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IN-VITRO GROWTH OF *CHLAMYDOPHILA ABORTUS* IN OVINE ENDOMETRIUM: EVIDENCE OF GROWTH SUPPORT IN STROMAL FIBROBLAST CELLS

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ABSTRACT

The aim of present study was to develope an *in vitro* model to investigate the mechanism of *Chlamydia abortus* (*C. abortus)* infection in endometrial cells. The cells were isolated from uterine tissue of cyclic ewes (n=6) through digestion by collagenase alone or collegians plus trypsin. Digestion with collagenase alone yielded a lower epithelial-stromal cell ratio compared to digestion with collagenase and trypsin. Monolayers grown from cells using both enzymatic digestion methods were provisionally identified as epithelial or stromal fibroblast. The epithelial and fibroblast cell monolayers were infected with 50 µl of McCoy cell lysate containing $3x10^4$ IFU/ml of *C. abortus.* No inclusions were observed in endometrial epithelial cells after 48-72 h post infection, however, typical inclusion bodies were observed in stromal fibroblasts. In vitro, *C. abortus* grew readily in endometrial stromal fibroblasts, the epithelial cells failed to support the growth of this organism. In conclusion, the disseverment protocols provide pristine populations of ovine endometrial epithelial and stromal cells and the cultured epithelial cells which may exhibit characteristics of *in- vivo* morphology and polarized function. This *in-vitro* method could accommodate as a consequential implement for further studies to investigate the different steroidal receptors in these cells at different stages of estrous cycle and their possible effects on susceptibility of the cells to *C. abortus*.

Keywords: *Chlamydophila abortus,* endometrium, epithelial cells, ovine, stromal fibroblast cells.

INTRODUCTION

The obligate, intracellular, gram-negative bacterium *Chlamydia abortus* (*C. abortus*) is recognised as a major cause of abortion and lamb loss throughout the world, with infection resulting in the disease known as Enzootic Abortion of Ewes

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(EAE) or Ovine Enzootic Abortion (OEA) (Longbottom and Coulter, 2003; Longbottom *et al*., 2013). Following natural or experimental infection through the oro-nasal route, the *C. abortus* is transmitted from the initial mucosal site to accumulate in the pharyngeal lymphoid tissues and tonsils and then spreads throughout the body by blood or lymph eventually reach its final target cells in the reproductive tract via blood stream during the latter stages of gestation (Jones and Anderson, 1988; Kerr *et al.,* 2005). Typically, following infection, a vaginal discharge containing large numbers of chlamydial elementary bodies (EBs) may be observed 24-48 hours before abortion, which occurs in the last two to three weeks of normal gestation, although in some cases it may occur earlier (Longbottom and Coulter, 2003). *C. abortus* passes from the maternal site to fetal chorionic epithelium when maternal haematoma develops at the materno-fetal interface in the hilus of each placentome. The organisms then localise in the placental chorionic epithelium (trophoblasts), replicate, and produce visible cytoplasmic inclusions (Studdert, 1968; Novilla and Jensen, 1970; Kerr *et al.,* 2005) within the haematophagocytic region. After 90 days of gestation in experimentally infected animals, the placenta is heavily infected with *C. abortus*. This is usually accompanied by focal placentitis spreading through the cotyledonary and intercotyledonary placenta and apposing endometrial epithelial damage, oedema and inflammation, which is characterized by reddening and thickening of the placental membranes and by concurrent focal necrosis in fetal liver and other tissues suggesting a haematogenous spread of infection from the placenta to the fetus (Buxton *et al.,* 1990; Buxton *et al.,* 2002; Miley *et al.*, 2009). During this period the pro-inflammatory response mounted by the dam leads to some destruction of the chorionic epithelial cells, followed by an imbalance in the progesterone: estradiol 17β ratio. The steroidal imbalance is then followed by an increase in PGE_2 production and result in abortion (Longbotton and Coulter, 2003; Kerr *et al.,* 2005). Uterine functions are influenced by the ovarian steroids, progesterone (P_4) and estrogen during the estrous cycle and pregnancy. During the early stages of the oestrous cycle, receptors reported to negatively autoregulate the expression of its own receptors, particularly in uterine luminal epithelium and glandular epithelium (Clarke, 1990; Spencer and Bazer, 1995). In mice, it enhances the infection of the uterus by chlamydiae in contrast to estradiol is thought to decrease susceptibility to intrauterine chlamydial infection by modulating the immune response (Kaushic *et al.,* 2000).

Hyperplasia of endometrial glands occurs during the first 15 to 50 days of gestation followed by hypertrophy, which increase surface area and allow for maximal production of enzymes, growth factors, cytokines, hormones, transport proteins, and other substances that are collectively termed "histotrophs" (Guillomot, 1995; Stewart *et al.,* 2000; Taylor *et al.,* 2000). The accompanying change in the endometrium during pregnancy may be conducive for bacterial growth. Furthermore, both fetal and maternal tissues remain under the strong influence of steroidal hormones during pregnancy. It is also known that during natural or experimental infection, the invasion of placental tissue by chlamydiae coincides with increased levels of progesterone during pregnancy (Kaushic *et al.,* 2000). However, where *C. abortus* resides before and during the early stages of pregnancy, what factors trigger its growth and multiplication in the endometrium

remain to be clarified. The present study was planned as preliminary step with the ultimate aim of developing an *in- vitro* model to investigate some of the mechanisms of infection of endometrial cells with *C. abortus*.

MATERIALS AND METHODS

Collection of uterus and preparation of endometrial cells

The uteri of sexually mature and cyclic ewes lambs (n=6) about 60 kg in weight, slaughtered for human consumption, were used for the isolation of endometrial cells. Uteri from these animals were aseptically collected during early winter and transported to the laboratory for the preparation of cells for *in vitro* culture. Endometrial cells were isolated by following methods:

Method 1

Uterine lumens were flushed and washed twice using sterile phosphate buffer saline (PBS, at pH 7.2) before ligating the uterine horns at fallopian end and adding 15 ml of 0.76% EDTA-PBS containing 0.1% (w/v) of collagenase type 1A (Sigma # C7661), 100µg/ml streptomycin, 100µg/ml vancomycin from the cervical end using sterile pasteur pipettes. The horns were then tied to retain the collagenase solution for digesting the epithelial cells. The whole tracts were placed in a wide-mouthed conical flasks containing PBS and incubated for 1 hour at 37°C under continuous shaking. After collecting the cell suspensions, the procedure was repeated and suspensions was stored at 4ºC before mixing and filtering the cell suspensions of both digestions through 100-µm nylon mesh (CLARCOR, UK). The cells were then pelleted at 100 x g for 10 minutes using a cold centrifuge and washed three times with PBS. The cell suspension was adjusted to make a final cell concentration of 10^4 viable cells/ml in DMEM/ Ham's F-12 (Sigma # D8437) culture medium supplemented with 5% FCS, 100µg/ml streptomycin, 100µg/ml vancomycin, 50µg/ml gentamycin and 5µg/ml amphotericin B (Sigma-Aldrich, UK). The cells were then seeded in 25cm^2 culture flasks and incubated at 37 ºC in humidified atmosphere and 5% CO_2.

Method 2

Uterine horns were opened longitudinally along the anti-mesometrial border; the epithelium from the inter-caruncular areas was then scraped using a sterile surgical blade, and placed into a flask containing 50 ml DMEM/ Ham's F-12 medium supplemented with 2 mM EDTA, 100µg/ml streptomycin, and 100µg/ml vancomycin. The cells were incubated at 37°C for 1 h and the supernatant collected in a 50ml sterile universal tube. Any visible epithelium remnant tissues were resuspended in a media supplemented with 0.1% w/v collagenase type 1A (Sigma #C7661) and 0.25% trypsin. Tissues were incubated similarly for digestion. Supernatant from both the digestions were then pooled and filtered through a sterile 100 µm sieve into 50 ml sterile culture tubes and centrifuged at 100 x g for 10 min and washed three times with PBS. The cells were counted with hemocytometer adjusted to make a final concentration of 10^4 viable cells/ml

in DMEM/Ham's F-12 culture medium supplemented with 10% FCS, 100µg/ml streptomycin, 100µg/ml vancomycin, 50µg/ml gentamycin, and amphotericin B. The cells were then seeded in 25 cm^2 culture flask and incubated at 37°C in humidified atmosphere in air supplemented by 5% CO_2.

Separation of fibroblast from endometrial cells

After 72 h of incubation, cells collected by Method 2 were incubated with prewarmed solution of 0.25% trypsin in PBS-EDTA for 3 minutes. This treatment resulted in the detachment of all fibroblasts while the epithelial cells remained attached.

Identification of cells type

Epithelial and fibroblast cells were identified on the basis of their morphological and growth characteristics or on their staining characteristics after treatment with antibodies against cytokeratin (mouse anti-cytokeratin-pan, clone Lu-5 # MS-744-A; NeoMarkers, Fisher Scientific, UK, which is specific to epithelial cells or antibodies against Vimentin (mouse anti-swine vimentin, M725, Dako, Hamburg, Germany), which is specific for fibroblasts and other mesenchymal cells. Immunostaining with these antibodies was carried out using the alkaline phosphatase anti-alkaline phosphatase complex method (APAAP, Cordell, 1984). For this purpose cell suspensions were fixed onto slides after cytocentrifugation. The cytospin slides were then fixed with 50% acetone-alcohol for 15 min and stored at -20 ºC until stained for the presence of cytokeratin and vementin.

***In vitro* cultivation of endometrial cells**

Separated fibroblast cells were resuspended in culture medium (DMEM/Ham's F-12) supplemented with 10% FCS, 100µg/ml streptomycin, 100µg/ml vancomycin, 50µg/ml gentamycin and amphotericin B. The cells were seeded into 25cm^2 culture flask at density of 10^4/ml and incubated at 37 ºC in humidified atmosphere in air with 5% CO_2. The growth medium was replaced with fresh medium every 72 hours until confluent monolayers were formed. The epithelial cells were maintained in medium containing 2% FCS, supplemented with insulin-transferin selenium solution (Gibco, life science technologies, US).

Infection of cells *in vitro*

From confluent (144 h) epithelial and fibroblast cells the growth medium was removed and the monolayers inoculated with one of our field isolates of *C. abortus* (ZW53), recently isolated from an aborted ovine placenta. Briefly, the epithelial and fibroblast cell monolayers were infected by adding 50 µl of infected McCoy cell lysate containing $3x10^4$ IFU/ml of *C. abortus*. After adding the inoculum, the cell monolayer was centrifuged at 1500 g for 45 min to enhance adherence of the bacteria as described by Rekiki *et al.* (2002). After adding 5 ml of new maintenance medium containing 2% FCS with or without supplementation with 0.5 µg/ml cycloheximide, the culture flasks were incubated at 37 ^{0}C in 5%

CO_2 for 48-72 hours. Cells in the infected monolayers were dispersed with a sterile glass Pasteur pipette and the cell suspensions collected in 20-ml sterile universals. To determine percent infectivity, cytospins were prepared on glass slides using a cytocentrifuge (Shandon, Thermoscience, USA), air-dried, and stained with Giemsa. Cells were examined for the presence of *C. abortus* inclusions under a light microscope using oil immersion and x100 objective lense.

RESULTS

Isolation and growth of epithelial and stromal fibroblast cells

Monolayers grown from cells isolated from ovine endometrium by both enzymatic digestion methods were provisionally identified as epithelial or stromal fibroblast on the basis of their morphological characteristics. The yield and the purity of endometrial epithelial and stromal fibroblast sub-populations varied according to the methods of isolation used. In cells obtained by Method 1, confluency was achieved within 6-7 days of incubation. After 24h of incubation clumps of cells with cuboidal or cobblestone-like morphology were observed; these cells were considered as epithelial cells (Figure 1A). After 24 to 48 h of culture spindle-like structures, became abundant and this type of cells grew faster, quickly surrounding the epithelial cells (Figure 1B). A 90% confluency of these fibroblast cells was apparent within 72-96 h (Figure 1C). After 144 hours, all the cells appeared spindle- or rod-shaped, usually overlapping each other. Abnormal intra-cytoplasmic changes such as intra-cytoplasmic vacuoles and black pigmentation were also observed in cells grown after two passages and became flattened. Approximately ninety five percent of cells obtained by the enzymatic digestion in method 2 appeared to have the morphological characteristic of epithelial cells reached the confluency in 9-12 days of incubation (Figure 2A). The cells initially appeared to be cuboidal, becoming spindle-shaped as soon as they became confluent. However, despite the good growth of epithelial cells by method 2, 1-2 % of the cells were fibroblasts, In order to reduce fibroblast contamination; the concentration of FCS was reduced upto 2%, after the initial use of 10% FCS for the first 24 hours of culture (Figure 3). After selective isolation of stromal fibroblast from co-culture of epithelial-stromal fibroblasts cells, stromal cell reached to confluent monolayer during 72 hours of incubation using growth media supplemented with 10% FCS. They became elongated spindle cells on crowding, were passage four times and retained their morphological appearance. Cells could not maintain their structure and morphology, became flatted and developed intra-cytoplasmic vacuoles.

Identification of fibroblasts and epithelial cells

Following the *in vitro* cultivation, both the fibroblast-like (stromal) and epithelial-type ovine endometrial cells were stained with antibodies and detected cytokeratin and vimentin. Monolayer of cells obtained by Method 1 had numerous cells exhibiting a relatively intense cytoplasmic reaction and thus identified as epithelial cells. The epithelial and stromal cells obtained by method 2 were also stained with the same markers; the cytokeratine, but not vimentin, was detected

in the cells with morphological characteristics of epithelial cells. In contrast positive staining for vimentin was demonstrated in cells with morphological features of stromal (fibroblast) cells (Figure 2A-E).

Growth of *C. abortus* in epithelial cells and fibroblasts

No inclusions were observed in ovine endometrial epithelial cells after 48-72 h post infection, typical inclusion bodies were observed in stromal fibroblasts in 48 hours post infection, by 72 h 75 to 80% of the fibroblasts being infected. The addition of cycloheximide had no significant effect on the growth of *C. abortus* in stromal fibroblast cells, the organism grew well in cells with or without treatment (Figure 3).

DISCUSSION

The main aim of the current study was to develop a method of isolating and growing primary ovine endometrial cells *in-vitro* and to investigate their susceptibility to infection with *C. abortus*. The *C. abortus* often resides in a latent form before it is released to infect the gravid uterus, but the site of latency and the trigger to its release are not known. Ewes infected with *C. abortus* as lambs or as gimmers didn't develop active infection, until about 90 days of gestation, the initial infection with *C. abortus* usually goes unnoticed (Buxton *et al.,* 1990; Papp and Shewen, 1996a; Sammin *et al.,* 2006). This delay in the development is thought to be related to changes in the endocrinal system during the various stages of pregnancy. The transformation of *C. abortus* from silent non-infectious stage to fully blown growth in the placenta resulting in serious pathological lesions, leading to abortion, is thought to be linked to changes in the balance between progesterone and oestradiol during the late stages of pregnancy (Kerr *et al.,* 2005).

The presence of various concentrations of progesterone, oestradiol and other hormones are likely to play a role in the pathogenesis of EAE. Endometrial cells collected from the intercaruncular area of the uterine epithelium of non-pregnant cyclic ewes are known to be under the influence of estradiol (E_2). However, in the present study the cells were neither supplemented with exogenous E_2 or progesterone and the concentrations of these hormones in the animals from which the cells were derived were not known but it has been reported that there were better yields of epithelial cells derived from the intercaruncular region of ewes treated with estradiol and progesterone compared to controls (Salamonsen *et al.,* 1993) and the presence of circulating endometrial steriods during the estrous cycle. In the present study, epithelial cells were shown to be surrounded by fibroblastic cells similar to those observed by Cherny and Findly (1990) and can partly be explained by the slow growth of epithelial cells than stromal fibroblasts. This could also be attributed to the cultivation on plastic surfaces, which were reported to affect the ability of epithelial cells to maintain their structural integrity (Salamonsen *et al.,* 1985).

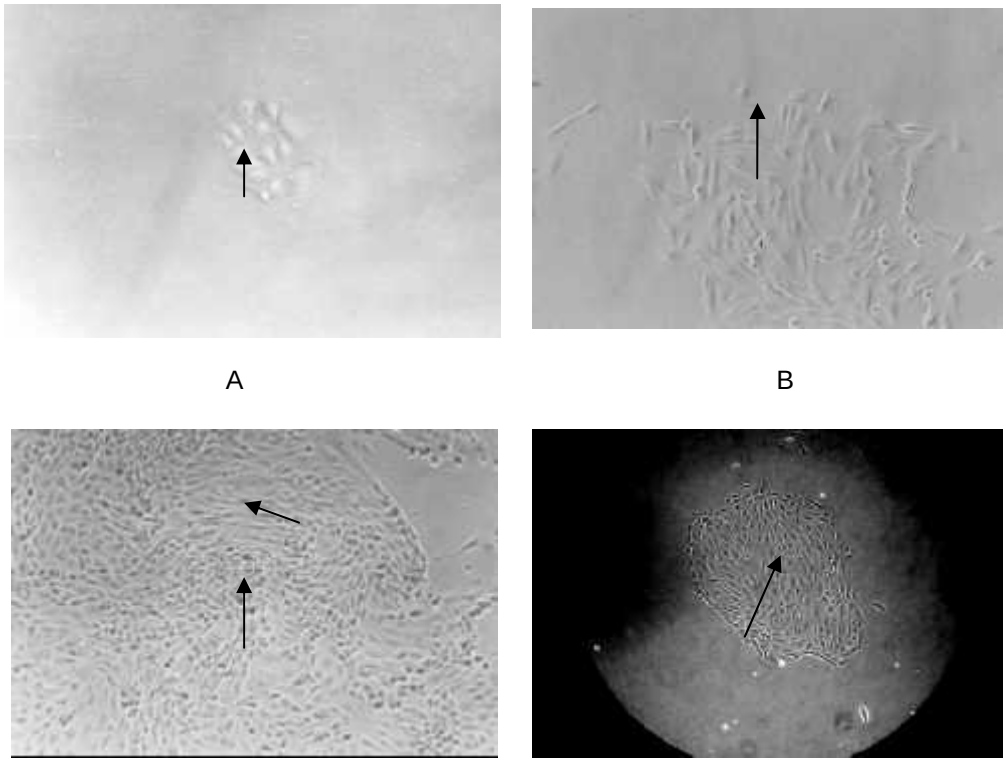

Figure 1. Ovine epithelial and stromal cells. (A) Unstained epithelial cells (early stage). (B) Unstained stromal cells (early stage). (C) Unstained mixed epithelial and stromal cells. (D) Unstained epithelial cells (7 days-old).

Proliferation of glandular epithelial cells is increased under the influence of E_2 (Salamonsen *et al.,* 1985) and although *in vitro* E_2 does not stimulate isolated uterine epithelial cells but the mitogenesis of uterine stroma (Cooke *et al.,* 1997). Furthermore, E_2 was reported to selectively increase the content of epithelial DNA in cocultures of uterine stromal and epithelial cells, but not in pure epithelial cells only. This suggests that E_2-induced epithelial mitogenesis is mediated indirectly via stromal cells. The fibroblast growth factor-10 (FGF-10) a novel endometrial stromal cell-derived mediator of uterine epithelial may have role in this regard (Chen *et al*., 2000).

In confluent cultures of bovine endometrial cells prostaglandin F2 alpha (PGF 2 alpha) production is greater than prostaglandin E2 (PGE2) production in epithelial cells, and the opposite is true in stromal cells, which suggest that epithelial cells of the endometrium may be a preferred target for the regulation of PG synthesis (Asseslin *et al.,* 1997). COX-2 may be involved in the mechanism by which oxytocin regulates $PGF_{2\alpha}$ production in the endometrium.

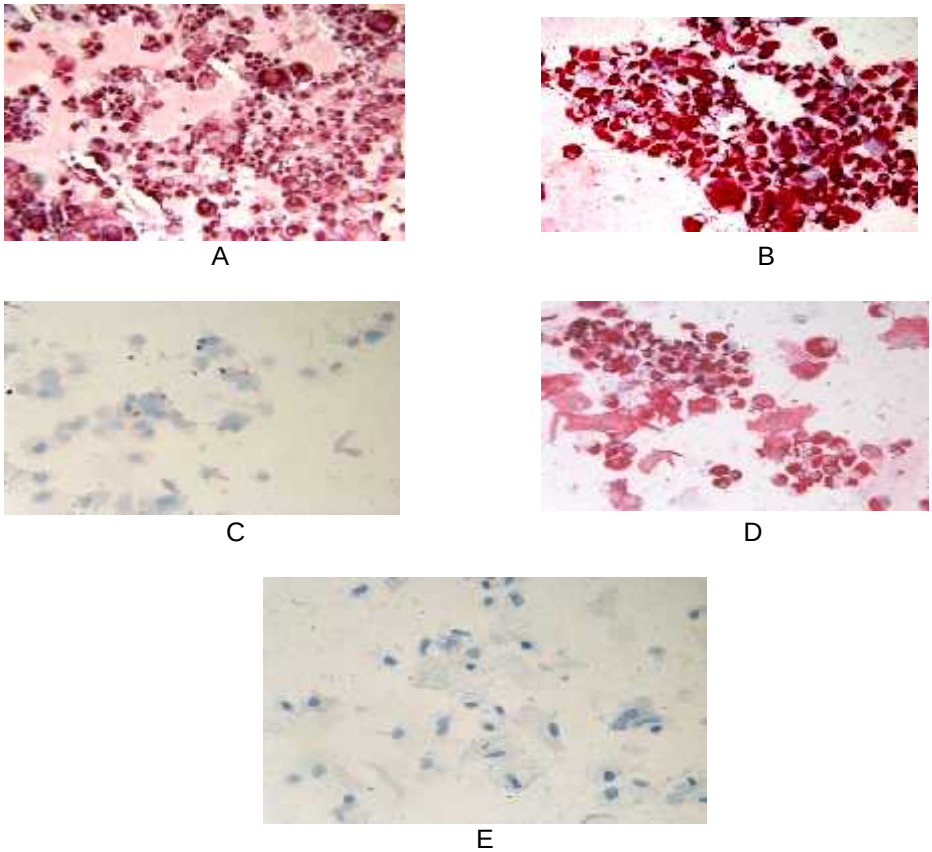

Figure 2. Ovine endometrial cells and McCoy cells stained with anti-cytokeratine and antivementine antibodies. (A) Vimentin+ve McCoy cells (B) Vimentin+ve endometrial stromal cells. (C) Vimentin-ve endometrial epithelial cells. (D) Cytokeratin +ve endemetrial epithelial cells. (E). Control epithelial cells not treated with anti-cytokeratine and antivementine antibodies.

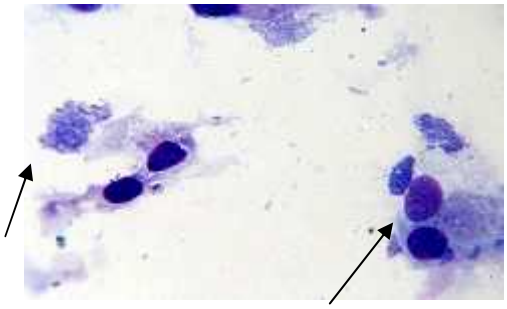

Figure 3. Ovine endometrial stromal cells infected with *C. abortus*.

In the present study, stromal fibroblasts supported the growth of *C. abortus*, and no evidence of infection in ovine endometrial epithelial cells was found. We have no explanation as to why the organism selectively grew in the stromal/fibroblast cells and not in the endometrial epithelial cells but there are several possible factors including the lack of specific receptors, the inhibitory effects of steroid hormones, prostaglandins, cytokines and enzymes which may be present in epithelial cells. Ovine endometrial cells isolated during the follicular phase (under the influence of E2) of oestrous cycles were allowed to grow *in vitro* before being inoculated with a strain of *C. abortus* of low passage. Whether or not the stromal cells, and not the epithelial cells, are targets of *C. abortus in vivo* remains to be clearly established but published data about the pathogenesis of *C. abortus* in pregnant and non-pregnant sheep indicated that the presence of high levels of progesterone stimulates its growth *in vivo*. High levels of E_2 increase the susceptibility of mice to infection with *C. abortus* compared to high levels of progesterone P4 (Kaushic *et al.,* 2000). Chlamydial lipopolysaccharide (LPS) is reported to induce the expression of COX-2, which in turn stimulates the synthesis of prostaglandins (PG) in endometrial epithelial and stromal cells (D'Acquisto *et al.,* 1997; Xiao *et al.,* 1998). E2 inhibits the production of $PGF_{2\alpha}$ and PGE_2 by down-regulating COX-2 expression in epithelial cells (Charpigny *et al.,* 1997) but it does not appear to affect the production and secretion of $PGF_{2\alpha}$ and PGE_2 by stromal cells (Goodwin *et al.,* 1980; Xiao *et al.,* 1998). P4 enhances $PGF_{2\alpha}$ production by downregualting COX-2 mRNA in stromal cells, but it can also have a stimulatory effect on PGE_2 production in stromal cells by up-regulating COX-2 mRNA. Inhibition of *C. abortus* in ovine endometrial epithelial cells could be possibly due to the downregulation of COX-2 activity by E2 (Xiao *et al.,* 1998)

Other possible factors responsible for restricting the growth of chlamydiae in the epithelial cells could be the production of various cytokines. The interactions between caprine endometrial stromal and epithelial cells can modulate the secretion of TGF-β1, TNF-α and IL-18 by endometrial epithelial cells exposed to E_2 and/or P_4 in vitro. In women the epithelial–immune cross-talk could promote the release of proinflammatory cytokines and enhance the barrier function of the endometrium against similar genus of bacterial cells (*C. trachomatis*) infection in the female reproductive tract (Meguel *et al.,* 2013). In contrast, stromal fibroblast cells in uterine endometrium were shown to be susceptible to *C. abortus* in the present study. The entry of infectious particles into fibroblasts and other cells is thought to be mediated by adsorptive pinocytosis or specific receptor mediated endocytosis (Besterman and Low 1983; Steinman *et al.,* 1983; Smyth and Warren, 1991). The absence of infection in epithelial cells in the present study may be due to loss of polarization on the epithelial cell surface, a process likely to affect endocytosis adversely (Tjelle *et al.,* 2000), as such polarization was shown to be necessary for the adhesion of chlamydiae to the cell surface (Escalante-Ochoa *et al.,* 2000). The lack of growth of *C. abortus* in ovine epithelial cells in the present study is in agreement with that reported by Escalante-Ochoa *et al.* (2000), which showed that *Chlamydophila psittaci* readily grew in fibroblasts (L cells) but not in BGM epithelial cells.

CONCLUSION

In conclusion the present study documents the first attempt to use primary epithelial and fibroblast cells isolated from ovine endometrium to grow *C. abortus in vitro.* The separation protocols provide pure populations of ovine endometrial epithelial and stromal cells and the cultured epithelial cells which may exhibit characteristics of *in vivo* morphology and polarized function. This *in vitro* method could serve as an important tool for further studies to investigate the different steroidal receptors in these cells at different stages of estrous cycle and their possible effects on susceptibility of the cells to *C. abortus*.

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