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Original article

HISTOMORPHOMETRIC ANALYSIS OF GOAT UTERINE TISSUE ON *IN VITRO* EXPOSURE WITH OVARIAN HORMONES AND MIFEPRISTONE

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Summary

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Uterus, the largest reproductive tract organ in female mammals, is the site of implantation of fertilised egg and foetus development. Uterus is a dynamic reproductive organ; its morphology alters with reproductive phase and steroidal cues. The aim of the present study was to assess the effects of progesterone (P_4), estrogen (E_2) and antiprogestogen i.e., mifepristone on goat's uterine histoarchitecture in *in vitro* short term culture. Uterine tissue slices were cultured in the presence of E_2, P_4 and mifepristone at the dose of 10^{-9} M, 10^{-7} M and 10^{-6} M respectively for 24 hours. Uterine morphology of E_2- and P_4-treated groups did not reveal marked changes from that of control group. Mifepristone treatment caused conspicuous changes in uterine histoarchitecture, led to congested endometrium, regressed uterine glands and constricted blood vessels. The changes observed in morphometry after E_2 and P_4 exposure included increased uterine gland diameter (47.00 and 45.95 µm respectively) and glandular epithelial cell height (18.37 and 17.43 µm respectively) while the mifepristone treatment resulted in significant reduction of gland diameter (34.95 µm) as well as epithelium height (14.25 µm) as compared to those in control group (39.9 and 15.56 µm respectively). These morphometrical changes revealed prominent regressive changes in anti-progestin treated group while E_2 and P_4 showed prolific effects in *in vitro* culture. Thus it is envisaged that E_2 and P_4 induced characteristic progressive changes in the histologic structure especially in endometrial glands of the goat uterus while anti-steroidogenic formulation i.e. mifepristone severely reduced the normal histoarchitecture of the uterus which is a prerequisite for implantation.

Key words: endometrium, estrogen (E_2), mifepristone, morphometry, progesterone (P_4).

INTRODUCTION

Uterus, the largest female reproductive organ primarily serves to support pregnancy by being the site of implantation as well as the home to developing embryonic and foetal life. The uterus in mammals is unique in having high regenerative potential. This organ undergoes various structural and biochemical changes in response

to cyclical ovarian hormones. In non-menstruating animals the endometrium undergoes cyclical proliferation, differentiation, and apoptosis rather than shedding like that in menstruating species (Gargett, 2007). During menstrual cycle the endometrium undergoes proliferation; it grows from 0.5–1.0 mm to 5–7 mm, differentiation, and then shed off (McLennan & Rydell, 1965). During pregnancy the uterus undergoes huge structural changes. Myometrial smooth muscle cells undergo hypertrophy and hyperplasia as a result of which uterine wet weight increases up to 15 fold (Shynlova *et al.,* 2006).

Cyclic sex steroids exposure is essential for periodic rejuvenation of the uterine tissue. In the proliferative phase, under the influence of estrogen, cellular components of the endometrium undergo proliferation evidenced by high mitotic indices especially in late proliferative phase and increased height of the tissue (Deligdisch, 2000). Estrogen priming is necessary for progesterone to act (Groothuis, 2007). Progesterone induces differentiation and secretory changes in the endometrium. It increases tortuosity of glands and also makes stroma more oedematous (Critchley & Saunders, 2009). Progesterone also reinforces the angiogenesis and thus increases vascularity by stimulating the endothelial cells to proliferate and this event is independent of estrogen priming or stimulation (Walter *et al.,* 2005). These changes are required to facilitate ovulation, embryonal development and to support pregnancy. During the estrogen dominant proliferative phase, frequency of mitoses in the epithelium and stroma is increased, endometrial glands become increasingly branched and an increased epithelial cell ciliation occurs while during secretory phase, glandular epithelial cells become vacuolated because of secretory material accumulation (Wang *et al.,* 2007).

Various anti-progestins or selective progesterone receptor modulators (SPRM) are used in clinical field for menstrual regulation, as emergency contraceptives, for termination of early pregnancies, and for treatment of uterine leiomyomata, endometriosis and breast cancer (Murdoch & Roberts, 2014; Goyeneche & Telleria, 2015). These antiprogestins acts by altering glandular and surface epithelium, vascular morphology and receptor expression of the endometrium. Mifepristone or RU486 is the first highly effective and widely used progesterone antagonist. Some of the PRMs exhibit non-competitive anti-estrogenic effects; mifepristone is one of them as it counteracts on estrogen-induced pathological conditions and also induces androgen receptors (Mutter *et al*., 2008). Anti-nidatory action of mifepristone involves endometrial desynchronisation with evident changes in glandular, vascular and stromal compartments of endometrium (Nayak, 1998).

Estrogen, progesterone and mifepristone, all the three are capable of affecting morphological features of endometrium. E_2 and P_4 stimulate endometrium growth while mifepristone causes anti-proliferative changes in this tissue structure (Peyghambari *et al.,* 2008; Spitz, 2010). On E_2 administration, the epithelium becomes pseudo-stratified having elongated nuclei. Morphometric changes including increased endometrial thickness and epithelial height also occur after E_2 supplementation (Girling *et al.,* 2000; Paulson, 2011). P_4 administration increases the weight and length of the uterine horns, as well as endometrium and myometrium thickness while it decreases the gland density (Bailey *et al.,* 2010). It also supports endometrial vascular development and

stimulates secretory activities in epithelial cells. In opposition to the ovarian steroid induced progressive changes mifepristone induces anti-proliferative changes including compact endometrial stroma, cystically dilated glands, decreased mitotic activity, non-secretory glands, defective blood vessels etc. These mifepristone-induced changes are more specifically called as PRM-associated endometrial changes (Horne & Blithe, 2007). However, these changes in endometrial structure vary from individual to individual as well as in different parts of the same tissue (Fiscella *et al.,* 2011).

Despite the fact that lots of work has been done on the effects of sex steroids and anti-sex steroids on uterine morphology and physiology, till now there is fragmentary available information on impact of hormones and anti-hormones in short-term *in vitro* culture conditions in small ruminants, which are primarily used for meat production and to some extent for milk, fibre and hide production. The present study was designed to investigate the *in vitro* effects of sex steroids and anti-sex steroids on the uterine tissue of *Capra hircus*. The *in vitro* system blocks peripheral systemic hormonal inputs and thus provides an opportunity to specifically analyse and point out the impact of estrogen, progesterone and progesterone antagonist on the uterus.

MATERIALS AND METHODS

Animal tissue collection

The uterus from 2–5 years old female goat (*Capra hircus*) of Jamunapari breed was obtained from slaughter houses around Chandigarh (30.73º N, 76.77º E) and was brought to the laboratory at 4 ^{o}C in normal saline fortified with antibiotics.

Estrous stage determination

For estrous phase determination gross morphology of ovary and fern pattern of cervical mucus were assessed. Ovaries possessing Graafian follicle of more than 5.0 mm diameter were considered as in proliferative/follicular phase while ovaries with corpus luteum of more than 5.0 mm diameter were considered in secretory phase/luteal phase. For further affirmation of estrous phase, fern testing was also performed for which cervical mucus was spread on a slide, then after air-drying it was observed under microscope. Mucus showing primary, secondary, and tertiary fern patterns was designated in follicular phase while vaginal smears showing primary and secondary or no fern pattern were categorised into luteal phase.

Experimental design

Uterine tissue collected from the follicular phase of five different goats was sliced into about 2 mm thick slices and was divided into four groups: control group, estrogen (10^{-9} M) treated group, progesterone (10^{-7} M) treated group and mifepristone treated group (10^{-6} M). Two or three tissue slices in all groups were cultured in Dulbecco's modified eagle medium (DMEM) along with specific hormonal and anti-hormonal formulation fortified with antibiotics at 39 ^{o}C with 95% humidity and 5% CO_2 in the CO_2 incubator for 24 h.

Histology

Histology was done according to the method devised by Pearse (1968). Uterine tissue was fixed in Bouin's fixative for 24 hours and then washing was given for around 4 hours to remove the excessive fixative. After that tissue pieces were dehydrated through different grades of alcohol, cleared with xylene and then embed-

ded in paraffin wax. The uterine tissue was sectioned serially at 5 µm thickness followed by stretching and dewaxing by xylene for 15 min and transferred to absolute alcohol.

For staining, the slides having stretched tissue sections were cleared in xylene twice each for 15 min. Then, these were rehydrated in 100%, 70%, 50%, and 30% alcohol series for 5 min in each. After that slides were passed sequentially through distilled water, haematoxylin stain, kept under running tap water (until the colour developed) then dehydrated in alcohol grades from 30% to 90%, placed in eosin stain for 2 min, again in 90% and 100% alcohol, then clearing was done in xylene for 15 min. Finally, slides were mounted with DPX (distyrene, a plasticiser, and xylene).

Morphometric measurements

Morphometric assessment was done with the help of ocular micrometer. First of all, the ocular micrometer was calibrated for different magnification with the help of stage micrometer (having 100 divisions, each of 10 µm). Readings were taken from five different fields in two random sections from two different blocks. Various parameters like glandular diameter, luminal epithelium height, glandular epithelial cell height; endometrial, myometrial and perimetrial thickness were quantified.

Statistical analysis

Data are expressed as Mean±SEM. Quantitative data were analysed using the t-test. P=0.05 was taken as limit of significance, data having $P>0.05$ were considered as non-significant while those having $P<0.05$ were considered as having significant difference (*$P<0.05$, **$P<0.01$, ***$P<0.001$) vs control group.

RESULTS

Histologically, the uterus in transverse section appeared to comprise serosa, a thin circular outer layer followed by a thick layer of muscular myometrium consisting of outer longitudinal muscle band, middle oblique and innermost circular muscle band, lying above the myometrium was the innermost layer of uterus i.e. endometrium including uterine glands and stromal tissue (Fig 1A). Control group revealed a lumen lined with simple columnar epithelium, well-marked round glands (Fig 1B), along with endometrial glands it had blood vessels (Fig 1C) and stromal cells which constituted areolar connective tissue. Estrogen-treated group led to thickened endometrium as well as myometrium with numerous circular glands (Fig 1D-F). Progesterone-treated group exhibited secretory transformation which was discernible through increased tortuosity of endometrial glands and myometrial thickness as compared to the control (Fig 1G-I). Antiprogestin (mifepristone) supplemented group divulged compact stroma, irregularly distributed degenerated endometrial glands and constricted blood vessels. Degenerative changes including desquamation of luminal as well as glandular epithelium were observed in all the groups (Fig 1J-L). These debilitating changes were more pronounced in mifepristone treatment group as compared groups while these were moderately lesser in progesterone supplemented groups.

Morphometric studies

Morphometric data of various parameters assessed are shown in Table 1. In estrogen

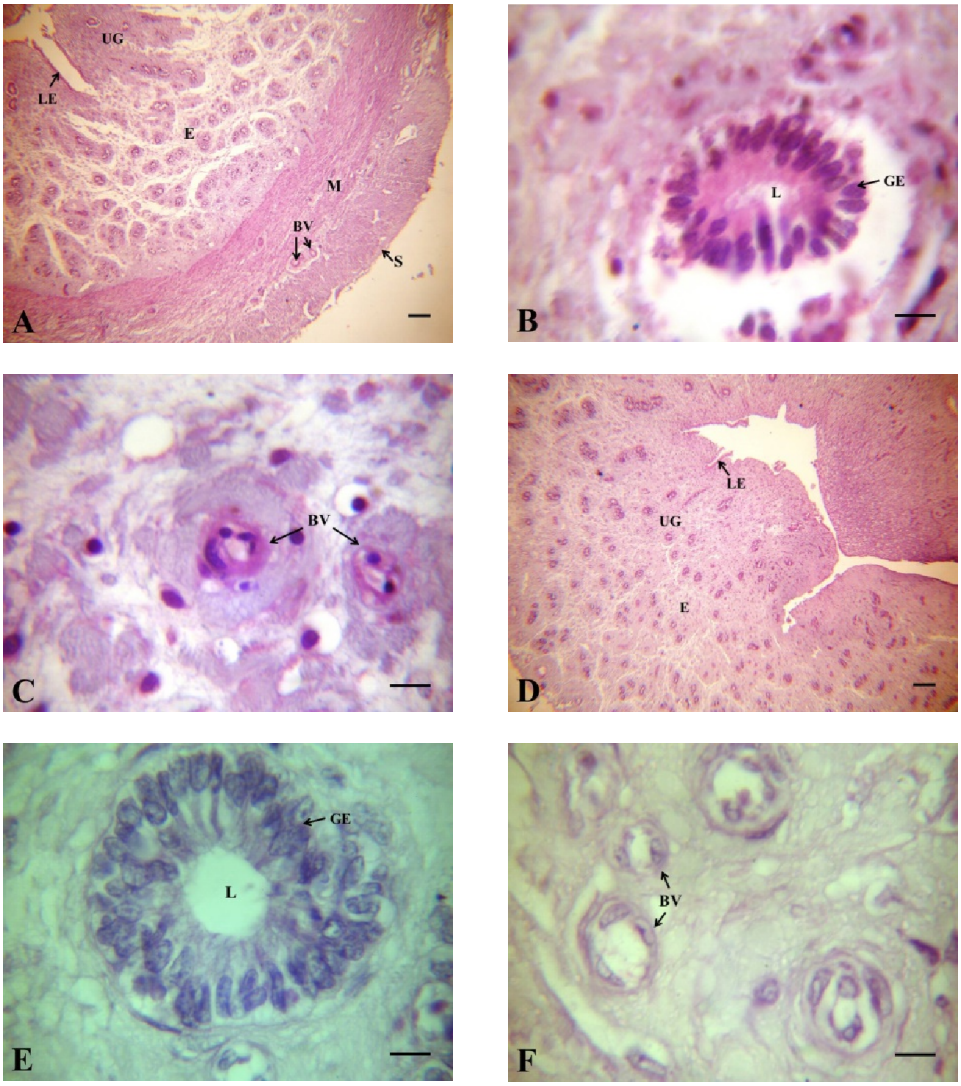

Fig. 1. Histomicrograph of uterine tissue stained with haematoxylin & eosin. A: control group revealing normal histoarchitecture with majority of round glands along with some elongated tortuous glands in stromal compartment of endometrium which is surrounded by myometrium and the outermost serosa (×40, bar=100 µm). B & C: Round gland with some pseudostratification and blood vessels from control group (×1000, bar=10 µm). D: E_2 administered group depicting round glands in thickened endometrium at (×40, bar=100 µm). E & F: Round gland with increased epithelial height and blood vessel respectively from E_2 treated group (×1000, bar=10 µm). BV – blood vessel, E – endometrium, GE – glandular epithelium, LE – luminal epithelium, M – myometrium, S – serosa, UG – uterine gland.

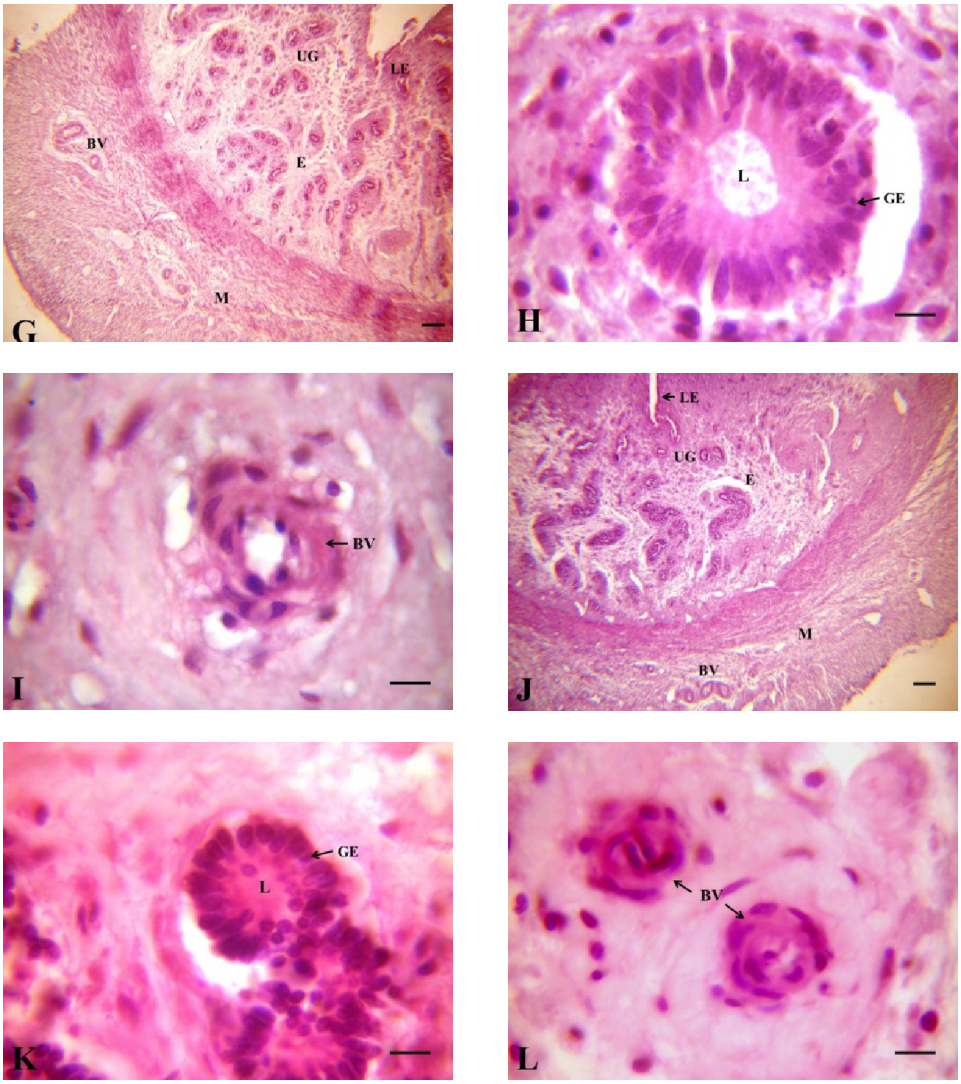

Fig. 1 (cont'd). Histomicrograph of uterine tissue stained with haematoxylin &eosin. G: P_4 treated group showing a mild increase in tortuosity of glands (×40, bar=100 µm). H & I: Rounded gland with wide lumen and elongated epithelium; and circular blood vessel from P_4 treated group (×1000, bar=10 µm) J: antiprogestin (mifepristone) supplemented group (×40, bar=10 µm) representing compact stroma, irregularly distributed regressed endometrial glands (*K*, ×1000, bar=10 µm) and constricted blood vessels (*L*, ×1000, bar=10 µm). BV – blood vessel, E – endometrium, GE – glandular epithelium, LE – luminal epithelium, M – myometrium, S – serosa, UG – uterine gland.

Table 1. Changes in different components of uterine tissue *in vitro* in control, estrogen-, progesterone- and anti-progesterone-treated groups. Values are expressed as mean±SEM (n=50)

Parameters	Groups			
	Control	Estrogen	Progesterone	Mifepristone
Gland diameter (μm)	39.9±2.11	47±1.64*	45.95±2.02*	34.95±1.43*
Gland epithelium height (μm)	15.56±0.67	18.37±0.54***	17.43±0.54*	14.25±0.40*
Luminal epithelium height (μm)	14.62±0.50	14.58±0.46	14.71±0.43	14.31±0.57
Endometrial thickness (μm)	1425±79.26	1931.25±107.34***	1463.75±60.49	1401.25±49.38
Myometrial thickness (μm)	897.5±20.69	1146.5±33.92***	1232.59±77.90***	857±12.69
Perimetrial thickness (μm)	39.01±2.92	47.82±5.01	53.46±4.61	47.58±4.52

*P<0.05; ***P<0.001.

and progesterone-treated group, the glandular diameter, glandular epithelium height, and myometrial thickness increased significantly as compared to control. Endometrial thickness increased significantly only in estrogen-treated group while other groups showed insignificant variations. In mifepristone-treated group significant reduction in gland diameter and gland cell height was observed from those of control group. Luminal epithelial height and perimetrial thickness didn't reveal any significant changes among different groups.

DISCUSSION

The results of the present *in vitro* study revealed that ovarian hormones and anti-progesterone (mifepristone) induced histopathological changes in uterine tissue of goat. Uterine tissue from control and estrogen-treated group revealed majority of pseudostratified round glands in endometrial stroma while in progesterone-treated group there was a mild increase in tortuosity of uterine glands, other structures were more or less similar in these three groups. Although morphological alterations were not very prominent, well-marked changes in morphometric attributes were observed. Glandular changes observed were more prominent among all the groups. There was significant increase in the glandular diameter, glandular epithelial height, endometrial and myometrial height in estrogen- and progesterone-treated group as compared to control group while the mifepristone treatment resulted in decrease of glandular diameter and also the gland cell height. Gibbons *et al*. (1986) reported that glandular diameter and gland cell height become increased in post-menopausal women receiving medroxyprogesterone acetate/progestin treatment. The present findings are also in agreement to the earlier findings of Peyghambari *et al*. (2008) who have reported that exogenous sex steroidal exposure resulted in altered endometrial morphometric indices – more specifically increased glandular diameter even on the

first day of the treatment. Teixeira *et al.* (2018) documented similar kind of changes in gland diameter and gland cell height after treatment with a progesterone analogue i.e. medroxyprogesterone in neonatal dogs. Newbold *et al*. (1993) documented that synthetic estrogen i.e. diethylstilbestrol induces proliferative changes in uterine tissue in terms of increased epithelial height and stromal density. E_2-induced thickness in endometrium was also observed by Girling *et al.* (2000). In our study, we found that P_4 stimulated increase in endometrium and myometrium thicknesses, in agreement with the study of Bailey *et al.* (2010).

Various selective progesterone receptor modulators (SPRM) are used to target progesterone receptors which finally leads to the blockade of progesterone action to treat various reproductive ailments like uterine fibroids, endometriosis etc. (Murdoch & Roberts, 2014). Mifepristone is one of them; it blocks the biological effects of progesterone by binding to the progesterone receptors. Mifepristone affects uterine morphological as well as biochemical attributes (Nayak *et al.,* 1998). Similar to findings of Qamar *et al.* (2012) epithelial height of uterus was decreased after mifepristone treatment. Our results are also in agreement with Ghosh *et al.* (1996) who documented a significant decrease in average diameter of glands after RU486 (mifepristone) treatment in rhesus monkey. Gland luminal diameter is also decreased on mifepristone administration (Chen *et al.,* 2011). Gafari *et al.* (2017) also concluded that antiprogesterons tended to down-regulate endometrial growth, these decreased the glandular diameter and glandular secretions.

We found that mifepristone led to antiproliferative alterations in endometrial morphology viz. compact stroma, defective blood vessels and endometrial glands. Our findings are in congruency with Kannan *et al.* (2018) who reported distorted glands, compact and nondecidualised stroma with thick-walled vessels on ulipristil acetate (UPA) – another SPRM. Spitz (2010) stated that mifepristone displays antiproliferative effects; it suppresses endometrial proliferation, mitotic activity and secretory activity and reduces endometrial thickness and wet weight.

In the present study all the above histometric alterations were accompanied by some degenerative changes as well like desquamation of epithelium which were most prominent in mifepristone-treated group. In explant culture of uterus disruptions started to occur by 12 hours of culture and these explants remained viable up to 48 hours only; progesterone showed protective effects against these necrotic changes (Bersinger *et al.,* 2009). Mifepristone had anti-progestogenic as well as anti-estrogenic activities as it downregulated the progesterone as well as estrogen receptors (Jiang *et al.,* 2002). Progestogenic and estrogenic activities are required for normal function and structural maintenance of uterus so the more prominent deleterious effects in mifepristone group were attributed to its anti-progestogenic and anti-estrogenic activities.

CONCLUSION

The present study revealed that sex steroids estrogen, progesterone and progesterone's antagonist acted on uterus and triggered morphological changes especially in uterine glands in *in vitro* short term culture. Alongwith these morphological changes, mifepristone-mediated degenerative changes were observed. The findings of such studies in goats can be utilised in veterinary medicine to improve

the reproductive health of uterus to enhance productivity.

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