Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 23 No.1 Ser. No. 78 2022

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Changes in surface morphology, lectin staining, and gene expression of caprine endometrium exposed to estradiol, progesterone, and mifepristone *in vitro*

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10.22099/IJVR.2021.41508.6036

(Received 18 Aug 2021; revised version 15 Nov 2021; accepted 12 Dec 2021)

Abstract

Background: In mammalian females, the uterine tissue is highly responsive to steroid hormones and their antagonists. **Aims:** In the present study, topographical, histoarchitectural, and gene expression changes in goat endometrium treated with estradiol, progesterone, and mifepristone for 24 h were investigated, *in vitro*. **Methods:** Scanning electron microscopy (SEM) was used for surface topographical analysis; WGA and DBA lectins were used for histochemical analysis; and qRT-PCR was done for the quantification of mRNA levels of MKI67 (marker of proliferation Ki67), ESR1 (estrogen receptor), PGR (progesterone receptor), CASP3 (caspase 3), and PDGFR-β (platelet derived growth factor receptor-β). **Results:** Few topographical alterations were observed in endometrial glands and the presence of scattered mucoid granules. A significant decline in WGA staining was reported only in the progesterone group. However, DBA binding was highest in the progesterone group and lowest in the mifepristone group. The expression of MKI67 gene declined to 79% in the mifepristone group, while in the estradiol and progesterone groups it elevated to 153% and 41%, respectively, than control; a similar trend was observed for PDGFR-β. The mRNA abundance for ESR1 declined to 59% in the progesterone group and 10% in the mifepristone group. However, a 100% increase occurred in the estradiol group. PGR expression followed the same trend as that of ESR1. CASP3 declined in the estradiol (50%) and progesterone (37%) group, but it showed a 67% increase in the mifepristone group. **Conclusion:** We concluded that the caprine uterus undergoes dramatic alteration in structure and functions in response to different kinds of steroidal environments.

Key words: Antiprogesterone, Endometrium, Gene expression, Goat, Uterus

Introduction

Ovarian steroids, including estradiol (E2) and progesterone (P4) have a specific influence in organizing the structural and functional changes in uterine tissue. Various uterine activities including proliferation, differentiation, apoptosis, angiogenesis, extracellular matrix turnover, and leukocyte infiltration occur in synchronization with cyclic fluctuations in sex steroid secretion (Evans *et al*., 1990; Jabbour *et al*., 2006; Henriet *et al*., 2012). E2 stimulates growth, vascularity, and edema of the endometrial tissue as well as proliferation of glandular epithelium, while progesterone elevates the proliferation of stromal cells, glandular cell differentiation and secretory activity in the endometrial glands (Ferenczy *et al*., 1982; De Cock *et al*., 1997; Vermeirsch *et al*., 2002; Bartel *et al*., 2013). These steroid hormones also influence the biochemistry of the uterine tissue. Administration of E2, and E2-P4 combination causes growth-related changes, like increased uptake of glucose, increased protein content in the tissues, changes in the activities of various enzymes, including hexokinase, glucose-6-phosphatase and glucose-6-phosphate dehydrogenase in rats (Moorthy *et al*., 2004). P4 administration in gilts increases the uterine weight, and also elevates total protein and 6-keto $PGF_{1\alpha}$ (a PGI_2 metabolite) level in the uterine lumen (Szymanska and Blitek, 2016). As an important mediator in cell recognition processes, the apical glycocalyx functions are assumed to be involved in interactions between the developing embryo and the uterus (Vierbuchen, 1991). In the endometrial epithelium, glycoconjugate expression alters in a cyclic manner and is probably a fundamental activity for maintaining uterine receptivity for the implanting embryo.

Various types of receptors, including progesterone (PR-A and PR-B), estrogen (ERα, ERβ), androgen (AR), and glucocorticoids (GR) receptors, are expressed within the endometrium (Bookout *et al*., 2006). In canine endometrium, different cell types show distinct expression patterns of ER and PR in response to fluctuating levels of ovarian steroids during the estrous cycle (Vermeirsch *et al*., 2002). An increase in the level of plasma estrogen causes an enhancement in the expression of ERs and PRs. However, increased plasma progesterone level leads to a decline in the expression of

ERs and PRs.

Selective progesterone receptor modulators (mifepristone, ulipristal, onapristone, aglepristone, etc.) are the steroid compounds which might be agonist or antagonist of progesterone (Wagenfeld *et al*., 2016). These exhibit clinical utility for the treatment of various hormone dependent conditions. Aglepristone (RU 46534), a compound similar to RU 486, is steroidal agent used to treat progesterone-induced pathological conditions in various animals like bitch, cow, rabbit, etc. (Gogny and Fieni, 2016). Mifepristone (RU486) is a renowned progesterone antagonist used for pregnancy termination. Besides, it successfully terminates the pregnancy in dogs (Linde-Forsberg *et al*., 1992). It also induces parturition in beef heifers when administered with relaxin (Dlamini *et al*., 1995). For induction of parturition, mifepristone is also given along with prostaglandin formulations. Prostaglandins are derivatives of cyclic carboxylic acids, derived from arachidonic acid or related fatty acids; these agents possess potent and diverse physiological roles (Sharma and Sharma, 2020).

In ruminants, specifically goats, scattered information is available about the impact of ovarian hormones and their antagonists on uterine physiology specifically in *in vitro* conditions. So, the present study was designed to investigate the alterations in the topography, glycoconjugate expression (by WGA and DBA), and gene expression in goat endometrial tissue *in vitro* treated with steroids, i.e., estradiol, progesterone, and mifepristone for 24 h.

Materials and Methods

Animal tissue collection

The uterine tissues from mature female goats (2-5 years old) were procured from the slaughterhouse of Chandigarh (30.73° N, 76.77° E). There is no ethical issue as per the University guidelines, because the tissue was procured from the slaughterhouse. Phase of the uterine cycle was determined according to the method of Sharma and Singh (2021). After confirming the stage of uterine tissue, tissue pieces were collected from uteri in the proliferative phase and were categorized into four groups according to the type of steroid exposure: one control group and three steroid-treated groups. Each treated group was administered individually with estradiol, progesterone, and mifepristone at the dose of 10^{-9} M, 10^{-7} M, and 10^{-6} M, respectively. All groups, having 5-6 tissue pieces, were cultured in DMEM supplemented with antibiotics and 5% FBS at 39°C with 95% humidity and 5% CO_2 in the CO_2 incubator for 24 h. A specified dose of the steroidal formulation was added in each group.

Scanning electron microscopy (SEM)

For SEM, the tissue samples were collected and washed in chilled 0.1 M phosphate buffer (pH = 7.2) and were fixed in 2.5% gluteraldehyde for 12 h. After fixation, samples were washed in 0.1 M phosphate buffer 3 times. Thereafter, the samples were dehydrated in ascending grade of acetone i.e. 30%, 50%, 70%, 80%, 90%, 95%, and 100% acetone (dry acetone) at 4°C. The dehydrated specimens were then dried in critical point drying apparatus containing acetone. Then, the tissues were placed in desiccators, mounted on aluminum stubs, sputters coated with a gold layer, and were viewed under EVO18 Zeiss scanning electron microscope.

Lectin staining

Lectin staining was performed to analyze the glycoconjugate expressions. Uterine tissue was fixed in Bouin's fixative for 24 h followed by washing under running tap water. After that, tissue pieces were dehydrated through alcohol grades (30%, 50%, 70%, 90%, and 100%), cleared in xylene, and then embedded in paraffin wax. The paraffin-embedded tissue slices were sectioned at 5-7 µm thickness. The slides having stretched tissue sections were deparaffinized in xylene. Sections were rehydrated through different grades of alcohol, including 90%, 70%, and then in distilled water. Unmasking of the antigens was performed by keeping the slides in citrate buffer for 15 min at 80-90°C in water bath. Thereafter, tissue sections were incubated with 2-3 drops of FITC-conjugated wheat germ agglutinin (WGA) (L4895-2MG from Sigma) in a moist chamber for 45 min at room temperature (RT) in a dark place. However, for fluorescein labeled *Dolichos biflorus* agglutinin (DBA) (FL-1031-2, from Vector Laboratories) an overnight incubation was performed in moist chamber. Slides were then washed three times in PBS for 6-7 min and were cover-slipped after mounting with glycerin, and were observed under the fluorescent microscope. The intensity of the reaction to each lectin was assessed with Image-Java software (Oracle, USA). Image intensity (pixel count) was done by selecting and specifying ROI (region of interest). Mean intensity was calculated from 10 different fields.

Gene expression analysis

Alterations in gene expression were analyzed using qRT-PCR. Endometrium (200 mg) was homogenized and total RNA isolation was done using Qiagen RNeasy mini kit (74136) as per the manufacturer's instructions. To remove the DNA contamination, the Qiagen column DNAse I enzyme was used to digest the remaining DNA. The quality and quantity of purified RNA was tested by qiaExpert spectrophotometer (Qiagen, USA). Thereafter, purified and good quality RNA of each sample was used for the preparation of cDNA using iScript™ cDNA Synthesis Kit (Cat No. 1708890) as per the instructions. RT-PCR reaction was performed using 0.7 µL c-DNA template, 2X Sybr Green Mater mix, and primers (Table 1) at optimized concentrations along with 10 µL MQ water. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the normalizer gene. Initial denaturation was done at 95°C for 3 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at57°C for 30 s, and then, extension at 72°C for 15 s. The final extension was done at 72°C for 5 min and held at

Table 1: Primer sequences used for qRT-PCR

Genes	Gene ID	Primer sequence 5´-3´	Amplicon size (bp)
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	100860872	F: TGAACGGGAAGCTCACTGG R: TCCACCACCCTGTTGCTGTA	187
ESR (estrogen receptor)	102189484	F: GTATGACCCTTCCAGACCTTTC R: CGCCAGACAAGACCAATCA	192
PGR (progesterone receptor)	102175599	F: CTCAGTGGTCAAGTGGTCTAAG R: CTCCTTCATCCGCTGTTCAT	193
CASP3 (Caspase 3)	102177031	F: CAGACCTGGACTGTGGTATTG R: CGAGCTTGTGAGCGTACTT	195
MKI67 (marker of proliferation Ki67)	102183289	F: CACTTCTTGGGAAGGTGGATAC R: TCCTTGGCCTCCTCTTACTT	195
PDGFR-β (platelet derived growth factor receptor-β)	102184426	F: GAGTCGGTGGACTATGTGCC R: CTGGTAGCTGAAGCCCACAA	189

12°C. For each sample, a melting curve was generated to ensure that a single product was amplified for the very reaction mixture type. For the quantification of various genes, comparative quantification cycle (Cq) method was adopted relative to that of GAPDH and control as the calibrator group. From, Cq value, relative quantification or fold change was derived for each gene.

Statistical analysis

Quantitative data was analyzed using ANOVA Tukey Post Hoc test. The values are expressed as mean±SEM. P=0.05 was taken as a limit of significance. The P-value >0.05 was considered non-significant, while P<0.05 was considered the significant difference.

Results

Scanning electron microscopy

Scanning electron micrograph of the control group showed the majority of empty gland openings, rarely scattered mucoid secretion appeared on endometrial surface, and a few gland bulges were also appeared (Fig. 1A). At higher magnification, this group revealed a lowly ridged cellular epithelial surface, while the majority of the glands appear devoid of any secretory substances (Fig. 2A). Estradiol treated tissue appeared to have more glandular openings, secretory material was found to be present in a few glands as well as scattered over the surface, and rare gland bulges (Fig. 1B); epithelial cells with low ridges were present (Fig. 2B).

In the progesterone administered group, numerous wide glandular openings containing mucoid material and a large number of surface secretory granules were also seen (Fig. 1C), the apical surface of epithelial cells appeared ridged (Fig. 2C). On mifepristone administration, endometrial surface appeared to have compressed glands (Fig. 1D) and high raised cellular ridges with many blebs (Fig. 2D).

Lectin staining

WGA staining

WGA binds with its complementary sugars β-N-acetyl-D-glucosamine and α-N-acetylneuraminic acid. It bound strongly to the luminal and glandular epithelial cells; however, the binding affinity was very weak in the

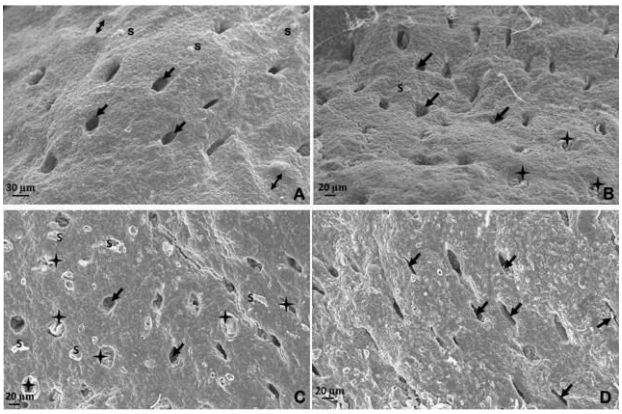

Fig. 1: Scanning electron micrograph of uterine tissue. (**A**) Control group showing empty gland openings (arrow), rarely scattered mucoid secretion (S), and gland bulge (double-headed arrow), (**B**) Estradiol treated tissue with more glandular openings; few glands are with secretory excretion (asterisk) as well as scattered mucoid secretion, (**C**) Progesterone group showing wide gland openings, most of them containing mucoid material, surface secretory granules are also numerous, and (**D**) Mifepristone treated tissue is with compressed glands (×500)

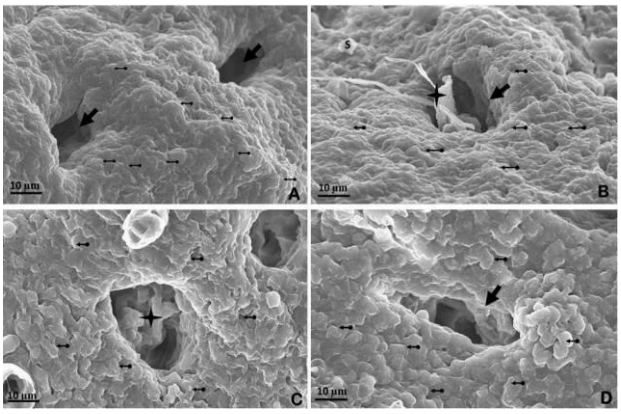

Fig. 2: Scanning electron photomicrograph of uterine tissue with higher magnification (×3000). (**A**) Control group showing empty gland openings (arrow), and ridged cell surface with low ridges (double-headed arrow), (**B**) Estradiol treated tissue with wide gland (arrow) having mucoid excretion (asterisk); epithelial cells with low ridges, (**C**) Progesterone administered group is showing wide gland openings containing secretory material, high ridged apical borders of epithelial cells (double-headed arrow), and (**D**) Mifepristone treated tissue depicting compressed glands, raised cellular ridges

stromal compartment for all the groups (Fig. 3). In estradiol treated group, the intensity appears brightest, however progesterone treated group is the least stained among the other groups. In endometrium, only superficial glands show a higher level of intensity, while the deeper glands possessed weaker binding. WGA exhibited apical-basal polarity in binding with the epithelial cells. It strongly binds on the apical side of the epithelial cells. The same pattern of lectin binding is followed in all the groups, but the staining intensity varied among the different groups (Fig. 5). Staining intensity in the control group was 52.67 ± 1.26. In estradiol treated group, the WGA staining intensity was non-significantly higher (54.90 ± 2.18) as compared to the control group. However, progesterone-treated group revealed the lowest (38.97 ± 1.57) level of staining among all the groups. In the mifepristone-treated group (47.45 ± 2.26), a non-significant decrease in intensity was observed compared to the control group.

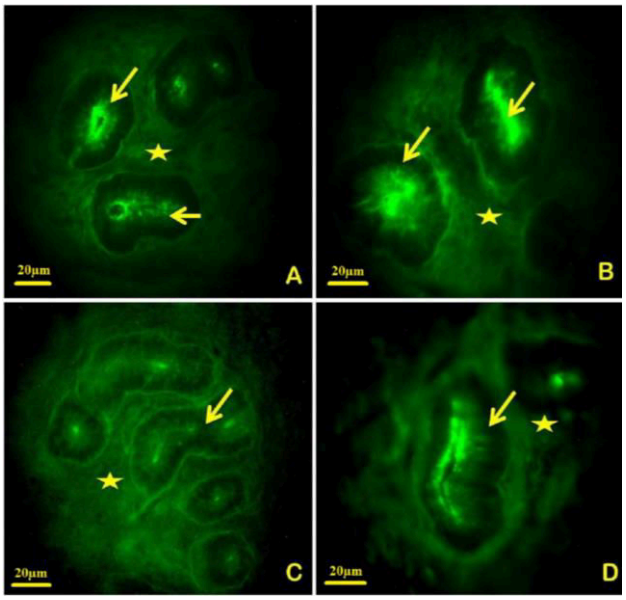

Fig. 3: WGA lectin histochemistry. WGA strongly binds with the glandular epithelium (arrow) with strong binding on apical surface, however the binding intensity with stromal compartment is weaker (star). (**A**) Control group, (**B**) Estradiol group, (**C**) Progesterone group, and (**D**) Mifepristone group

DBA staining

DBA binds with its complementary sugar α-N-acetylgalactoseamine. Similar to WGA binding, DBA was also bound strongly to the luminal and glandular epithelial cells. It possessed a weaker binding in the stromal compartment. The pattern of DBA staining was also similar in all groups (Fig. 4). Control and estradiol treated group shows a similar level of DBA staining (Figs. 4A and B). Staining intensity appeared highest in progesterone treated group (Fig. 4C) and lowest in the mifepristone treated group (Fig. 4D). Similar to WGA, the fluorescent intensity of the lectin varied among different groups (Fig. 5). Estradiol-treated group (51.86 ± 1.99) revealed non-significantly higher staining intensity as compared to the control group (49.18 ± 1.19). Progesterone-treated group (66.82 ± 4.06) possessed a much higher intensity ($P<0.05$) in comparison to the control group. However, in the case of mifepristone-treated group (39.09 ± 1.46), a significant decline was observed as compared to all the other groups.

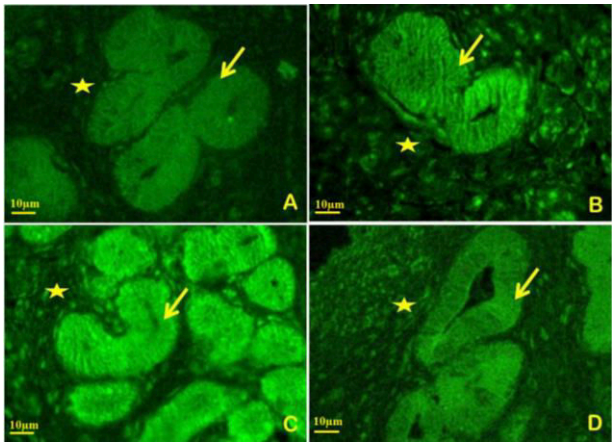

Fig. 4: DBA lectin histochemistry. WGA strongly binds with the glandular epithelium (arrow), however the binding intensity with stromal compartment is weaker (star). (**A**) Control group, (**B**) Estradiol group, (**C**) Progesterone group, and (**D**) Mifepristone group

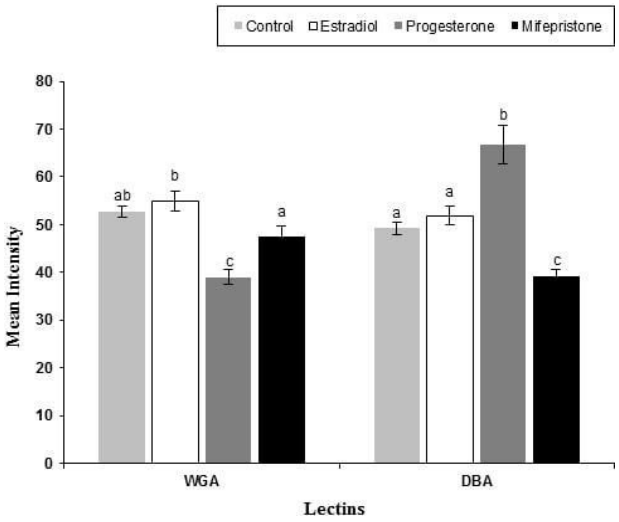

Fig. 5: WGA and DBA staining. Values are expressed as mean±SEM, $P<0.05$ (a, b, c, and d are showing the different levels of significance

Gene expression analysis

Expression levels of *MKI67*, *ESR1*, *PGR*, *CASP3*, *PDGFR-β* were analyzed using qRT-PCR for various groups, i.e., control, estradiol treated, progesterone treated and mifepristone treated. The relative mRNA abundance for ESR1 was declined to the lowest level (59% decline) in progesterone group (Fig. 6). However, its expression increased to 100% in estradiol treated group. In case of the mifepristone group, ESR1 level non-significantly declined to 10% compared with the control group. Similar to ESR1, the level of PGR gene expression was highest in estradiol group, and it reached

to 91% increase as compared to the control group (Fig. 6). Its level declined significantly in progesterone (37%) and mifepristone (30%) treated groups to a similar level. The relative mRNA abundance of MKI67 gene was lowest in mifepristone group (79% decrease), while in estradiol (153% increase) and progesterone (41% increase) groups its level is significantly higher compared with the control group (Fig. 6). The level of PDGFR-β mRNA increased in estradiol-treated (209%) and progesterone-administered (20%, non-significant) groups. Mifepristone treated group revealed a significant decline to 53% compared to the control group. The expression level of apoptotic gene, CASP3 declines in estradiol (50%) and progesterone (37%) group to a significant level in comparison with the control group (Fig. 6). However, in mifepristone group a 67% increase in CASP3 expression was recorded.

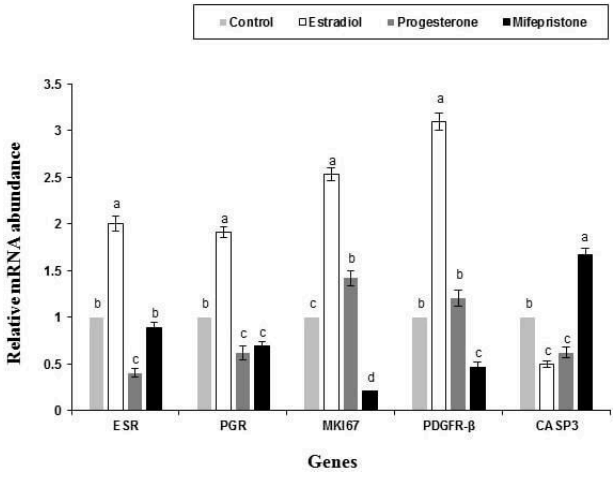

Fig. 6: Relative mRNA expression level of *ESR*, *PGR*, *MKI67*, *PDGFR-β*, and *CASP3* genes. Values are expressed as mean±SEM, P<0.05 (a, b, c, and d are showing the different levels of significance)

Discussion

The mammalian uterus undergoes cyclical alterations in terms of its structural and functional parameters. The goat uterine tissue undergoes morphometric alterations in response to the exposure of different steroids, estradiol, progesterone and mifepristone for 24 h in *in vitro* conditions (Sharma and Singh, 2021). In the present study, we reported that the topography of the endometrium gets transformed according to the type of steroidal exposure. In the control group (collected from uterus in proliferative stage), gland bulges without luminal orifices appeared on the endometrial surface. This is concordant with the study of Spornitz *et al*. (1999), who reported the appearance of gland bulges on the endometrial surface during the proestrus phase of the estrous cycle in rats. Spornitz *et al*. (1999) described that the gland bulges are actually real glands which remain covered with a very thin epithelial layer. Bulges are the sites where the mouths of glands appear during later proliferative or secretory (progesterone-dominated) phase. In the estradiol-administered group, we observed many gland openings; few of them contained the oozing out mucoid secretion; however, in the progesterone group there were majority of the glandular openings with exuding secretions. Mifepristone treatment made the uterine glands compressed and devoid of secretory substances. Motta and Andrews (1976) also documented the presence of numerous glands openings and secretion containing protrusions on endometrial surface in progesterone commanded secretory phase of rabbit. In goats, the estrous cycle (21 days duration) and pregnancy is accompanied by histochemical alterations in glycogen and lipid distribution in the endometrium (Tokashiki *et al*., 1996). During non-breeding season, the surface epithelium possesses a small amount of lipids and glycogen, however during pregnancy the expression of these substances elevates. In contrast to lipids and glycogen, the pigment cells appear during the cycle but disappear during pregnancy. Newton *et al*. (2019) demonstrated that the expression of the fucose-containing oligosaccharides, fucosyltransferase (FUT) and their enzymatic product, and H-type 1 antigen (HT1) in the luminal epithelium of the uterus is critically needed for the establishment uterine receptivity and placenta in goats. The expression of HT1 antigen in endometrium alters dramatically during the peri-implantation period from Day 15-19. In these ruminants, the FUT1 is primarily responsible for high levels of HT1 antigen present on the uterine luminal epithelium between days 5 and 11 of the estrous cycle and early pregnancy.

Lectin staining is practiced for the analysis of carbohydrate pattern; specific glycoproteins have been reported in uterine tissue that helps in cell recognition and adhesion (Sant'Ana *et al*., 2006). Lectin binding varies with the phase of reproductive cycle and pathological condition. In the current study, WGA staining was very strong in epithelial compartment, but it was weaker in stromal compartment of the endometrium. It showed an apical-basal polarity in binding to the epithelial cells; stronger binding was observed on apical surface than basal portion. The present findings are in congruence with Bartel *et al*. (2013), reporting a similar apical-basal polarity in WGA binding in the case of pigs. WGA was non-significantly higher in estradiol exposed group, while it declines significantly in the progesterone-treated group. In porcine uterus, during follicular phase, WGA stains the epithelial compartment strongly, while in secretory phase a decline in the staining occurs (Walter and Bavdek, 1997). Present observations in goats are contrary to that in humans. In humans, WGA binds prominently on the apical surface of the epithelium, but it binds almost equally to the epithelium in estradiol as well as progesterone influenced phase (Klentzeris *et al*., 1991). These differences might be due to species specific variations in glycoconjugate expression in uterine tissue. We documented a decline in WGA staining in mifepristone group.

DBA lectin also exhibited a strong affinity for epithelial compartment than stromal compartment, but

DBA did not reveal any apical-basal polarity. However, our results are contradictory to the findings of Bailey *et al*. (2010) in pigs, who reported a higher binding intensity on apical surface of the epithelial cells. They also reported that progesterone administration minimizes the DBA affinity. Munson *et al*. (1989) observed a higher DBA intensity in pregnant bovine endometrium than non-pregnant ones. The pregnant endometrium remains under the influence of far more progesterone compared to the non-pregnant endometrium (Verstegen-Onclin and Verstegen, 2008). Jones *et al*. (1998) also described that DBA staining remains almost absent under the influence of estradiol. However, it elevated on progesterone exposure. Similar to the findings of Danielsson *et al*. (2003), we also documented a significant decline in DBA binding on mifepristone administration.

In the present investigation, relative mRNA expression analysis of five different genes including *MKI67*, *ESR1*, *PGR*, *CASP3*, and *PDGFR-β* was also recorded. The MKI67 gene is a marker of proliferation and its expression analysis is performed to monitor mitotic activity or growth rate in a particular tissue type (Gerstenberg *et al*., 1999). MKI67 is expressed in all cell cycle phases except the resting phase. In the present study, estradiol and progesterone treatment elevates MKI67 expression compared to the control group. However, in mifepristone group, a significant decline (59%) was observed. Benbia *et al*. (2020) reported an increase in MKI67 expression during estradiol influenced follicular phase, but its level declined in the secretory phase. In contrast to Benbia *et al*. (2020), Dahmoun *et al*. (1999) described an increase in MKI67 marker expression during latter part of the secretory phase. Our finding is concordat with the investigation of Narvekar *et al*. (2004), who reported a decline in Ki67 expression on mifepristone exposure. We have reported a similar pattern of gene expression for *PDGFR-β* to that of MKI67. Various studies revealed that estradiol leads to an elevation in *PDGFR-β* expression (Rodriguez-Martinez *et al*., 1992; Yanaihara *et al*., 2005). *PDGFR-β* acts as a mitogen and contributes in proliferative activities, and thus its increase in exposure to estradiol is obvious. However, Chegini *et al*. (1992) observed that the different isoforms of PDGFR display different expression patterns in different phases of the reproductive cycle; PDGFRB expressed strongly in proliferative phase, while PDGFRAB appears at an equal level in both of the phases.

Estradiol and progesterone exhibit opposite effects in regulating the ESR1 and PGR expression; estradiol up-regulates the level of both receptor proteins, while the progesterone leads to a decline in their content (Casanas-Roux *et al*., 1996; Peralta *et al*., 2005). We also observed the same pattern of gene expression for these receptors. Ing *et al*. (1996) showed an increase in ESR1 and PGR mRNA expression in ovariectomized ewes exposed to estradiol. The present study revealed a decline in both of these receptors on exposure to mifepristone in consonance with the studies of Maentausta *et al*. (1993). In contrast to our study, some studies documented an increase in ESR1 and PGR on mifepristone exposure (Chen *et al*., 2011; Fiscella *et al*., 2011). *CASP3* belongs to the cysteine-aspartic acid protease family, and it is a key player in programmed cell death or apoptosis (D'Amelio *et al*., 2012; Tian *et al*., 2020). We observed that estradiol and progesterone both declined the level of CASP3 expression and, thus displayed a tissue-protective effect. Monroe *et al*. (2002) and Elkabes and Nicot (2014) also concluded that ovarian sex steroids are anti-apoptotic in nature. However, the antiprogestogen, mifepristone acts as an apoptosis inducer (Jang *et al*., 2013; Wang *et al*., 2014). We also observed a substantial increase in the level of *CASP3* on mifepristone exposure.

The results obtained in the present study revealed that the uterine tissue undergoes dynamic changes in topography, histo-architecture, and gene expression in response to hormones and anti-hormone exposure. Toporgraphical changes were not very prominent, but the differences were in the presence of mucoid secretion, gland openings, and ruffled cellular surface. Alterations in WGA and DBA signify the changes in chemical microenvironment of endometrial compartment in response to specific steroid presence. These differences might be required for carrying out specific activities like endometrial growth and differentiation. Growth and differentiation responses are further supported by specific gene expressions.

Acknowledgements

The authors are grateful to the CSIR for the financial support granted as Senior Research fellowship to Ms. P. Singh. We are also thankful to the Department of Zoology, Kurukshetra University, Kurukshetra, India, for UGC-SAP sponsored laboratory facilities.

Conflict of interest

There is no conflict of interest regarding this article.

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