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S. BENBIA, Y. BELKHIRI, M. YAHIA

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Research article
Ερευνητικό άρθρο

Apoptotic Cell Death in Ewe Endometrium during the Oestrous Cycle

S. Benbia[1]*, Y. Belkhiri[1], M. Yahia[2]

[1]Department of Biology of organism, Faculty of Life and Natural Sciences, University Batna 2, Algeria.

[1,2]Laboratory of Biotechnology of the Bioactive Molecules and the Cellular Physiopathology, Department of Biology of organism, Faculty of Life and Natural Sciences, University Batna 2, Algeria

[2]Department of cellular and molecular biology, Faculty of Life and Natural Sciences, University Abass Laghrour , Khenchela, Algeria.

ABSTRACT: We hypothesized that endometrial tissues from ewes undergo spatial and temporal changes. Thus, two regulatory events were investigated in this study: cell death (apoptosis) and cell proliferation. Uteri were obtained from healthy ewes at Batna abattoir (Algeria). Based on macroscopic observation of the ovaries and plasma progesterone, uteri were assigned to follicular, early and active luteal phases. Apoptosis and proliferation were assessed by detection of cleaved caspase-3 and Ki-67, respectively. Ki-67 and cleaved caspase-3 (CCP-3) were expressed in both phases of the oestrous cycle and all endometrium cells types [luminal epithelia (LE), superficial gland epithelia (SG) and deep gland epithelia (DG)]. Immunohistochemistry for cleaved caspase-3 revealed few or no apoptotic stained cells in all endometrium locations during the entire oestrous cycle. However, Ki-67 was significantly higher in the follicular phase than in the early and active luteal phase. Besides, expression of CCP-3 in LE was higher than in SG and DG at the follicular phase and early luteal phase. However, Ki -67 and CCP-3 levels in all endometrium cells types did not significantly change at active luteal phase. Therefore, it is concluded that apoptosis and proliferation were occurred in ewe endometrium in a cyclic pattern and under the influence of the endocrine profile.

Keywords: ewes; endometrium; apoptosis; proliferation; oestrous cycle.

Corresponding Author:
Souheyla Benbia, Laboratory of Biotechnology of the Bioactive Molecules and the Cellular Physiopathology, Department of Biology of organism, Faculty of Life and Natural Sciences, University Batna 2, P.O. Box 05000, Algeria
E-mail address: s.benbia@univ-batna2.dz

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INTRODUCTION

It is well known that the major reason for the failure of the embryo implantation is abnormal endometrial receptivity. During this period, morphological and biochemical changes occur in mammalian endometrium. These modifications are regulated by a plethora of factors, including oestrogen, progesterone, and their receptors, transcriptional factors and many others (Lonergan and Forde, 2014; Simmons et al. Recent investigations have demonstrated the importance of apoptosis in the processes of endometrial tissue remodeling during the oestrous cycle (Arai et al., 2013; Simmons et al., 2009). The balance between cell proliferation and apoptosis regulates the periodic repair and shedding of endometrial cells and leads to the menstruation (in the woman) or prepare the mucosal layer of endometrium for the implantation of the embryo(Stewart et al., 1999; Antsiferova and Sotnikova, 2016).

Apoptosis is a form of programmed cell death in which cells condense and fragment their nuclear material, condense their cytoplasmic material and then release their contents in membrane-bound apoptotic bodies (Mcilwain et al., 2013). Cells are induced to undergo apoptosis through either extrinsic or intrinsic pathways, which are controlled by the expression of a number of regulatory apoptosis-related genes, such as bcl-2 family (Li et al., 1998; Mcilwain et al., 2013), death receptor and caspases (Li et al., 1997; Mcilwain et al., 2013). Caspases, a family of aspartic acid-specific cysteine proteases are considered a key mediator of apoptosis in various cells (Brenner and Mak, 2009). Caspase-3 is the best-identified protein among the 14 caspase family members (Brenner and Mak, 2009; Mcilwain et al., 2013).

Ki-67 is a non-histone nuclear protein observed in proliferating cells and found in all phases of the cell cycle, except of G0 phase (Gap 0), and comprises two molecules of 345- and 395-kD weight; its gene is located on human chromosome 10.This marker is expressed during G1(Gap 1), S (Synthesis), G2, and M (Mitosis) phases of cell cycle. In the first phase, Ki-67 is frequently revealed neari the nucleus. During S and G2 phases, this protein is detected throughout the nucleus (Verheijen et al., 1989a). Generally, Ki-67 expression was observed during mitosis phase (Verheijen et al., 1989b).

Although cell proliferation has already been verified in the sheep endometrium (Zheng et al., 1996; Johnson et al., 1997a, 1997b), to our knowledge no study data on apoptotic cells are available. The aim of this investigation was to examine the occurrence of endometrial apoptosis and proliferation at defined stages of the oestrous cycle of the ewe, through the assessment of active caspase-3 and Ki-67, respectively.

MATERIAL AND METHODS

Collection of endometrium and blood samples

Twenty five normal uteri without a visible conceptus were collected from ewes at commercial abattoir, Batna (Algeria). Based on the methodology described previously by Benbia et al. (2017), ten of the ewes were classified in follicular phase and fifteen were in luteal phases (six at ELP and nine at ALP). Endometrial biopsies were obtained from the uterine horns, and then fixed in formol 10% before embedding. Jugular blood samples were collected into EDTA tubes from all animals before slaughter; plasma was stored at -20°C until assay.

Immunohistochemistry and scoring

Sections of 3-4 μm were placed on Super Frost slides (Menzel-Glaser, Freiburg, Germany). Slides were deparaffinized and subjected to antigen-retrieval at 95°C for 20 minutes on a Dako PT Link Envision Flex-Target retrieval solution at low pH (pH 6.0). Thereafter, slides were cooled and incubated for 10 min in Envision Flex Washing buffer.

Sections were stained in an automatic immunohistochemical staining device (Dako Autostainer Link 48) with mouse monoclonal anti Ki-67 antibody, clone MIB-1 (M7240; Dako-Cytomation, Glostrup, Denmark) as the proliferation marker and rabbit polyclonal anti cleaved caspase 3 (Asp175) antibody (#9661; Cell Signalling Technology, Boston, USA) as the apoptotic cell marker. Normal mouse or rabbit serum was used instead of the primary antibody (for negative control). Sections were incubated with secondary antibodies (EnVision FLEX; Dako) for 20 minutes. Staining was performed with 3,3-Diaminobenzidine (DAB) as chromogen for 20 minutes. Finally, sections were counterstained with Mayer's hematoxylin, in a graded series of ethanol, cleared in xylene, and covered with a cover slip using a monted medium.

Staining was assessed at: luminal epithelium (LE), superficial gland epithelium (SG) and deep gland epithelium (DG). Immunoreactivity of both markers was calculated using Quick Score (QS). The following formula was used for the evaluation of QS as

described by Sağsöz et al. (2011)stromal cells and smooth muscle cells. Generally, in the cervix, ERα immunoreactivity was more intense in the epithelial and smooth muscle cells during the follicular phase and in the epithelial cells during the luteal phase ($p < 0.05$:

QS= Intensity score (IS) + Proportional score (PS).

The IS was defined as the intensity of brown colour of nuclei. The intensity of staining for each protein was quantified using Image-Pro Plus 6.3 image processing and analysis software according to the manufacturer's instructions (Media Cybernetics, Inc., Bethesda, MD, USA). The PS was evaluated by manually counting the number of cells showing nuclear staining in 100 epithelial cells. The Immunoreactivity was categorized as described in table 1.

Hormone assessment in blood

Plasma progesterone concentration was determined using a radioimmunoassay kit (ImmuChem™ Double Antibody Progesterone I^{125} RIA Kit, MP Biomedicals, USA). The sensitivity of this assay was 0.18 ng/ml. Plasma concentrations of oestradiol 17β was assessed using E2 Diasorin RIA kit (Sorin Diagnostic, Antony, France). The sensitivity of the assay was 0.07 pg/ml.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism 6 (ver. 5.02, GraphPad Software, Inc., CA, USA). Initially, data were tested for normality and homogeneity (Bartlett test) of variances. The factors in the procedure were: stage of oestrous cycle (FP, ELP and ALP), and endometrium cells types (LE, SG and DG).

Effects of stage of the oestrous cycle on cell-specific expression of Ki 67 and CCP-3 were analyzed using two-way ANOVA. Comparison of means was performed by Bonferroni post hoc test. Possible correlations among cell apoptosis (CCP-3), cell proliferation (Ki-67) and plasma oestradiol 17β concentration, in the three endometrium cell types were tested using spearman correlation. Statistical significance was considered as $P<0.05$.

RESULTS

The plasma concentration of P4 was 3.2 ± 0.06 ng/ml in the active luteal phase and decreased ($P<0.05$) to to 1.1 ± 0.49 ng/ml in the next phase. The lowest P4 concentration was observed in the early luteal phase (0.5 ng/ml).

Localization and expression of Ki-67-positive cells throughout the oestrous cycle

As shown in Figure 1, endometrial cell proliferation and apoptosis were detected during the oestrous cycle of the ewes. Even though Ki-67 and CCP-3 were expressed in both follicular and luteal phases of the oestrous cycle, the Quick score of cells expressing Ki-67 was significantly higher than CCP-3 at the FP and ELP ($P<0.05$) (Fig1), while CCP-3 was significantly higher in FP compared to ALP. Furthermore, Ki-67 marker was higher at the early luteal phases compared to the active luteal phase ($P<0.05$) (Fig 1). Regarding cleaved caspase 3, no difference was observed among luteal phases ($P= 0.95$) (Fig1).

Table 1: Immunosensitivity categorization - Grading of Ki-67 and CCP-3.

IS		PS	
0	Absent staining	**1**	<1%
1	Weak staining	**2**	1–10%
2	Moderate staining	**3**	11–33%
3	Strong staining	**4**	34–66%
		5	67–100%

IS: intensity score.; **PS**: proportional score (%)

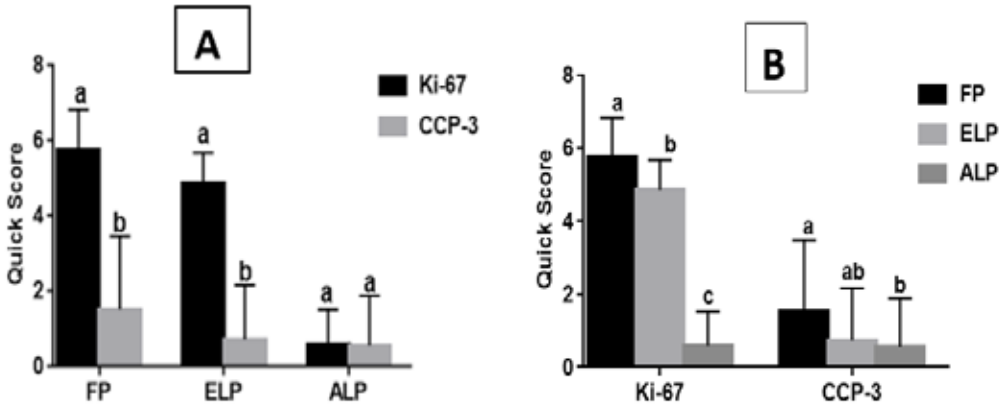

Figure 1. Comparaison between total Quick score of CCP-3 (mean±SEM) and Ki-67 (mean±SEM) expression in sheep endometrium during the oestrous cycle Values with different superscript letters differ significantly (P<0.05), between Ki-67 and CCP-3 (figure 1.A) and among the FP, ELP and ALP (figure 1.B).

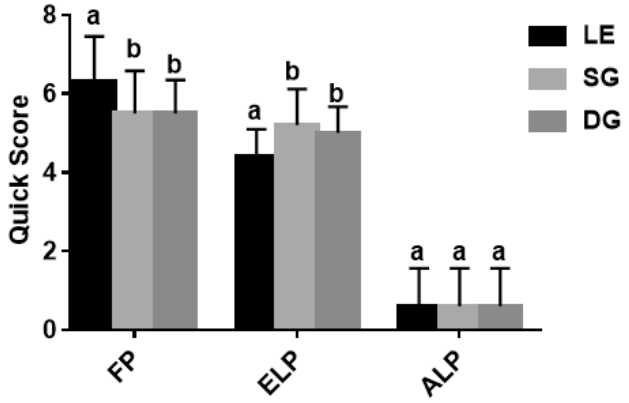

Figure 2. Quick score of Ki-67 (mean±SEM) **in different sheep endometrial compartments (luminal epithelium, superficial and deeper glands) at the follicular phase and at different stages of the luteal phase. LE:** luminal Epithelium; **SG:** Superficial Glands; **DG:** Deeper Glands; **FP:** Follicular Phase; **ELP:** Early Luteal Phase; **ALP:** Active Luteal Phase. Values with different superscript letters differ significantly, among the endometrium cell types (LE, SG, DG).

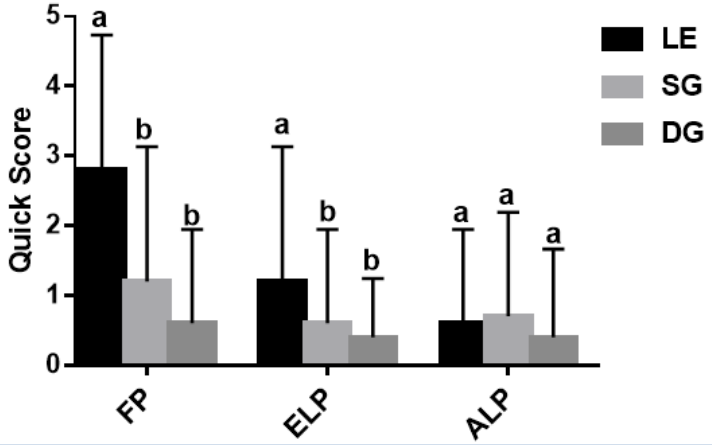

Figure 3. Quick score of CCP-3 (mean±SEM) in different sheep endometrial compartments (luminal epithelium, superficial and deeper glands) at the follicular phase and at different stages of the luteal phase. LE: luminal Epithelium; SG: Superficial Glands; DG: Deeper Glands; **FP:** Follicular Phase; **ELP:** Early Luteal Phase; **ALP:** Active Luteal Phase. Values with different superscript letters differ significantly, among the endometrium cell types (LE, SG, DG).

Ki -67 nuclear expression in different endometrial cells (LE, SG, DG) throughout the follicular and luteal phase are presented in Figure 2. Ki -67 stains all endometrium cells with high intensity, so intensity score was always as grade 3. The proliferative activity of luminal epithelium (LE) was significantly higher compared with endometrial glands (SG and DG) at follicular phase (P<0.01) (Fig 2). The immunoreactivity of cells expressing Ki-67 protein was significantly increased in the follicular phase and early luteal phase than in the active luteal phase. Besides, expression of this marker in LE, SG and DG was higher during the early luteal stage than in the active luteal phase (P<0.05) (Fig 2). Furthermore, Ki -67 nuclear expression in endometrial glands (SG, DG) did not significantly change during the follicular and early luteal stage transition (Fig 2).

Expression Ki -67 in uterine epithelium (LE, SG and DG) did not significantly change at active luteal phase.

Localization and expression of CCP-3-positive cells in ewe endometrium

Active CCP-3 expression was detected in both luminal and glandular epithelial cells in the sheep endometrium during all phases of the oestrous cycle (Fig 3). CCP-3 was more expressed in the luminal epithelium than in the glandular epithelial cells at the follicular phase and early luteal phase (P<0.05); however, the active luteal phase endometrium cells types did not affect the expression of this protein (Fig 3).

Relationship between cell apoptosis (CCP-3) and cell proliferation (Ki-67)

Table 2 shows that a negative correlation was revealed between CCP-3 and Ki-67 expression; this correlation was significant during the ELP (P=0.02) and approached significnace during FP (P=0.05).

Relationship between cell proliferation and plasma oestradiol 17β concentration

As shown in a Table 3, positive approached significant correlation was found between Ki-67 expression and plasma oestradiol 17β concentration in ewe endometrium in LE (ELP, P=0.06; ALP, P=0.05) and SG (ELP, P=0.08) during oestrous cycle phases (P>0,05) (this taken from the limited number of samples in this work).

Table 2. Correlation between cell apoptosis (CCP-3) and cell proliferation (Ki-67), during the oestrous cylcle.

	Ki-67 Quick score					
	PF		ELP		ALP	
CCP-3 Quick score	r		r	P	r	P
	-0.62	0.05	-0.751	0.02	0.334	0.2

FP: Follicular Phase; **ELP**: Early Luteal Phase; **ALP**: Active Luteal Phase; r: correlation coefficient; P: Probabilities significant (P<0.05)

Table 3. Correlation between cell proliferation and plasma oestradiol 17β concentration, in the three endometrium cell types during the oestrous cylcle.

		Plasma oestradiol 17β concentration					
		FP		ELP		ALP	
Ki-67 Quick score		r	P	r	P	r	P
	LE	0.691	0.059	0.501	0.06	0.689	0.05
	SG	0.61	0.063	0.761	0.08	0.565	0.084
	DG	0.40	0.19	0.39	0.49	0.32	0.33

FP: Follicular Phase; **ELP**: Early Luteal Phase; **ALP**: Active Luteal Phase; LE: luminal epithelium; SG: superficial gland; DG: deeper gland; r: correlation coefficient; P: Probabilities significant (P<0.05).

DISCUSSION

The present study examined, for the first time, the occurrence of apoptosis (CCP-3) in the various cells types of sheep endometrium, during the stages of the oestrous cycle. In many mammals, endometrium function and homeostasis is an important physiological phenomenon that ensures a dynamic balance between cell proliferation and cell death (apoptosis) throughout the oestrous cycle (Johnson et al., 1997b, 1997a) on cow (Arai et al., 2014, 2013) , goat (Zhang et al., 2018, 2019) and equine (Costa et al., 2007), but, to our best knowledge, not in sheep.

According to our results Ki-67 and cleaved caspase-3 were differentially expressed in endometrium of sheep, and this expression exhibited spatial and temporal changes. It was noteworthy that there were few or no apoptotic stained cells in all endometrium locations during the entire oestrous cycle.

In this research, we found higher levels of Ki-67 in the follicular and early luteal phase, compared with active luteal phase endometrium when low expression of this protein was observed in the various endometrium cell types. These Ki-67 patterns during luteal phase are in agreement with the results of García-Palencia et al. (2007), who demonstrated that intra-vaginal progestogen treatment had a great effect on the immunoexpression of cell proliferation marker in sheep endometrium.

In the present study, cleaved caspase-3 expression increased significantly in the follicular phase and decrease in early and active luteal phase. Additionally, we revealed that CCP-3 is differentially expressed in the various cell types of sheep endometrium. It was well-known that the main executioner involved in the cell death pathway is caspase-3 (CPP3), which is activated during the early stage of apoptosis in rat (Alan and Liman, 2016). The assessment of the active form of this protein is one of the most exploited diagnostic methods for revealing apoptosis in various cell types (Jin and El-Deiry, 2005). Likewise, it has been suggested that endometrial apoptosis was regulated by the expression of active caspase-3 during the oestrous cycle in goat and cow (Zhang et al., 2018; Arai et al., 2013) and during early pregnancy in the rat (Öner et al., 2010).

Recently, it was indicated that, several regulators of programmed cell death and cell proliferation, such as PTEN, PTEN/PI3K/AKT, Bcl-2 family, FAS, p53 and Caspase-3 are implicated in the apoptosis and proliferation of the endometrium cells of dairy goats in vitro, (Zhang et al., 2019). For example, Zhang et al. (2018) reported that miR-26a mimic increased the Caspase-3 protein levels in endometrium epithelial cells, but it decreased the expression of this protein in endometrium stromal cells of dairy goats (Zhang et al., 2018) . However, no such research has been performed in the endometrium of sheep. These in vitro studies suggested that there were enormous and complicated regulatory networks expressed differentialy in cell types and functions in the endometrium of ruminants. Except the oestrogen and progesterone, a variety of factors contributed in this regulation, such as growth factors (Stevenson et al., 1994; Md. Rashedul Islam et al., 2016) and integrins (Park et al., 2017).

In this study, we found that the expression of CCP-3 and Ki-67 were highly regulated temporally and among cell types, mainly in uterine luminal epithelium. Surface epithelium is the first contact point of the embryo during the early stage of implantation (Bazer, 2013). Our result suggest that apoptosis may be indispensable for the establishment of endometrial function in sheep.

Previous investigations in several mammalian species have revealed an inverse correlation between cell apoptosis and cell proliferation in the uterus (Harada et al., 2004; Costa et al., 2007; Okano et al., 2007) which was consistent with the results of the present study in the follicular phase and early luteal phase endometrium of sheep.

In the present study, a positive correlation was suggested between oestrogen concentration and cell proliferation score (approach significant) and was significant during early luteal phase in LE and SG cells. Thus, Thus, most proliferation occurred during the follicular and early luteal phases, when follicular growth and maturation result in the highest production of E2, followed by the second E2 peak during metestrus(Robinson et al., 2001).

Endometrial epithelial cells proliferation decreased during the active luteal phase when circulating P4 levels were high.. Shiozawa et al. (2001) hypothesized that in the luteal stage, P4 can raise the levels of some negative regulators, such as p27 (Shiozawa et al., 2001) and cyclin G1(Yuan et al., 2014) required for the proliferation inhibition as it has been observed in human endometrial epithelial cells. Recently Arm-

strong et al. (2017) revealed that apoptosis was rapidly induced after the progesterone-withdrawal in mouse and human endometrium (Armstrong et al., 2017). We hypothesized, probably, that a similar mechanism might also apply in the case of sheep.

CONCLUSION

It is concluded in the present report that both Ki-67((proliferation) and cleaved caspase-3 (apoptosis) were expressed in the endometrium of sheep, and this expression exhibited spatial and temporal variations. Tissue remodeling was mainly observed in luminal epithelium cells of endometrium.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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