro techniques remain a valuable tool in reproductive science.

There are two main cell types in the endometrium, epithelia cells (luminal epithelia (LE) and glandular epithelia, (GE)) and stroma (ST). Other cell types include immune and endothelial cells. Prostaglandins (PG) play crucial roles in many reproductive processes. In the non-gravid endometrium, $PGF_{2\alpha}$ is principally produced by epithelial cells and PGE_2 by stromal cells (Weems *et al*., 2006). Towards simulating physiological endometrial cellular components and milieu, attempts were made to isolate endometrial epithelial and stromal cells *in vitro*. Mammalian endometrial cell culture system was first established using endometrial cells from adult rabbit uterus (Whitson & Murray, 1974). Endometrial cell culture of either single or mixed culture of LE and ST in other mammals such as mice, guinea pigs, pigs, human, cows and sheep have successively followed (Kleinman *et al.,* 1983; Fortier *et al.,* 1988; Refaat *et al.,* 2012). The limitation of endometrial cell culture system is that, they do not replicate in totality of the micro-architectural configuration of intact endometrium. Of recent interest is the development of endometrial culture systems using advanced technologies such 3-dimensional (3-D) culture systems and cell lines (Haycock*,* 2011). Also, the immortalised cell lines of LE, GE and ST have been generated and characterised in many mammals including sheep (Johnson *et al.,* 1999). In one study, a 3-D system of human endometrial cells and cell lines was constructed with fibrin-agarose as matrix scaffold, upon which trophoblast-like Jar spheroids were superimposed (Wang *et al.,* 2012). However, based on the results of previous studies in our laboratory, the immortalised LE failed to exhibit certain characteristic features which are distinct of the cell type (Raheem, 2013). They lacked progesterone receptor, cyclooxygenase-2 (COX-2) and did not respond to oxytocin challenge despite their high rate of proliferation. In addition, the use of these advanced techniques can further complicate analysis of results of an intrinsically complex phenomenon like implantation. Therefore, despite the resemblance of endometrial cells (derived from these advanced systems) to the normal endometrial structure, these techniques are yet to be accepted generally as standard models in studying implantation. The advantages of using endometrial cells *in vitro* in studying implantation include the ability to manipulate cells and streamlined experimental content/context and environment to study specific cell types and their functions. In addition, endometrial cells *in vitro* enables the opportunity to eliminate or add co-factors in a step-wise manner to determine if an outcome is actually due to action of a specific factor.

Many studies have described method of isolating endometrial cells *in vitro* (Whitson & Murray, 1974, Haycock, 2011). Most of these either used sophisticated equipment or were cumbersome with multiple digestions. In this study, we have described a simple method of isolating individual or mixed endometrial cells that comprise luminal epithelial (LE) and stromal cells (ST) using a single digestion. The cells were characterised using morphology, expression of cytoskeletal proteins, progesterone and oestrogen receptors and leucocyte maker, CD45.

Materials and methods

Experimental design

This study aims to provide a simple method of cell isolation and culture using abattoirs uterine samples. The digestion was done once. LE and ST were identified in the mixed endometrial cells culture using expression of cytoskeletal proteins by immunocytochemistry. Endometrial cells meant for immunocytochemistry were cultured in a four-well chamber slide (Fischer Scientific, Loughborough, UK). The chambers could be easily freed from the slide for immunostaining after fixation. The cells were grown to 70-100% confluence and then fixed with ice-cold acetone for immunocytochemistry. Also on day 1 of the culture, some cells were snap-frozen and then stored at -80^0C for mRNA extraction at a later date. The latter were used to determine the presence of leucocytes in the culture by looking at expression of a leucocytes marker CD45 (receptor-type C tyrosine-protein phosphatase) using conventional PCR. Some cells cultured in a 24-well microplate were harvested at the time of 100% confluence usually on day 7-8 to determine the expression of oestrogen and progesterone receptors in the cell culture system. The cell population was determined by i) cell morphology as was described in previous study (Fortier *et al*., 1988) and ii) immunocytochemical staining with cell specific staining of cytokeratin and vimentin following the method described previously (Arnold *et al*., 2001). The cells yield, ratio of LE and ST in the mixed culture**,** percentage purity of individual LE or ST

isolated and expression of CD45 in this methods were compared to other similar methods of endometrial cell culture.

Cells isolation and culture

Primary endometrial cells were isolated and cultured as previously described (Swangchan-Uthai *et al.,* 2012) with some modifications. Fresh ovine uteri samples in the early luteal phase of the oestrous cycle were collected from a local abattoir and transported to the laboratory in an ice pack inside a thermos flask within 60-70 min of animals' slaughter. The recognition of this stage with the aid of corpus haemorrhagicum brought consistency in the uteri sample collected from the abattoir (Ireland *et al.,* 1980). Endometrial lining were sliced out and after washing in Hanks Balanced Salt Solution (HBSS, Sigma, Aldrich) a couple of time the tissue samples were digested in 50 ml of digestive solution consisting of 25 mg of trypsin III (Roche, Welyn, UK), 25 mg of collagenase II (Roche), 50 mg of bovine serum albumin (BSA, Sigma) in 50 ml of HBSS. The tube was incubated for 90 min at 37^0C in shaking water adjusted to 100 moves/min. It was vortexed every 30 min for 30s at medium speed. After the digestion, the cell suspension was filtered through 70 µm mesh size filter cell strainer (Fisher Scientific, Loughborough, UK) into a new 50 ml tube. Enzymatic digestion was stopped by adding 5 ml FBS to the filtrate resulting in a concentration of 10% FBS. Then, 100 ul of DNase (Sigma, DN25; 40 mg/ml) was added and the filtrate was centrifuged at 300 g for 10 min. The supernatant was discarded and onto the pellet, 5 ml of sterile distilled water was added for 10 s to the cells pellets to wash and remove blood cells contamination as the blood cells undergo haemolysis in the water. Immediately after, an equal amount of 2 × PBS was added to equilibrate osmolarity of the solution and the tube was centrifuged again at 300 g for 10 min.

After the second centrifugation, the cells pellet was mixed with 5 ml of culture media. Cell viability and concentration were determined using the trypan blue exclusion method with the aid of a Neubauer haemocytometer chamber under light microscopy in accordance with guidelines supplied by Cascade Biologics, Inc. (2002). The concentration of the washed pellet was adjusted with Dulbecco Modified Eagle/F12 medium (DMEM, Sigma, Aldrich) containing 50,000 U of penicillin and streptomycin and 10% fetal calf serum (Sigma) to 5×10^5 cells/ml. Following several pipetting, 1 ml of the cell suspension was added per well in a 24-well Nunc® microplate (Thermo Scientific, Roksilde, Germany). The plates were then incubated in a humid atmosphere at 37^0C with 5% CO_2. The isolated endometrial cells contained population of both uterine LE and ST cells. To separate the LE cells from the ST, the differential attachment method was employed (Fortier *et al.,* 1988). It is expected that the ST cells attach to the bottom of the wells whereas the LE cells require longer duration for attachment. Preliminary works in our laboratory showed that the stroma would have firmly attached to the culture plate after 18 h of culture and can be separated from the floating LE cells. At 18 h of culture, the LE cells were collected with the media and re-plated into a new plate. The culture media was then changed for both individual and mixed LE and ST every 48 h for 7-8 days for the cells to attain 100% confluence.

Immunocytochemistry

Cells for immunocytochemistry (ICC) were cultured in a 4-well chamber slide (Themo Scientific, Germany) usually at a concentration of 100,000 cells per well in a 250 µl volume of media. At the end of the culture, the culture media were removed from the wells and rinsed with PBS, followed by fixing the cells with cold acetone incubation for 10 mins at -20^0C. Then, the slide was freed from the attached well chamber component for easy handling. The IHC procedures for cytoskeletal proteins had been described elsewhere (Krishnaswamy *et al.,* 2009) using mouse anti-vimentin (Sigma, Aldrich) for stroma and mouse anti–cytokeratin (Sigma) for luminal epithelial cells. Briefly, after rehydration of the cells in PBS, nonspecific binding was blocked using normal donkey serum (Santa Cruz, Heidelberg, Germany) for 90 min in a humidified chamber. This was followed by incubation with primary antibodies, mouse anti-cytokeratin (Sigma, 1:100) and mouse anti-vimentin (Sigma Aldrich, 1:100) at 4^0C overnight. The control slides were treated with PBS and mouse immunoglobulin (Santa Cruz) as negative and isotype controls respectively. Washing in PBS was done after each step following the primary antibodies incubation. The secondary antibodies was FITC-conjugated donkey anti-mouse IgG (Jackson Immunology, West Baltimore Pike, West Grove, USA) and was applied under a dark condition for 1 h duration. Following Hoechst (Thermo scientific) incubation for 5 mins, the slides were mounted with Vectashield (Vector Lab., Burlingame, USA).

RNA extraction

For the endometrial cell culture, RNA was extracted using a commercial kit (RNeasy Mini Kits; QIAGEN Ltd, West Sussex, UK) according to the manufacturer's instructions (QIAGEN, 2013a). At the end of the culture, media from each well were collected into a 1.5 ml ependorf and stored at -20^{0}C until further analysis. The cells were rinsed with PBS and then harvested directly from the plate by scraping the cells with the rubber plunger of a sterile tuberculin syringe after adding 120 µl highly denaturing guanidine isothiocyanate (GITC)-containing buffer (RLT buffer, RNeasy Lysis Buffer, supplied in the kit). The latter had been previously mixed with 10 µl β (2)-Mercaptoethanol (Sigma) per 1 ml RLT buffer. The mixture (of cells and the buffer) was pipetted several times through a 26' gauge needle to make cell lysates. An equal amount of ethanol (120 µl) was added to each well, while cell lysate from three wells (720 µl) for each treatment were pooled together. The cell lysate was then put into a column tube provided with the Kits and centrifuged at 300 g for 15 s. Having washed with the provided 'RW1' and 'RPE' buffers which contained a guanidine salt and ethanol respectively, the column was dried of ethanol by centrifugation at 300 g for 15 s and put on top of a new ependorf tube. In the final stage, the isolated RNA attached to the column membrane was eluted with 30 µl RNase-free water and collected into a sterile eppendorf tube after centrifugation at 300 g for 1 min.

Reverse transcription and conventional PCR

Total RNA (500 or 1000 ng) from each sample was treated for potential genomic DNA carry over in a single reaction with DNAse in accordance with manufacturer instruction (Promega Corporation, Madison, WI). For the CD45 control, the whole blood of sheep was used for DNA extraction with the aid of DNA kit (QIAGEN Ltd, West Sussex, UK) according to the manufacturer's instruction (QIAGEN, 2013b).

DNase-treated 1 µg RNA of endometrial cells from the cell culture was reverse transcribed using random hexamer primers and processed accordingly (Reverse Transcription System Kit; Promega) in a 20 µl reaction using a 200 µl ependorf tube as was described previous study (Raheem *et al.,* 2013). It was assumed that the reaction was 100% efficient. The double stranded cDNA (500 ng/20 µl or 1000 ng/20 µl) was centrifuged and diluted to 5 ng/ µl. This may be aliquoted into 5 µl per tubes and kept at -20^{0}C until usage for conventional PCR

The forward and reverse primers for progesterone receptor were 5' ATTGTTGATAAAATCCGCA '3 and 5' GAGGTATCAGGTTTGCTGTTGTC '3 respectively while that of the oestrogen receptor were 5' ATGACCCTACCAGACCTTTCAGT '3 and 5' ATTTGAGGC ACACAAACTCTT C '3 respectively. The PR amplicon size was 380 base pair while that of ER was 357 bp. For CD45, the primers were forward 5' TGACATCAATAGCCCTGCT '3 and reverse 5'ATCCCTTTCAACGAGT TCCT '3 with 101 bp amplicon size. The primers were used to run a conventional PCR using a Multiplex kit (QIAGEN Ltd, West Sussex, UK) according to manufacturer's instruction. Briefly, a master mix of 50 µl per reaction was constituted into a 200 µl ependorf tube containing the following; 25 µl Multiplex, 10 µl Q-solution, 5 µl primer (2uM, PR or ER), 10 µl RNase-free water and 5 µl cDNA of the test sample. In the negative control, the cDNA was replaced by nuclease free water. The mixture was vortexed and centrifuged before being run in a thermal cycler (Techne PCR Machine TC312; Scientific Laboratory Supplies, Yorkshire, UK) under the following conditions; Taq activation 94^{0}C (2 min), denaturation at 94^{0}C (30 s) and a varying annealing temperature for 30 s (PR = 59.5^{0}C; ER = 61^{0}C, CD45=61^{0}C). The polymerase extension and final extension were done at 72^{0}C for 30 s and 5 min respectively.

The amplicons were visualized by electrophoresis on 1% agarose gels with the aid of nucleic acid stain, ethidium bromide (Safe View; NBS Biologicals, Huntingdon, UK) as described in earlier study (Raheem, 2013). At the end of the 45-60 min run, the gels were placed on a UV illuminator (Image Master Video Documentation System, IMVDS; Pharmacia Biotech) for imaging. The images were saved as TIFF/JPEG files for further assessment of PR and ER in the culture system.

Results

Identification of endometrial LE and ST in the cell culture system

The mean yield was 49 × 10^6 cells per 50 gm of digested endometrial tissue. Under phase contrast microscopy, epithelia appeared cuboidal to columnar in shape while stromal cells were spindle shaped which are more distinct in the separated individual LE and ST (Plate 1A and 1B respectively) than the mix culture of both (Plate 1C). The cells were found forming colonies and most often, different colonies of LE or ST were observed with few other cell types within a colony. Few glandular epithelial cells were observed amidst colonies of ST or LE (Plate 1D).

In the single staining, LE cells were stained positive with the anti-cytokeratin (Plate 2A). Stromal cells expressed anti-vimentin with characteristic spindle shaped features (Plate 2B). The percentage expression of cytokeratin staining in the stroma and that of vimentin in the epithelia represented the 'contamination' with other cells type in the separate/individual LE or ST cell culture. The negative controls did not show expression of either of the two cells type with their nuclei showing bluish colouration of DAPI (Plate 2C). Estimation of each cell type in the mixed culture was determined by counting LE and ST from at least 20 different fields at 40 × objective of Fluorescence microscope of cytokeratin and vimentin stained cells respectively. Separated stromal cells were of higher purity percentage (ST; 90 ± 4%; against LE; 10 ± 4%) than epithelial cells which tended to have more stroma (LE; 83 ± 10%, against ST; 17 ± 10%). At 100% confluence (usually day 7-8), the mean percentages LE and ST in the mixed culture were 59% and 41% respectively.

Expression of CD45 marker

The isolated endometrial cells at the time of plating the cells failed to show expression of CD45 mRNA while the positive control leucocytes showed a distinct band of CD45 marker (Plate 3).

Expression of progesterone and oestrogen receptors

The presence of PR and ER mRNA in the endometrial cells *in vitro* was demonstrated by gel electrophoresis of PCR amplicons (Plate 4). The results revealed that PR and ER were expressed in the cell culture system. The template negative control in which the RNA was replaced with RNeasy free water did not show any band.

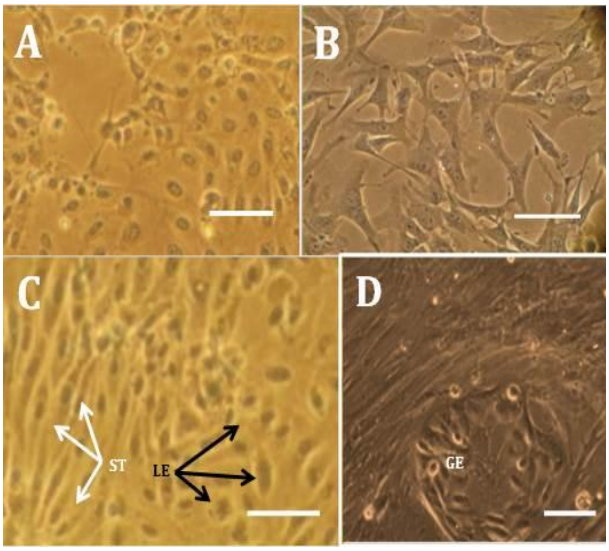

Plate 1A, B, C & D: Phase contrast microscopy of isolated luminal epithelia (LE) and stroma (ST) showing morphology. Individual LE (A), ST (B) and mixed culture of LE and ST and glandular epithelia (GE) observed in middle of ST colony (D) (Bar = 25 µm).

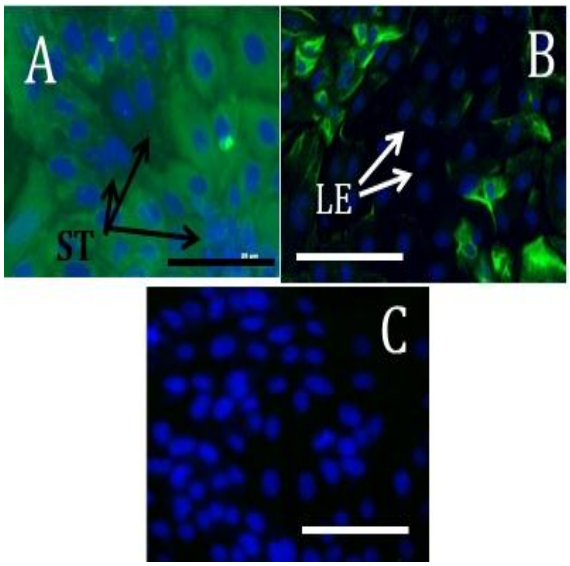

Plate 2 A, B, C & D: Fluorescence microscopy of isolated luminal epithelia (LE) and stroma (ST) in the mix culture showing expression of cytokeratin and vimentin in the LE and ST respectively. The black arrows showed the ST within LE (A), while the white arrows indicated LE within ST (B). IHC negative control stained with isotype mouse IgG showed only bluish nuclei DAPI stains (C). At 100% confluence (usually day 8), the mean percentage LE in the mixed culture was 59 % while ST was 41% (Bar = 50 µm)

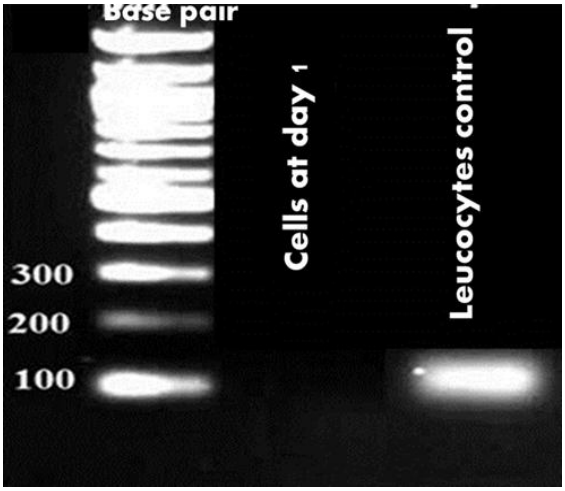

Plate 3: One percent gel electrophoresis of CD45 (receptor-type C tyrosine-protein phosphatase) cPCR product (101bp) in the endometrial mixed cell culture of luminal epithelial and stromal cells at day 1 of culture and the sheep leucocytes positive control

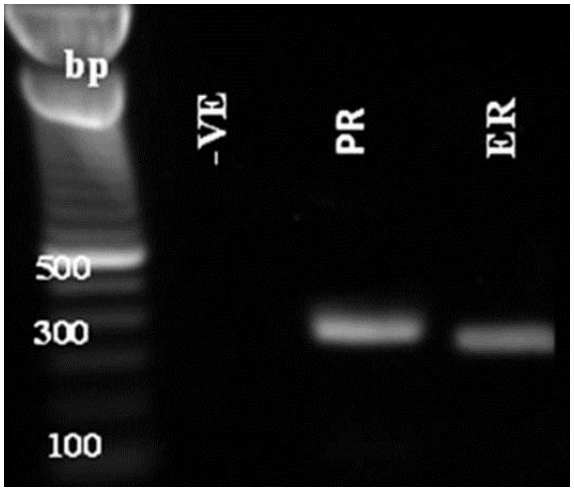

Plate 4: Expression of progesterone (PR, 385 bp) and oestrogen receptor (ER, 357 bp) in the endometrial cell culture. Legend: PR; progesterone receptor, ER; oestrogen receptor, bp; base pairs

Discussion

Uteri at the early luteal phase of oestrus were used for the study based on a number of reasons. Firstly, the recognition of this stage with the aid of corpus haemorrhagicum (Ireland *et al.,* 1980) brought consistency in the uteri sample collected from the abattoir. Secondly, the stage corresponds to pre-implantation stage in a conceptive cycle during which the hormonal profiles and other complemented factors as well as the maturation of endometrial epithelia are inclined towards endometrial receptivity. Total separation of individual LE and ST was difficult. However, with this protocol, there was a high purity of isolated ST (mean 90%) and LE (83%) and substantial cells yield with a mean value of 49×10^6 cells per 50gm of digested endometrial tissue. Stromal cells were of higher purity compared to epithelia which tended to have more stroma. This may be attributed to adhesion of some stromal cells to the plate before the collection of ST for re-plating. Epithelial cells appeared cuboidal or columnar while the ST were spindle in shape as was reported in earlier studies (Varma *et al.,* 1982; Arnold *et al.,* 2001). Phenotypic markers- vimentin and cytokeratin are commonly used to distinguish epithelia and stroma cells respectively (Zhang *et al.,* 1995; Johnson *et al.,* 1999), whose expressions in this study are at par with earlier report (Swangchan-Uthai *et al.,* 2012).

We are inclined towards the mixed culture system rather than a separate LE and ST cell culture for a number of reasons. A 100% separation was not attainable. The two basic cell types in the endometrium are epithelial and stromal cells with distinct cell features and characteristics as observed above. Evidence of previous experiment in our laboratory including the pilot study in this work revealed that the mixed culture system facilitates interaction between these cells in order to produce optimal effect due to a particular treatment (Swangchan-Uthai *et al.,* 2012). Besides the co-culture of both LE and ST where the two cells communicate and interact simulates or mimics the *in vivo* condition better than the single culture (Yoshinaga, 2008; Miki *et al.,* 2012). The paracrine action of the ST was demonstrated to upregulate the growth of epithelia (Arnold *et al.,* 2001). It was on this premise that the mixed culture containing both LE and ST was preferred. The mean percentage of epithelia and ST in the cell culture was 59% and 41% respectively as at the time of 100% confluence usually on day 8. This ratio is higher than that reported in bovine by Swangchan-Uthai *et al.* (2012). The difference might be due to different species and double digestion used in that study as against a single digestion in the present study. Possibly, the second digestion seemed to generate more of epithelia cells that ST. Few GE were observed in the middle of either ST or LE colony as was reported in previous study (Wang *et al.,* 2012).

Prostaglandins (PGs) play essential roles in female reproduction (Weems *et al.,* 2006). Since PGs may be part of the genes to be investigated with the isolated endometrial cells in future study, it was essential to rule out any other extra-source of PGs within the cell culture system apart from the endometrial cells. The absence of CD45 confirms the purity of the cells and rules out the presence of leukocytes which may also produce PGs (Harris *et al.,* 2002), thereby eliminating any extra-source of PGs apart from the LE and ST. In this context, this differs from the report of Swangchan-Uthai *et al.* (2012) where leucocytes though extremely very minimal were detected in bovine endometrial cell culture. It is possible that the detection method used in this study (cPCR) is not robust enough to detect the small number of leucocytes that might be present in the cell culture as against qPCR used by Swangchan-Uthai *et al.* (2012). In the present study, blood cells were lysed by addition of sterile water which was quickly followed by adding 2 × PBS. Haemolysis of blood cells occurs in hypotonic solution like water. In addition, extreme care was exercised to isolate only the endometrial layer, without going deep enough into the vascularised muscular layer.

Dynamic changes in the endometrium during oestrous cycle and pregnancy are associated with progesterone and oestrogen, whose effects are mediated via their respective receptors (Gadkar-Sable *et al.,* 2005). Therefore, it becomes incumbent to demonstrate that these cells possess steroids receptor so that their prospective biological effects via their receptors in the cell culture system are guaranteed. ER and PR were expressed by the cells. There are two forms of ER, ERα and ERβ, while the two isoforms of PR are PRA and PRB (Meikle *et al.,* 2004). It is possible that these different isoforms are present in the cells culture despite that no attempt was made to specify a particular isoforms expressed in the culture system.

In conclusion, we have described in this study a simple method of isolating endometrial LE and ST from uterine samples obtained from the abattoir. The method generated a substantial number of endometrial cells with characterised features similar to the same cells *in vivo.* They express cytoskeletal proteins (LE are cytokeratin positive while ST expressed vimentin marker). The isolated cells also express receptors for progesterone and oestrogen. Thus, the method described here and the characteristics of the isolated endometrial LE and ST cells indicate that this method could be useful in future studies related to the effect of progesterone and oestrogen on the endometrium. This is of particular importance especially in developing countries where availability of facilities such as those used in other cell culture system is a major bane of conducting research.

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