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Research Article

Histomorphometric and Immunohistochemistry Studies of the Corpus Luteum of Bedouin Goats Reared in Arid Environment

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Abstract: The aim of this work was to study the histomorphometry and the immunohistochemistry of the Corpus luteum (CL) of Bedouin goat living in arid zones. Pregnant and non-pregnant females ovaries were collected from slaughterhouses, weighed, measured and then fixed in buffered formalin for histological, histochemical and immunolocalization of Ki-67, active caspase-3, aromatase and progesterone receptor. Our results showed that CL affected significantly the ovarian weight (P<0.001). The CL (P<0.05) and the large luteal cells (P<0.001) diameters were higher in pregnant compared to the non-pregnant CL. In the non-pregnant CL, the immunostaining of Ki-67 was only observed in the small luteal cell's nuclei whereas the active caspase-3 was detected in the large and small luteal cells cytoplasm. The aromatase was also detected in the CL and capsule cells cytoplasm, in contrast the progesterone receptor was observed in all luteal cell's cytoplasm and in some luteal cell nuclei. We conclude that in the Bedouin goat, the CL affects the ovarian biometric parameters. The development and luteolysis of non-pregnant CL seem to be under the balance between luteotropic and luteolytic markers, with luteolysis occurring by apoptosis via the caspase-3 pathway.

Keywords: *Active caspase-3, Aromatase, Corpus luteum, Goat, Histomorphometry, Immunohistochemistry, Progesterone receptor*

Kurak Ortamda Yetiştirilen Göçebe Keçilerin Corpus Luteum'u Üzerine Histomorfometrik ve İmmünohistokimyasal Çalışmalar

Öz: Bu çalışmanın amacı, kurak bölgelerde yaşayan göçebe keçilerin Corpus luteum'unun (CL) histomorfometrisini ve immünohistokimyasını incelemektir. Gebe ve gebe olmayan keçilerin ovaryumları mezbahalardan toplanmış, tartılmış, ölçülmüş ve daha sonra Ki-67, aktif kaspaz-3, aromataz ve progesteron reseptörünün histolojik, histokimyasal ve immünolokalizasyonu için tamponlu formalin içinde sabitlenmiştir. Bulgular, CL'nin ovaryum ağırlığını önemli ölçüde etkilediğini göstermiştir (P<0.001). CL (P<0.05) ve büyük luteal hücrelerin (P<0.001) çapları gebe CL'de gebe olmayan CL'ye kıyasla daha yüksekti. Gebe olmayan CL'de, Ki-67'nin immün boyanması sadece küçük luteal hücre çekirdeklerinde gözlenirken, aktif kaspaz-3, büyük ve küçük luteal hücre sitoplazmalarında tespit edilmiştir. Aromataz, CL ve kapsül hücrelerinin sitoplazmasında tespit edilmiş, buna karşın progesteron reseptörü tüm luteal hücrelerin sitoplazmasında ve bazı luteal hücre çekirdeklerinde gözlenmiştir. Göçebe keçilerinde, CL'nin ovaryum biyometrik parametrelerini etkilediği sonucuna vardık. Gebe olmayan CL'nin gelişimi ve luteolizi, luteotropik ve luteolitik belirteçler arasındaki denge altında görünmektedir ve luteoliz, kaspaz-3 yolu üzerinden apoptoz ile gerçekleşmektedir.

Anahtar sözcükler: *Aktif kaspaz-3, Aromataz, Korpus luteum, Keçi, Histomorfometri, İmmünohistokimya, Progesteron reseptörü*

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Introduction

The Algerian Sahara is reputed to be the hottest and most deserted region in the world. Despite this hostile conditions, Bedouin goat, a small ruminant perfectly adapted to its environment [1], manages to maintain a good reproductive performance. This testifies to their great capacity for adaptation, the mechanism of which remains poorly understood. These species play an important economic role for the population living in arid regions. The Bedouin goat was known for its high fertility, fecundity, prolificacy rates and seasonal breeding activity [2-5], to ensure the survival of the offspring by coinciding parturition with the adequate period of nutrition and climatic conditions [6].

The corpus luteum (CL), called "temporary endocrine gland" is an important ovarian structure resulting from the differentiation of follicular cells from the ovulatory follicle after the expulsion of the oocyte [7]. The presence of CL characterizes the luteal phase in the non-pregnant female and determines the appropriate course of the oestrous cycle; it also ensures the subsequent maintenance of pregnancy [8]. Pregnancy in the goat has been shown to be dependent on the presence of CL even after mid-gestation due to the low amount of progesterone produced by the goat's uterus. Indeed, the goat placenta of the goat does not produce progesterone in sufficient amounts to support pregnancy [9]. The lifespan of CL is a function of luteotropic and luteolytic factors [10]. Many studies have reported that the CL regulation is under hypothalamic and pituitary-gonadal hormones, progesterone, aromatase, and oestrogen cited as local regulatory hormones that acting as paracrine and/or autocrine factors and angiogenic factor like Ki-67, their activity is a sign of maintained luteal activity and CL progression [11]. Luteolysis is classified into two forms; functional and morphological luteolysis. Functional luteolysis is the underlying deterioration of progesterone discharge while morphological luteolysis is the consequent change in the CL [12]. It was reported that apoptotic cell death are mediated by locally produced factors such as caspase family molecules [13]. However, the process of CL growth and regression remains poorly understood in the goat due to lack of evidence. Some aspects of ovarian structure in the Bedouin goat have been carried out in previous studies [3,14]. The CL homeostasis must be maintained in order to avoid any reproductive issues. Indeed, luteolysis is involved in the maintenance of pregnancy if this later took place, otherwise the CL must regress in order to give the female the opportunity to start another ovarian cycle and thus become pregnant.

The aim of the current study was to provide more information on the histomorphometric analysis of non-pregnant and pregnant CL and also the immunolocalization of Ki-67, active caspase-3, aromatase (P450-Arom) and progesterone receptor (PR) in the non-pregnant CL of the Bedouin goat which will allow us to understand the molecular mechanisms that regulate the function of the non-pregnant LC

Material and Methods

Ethical Statement

This study was approved by the Algerian Ministry of Higher Education and Scientific Research (Executive Decree 10-90 supplementing the Algerian government decree 04-82) and the AASEA (45/DGLPAG/DVA. SDA.14).

Animal Protocol and Sample Preparation

A total of 29 adult goat, pregnant (n=12) and non-pregnant (n=17) aged from 2 to 5 years were used in this study. The diagnosis of pregnancy was carried out in post-mortem. After the slaughtering of goats at Bechar slaughterhouses (South West of Algeria, 31°62'N, 2°22'W), the genital tract was removed, examined and ovaries were excised immediately, washed with phosphate-buffered saline (PBS), classified according to the presence or absence of CL and then measured by estimating the weight, length, width and thickness. The diameter of non-pregnant and pregnant CL was measured after longitudinal sectioning, along the axis of the helium, using the caliper *(Fig. 1)*. Then, the ovaries were fixed in buffered formalin (10%) to perform histological, histochemical and the immunohistochemical technics.

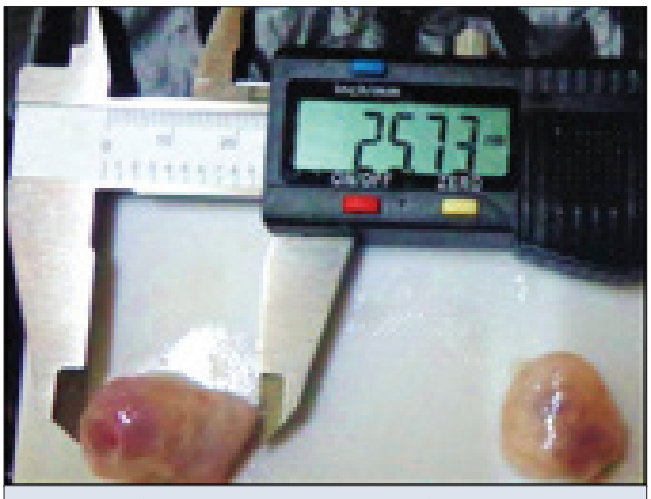

Fig 1. Ovarian biometric measurements using caliper

Histological Preparation

The fixed and sectioned ovaries were dehydrated in a graded series of ethanol (70, 80, 90 and 100°), clarified in xylene, embedded in paraffin and sectioned at 4 µm. Sections were used for the histological technique, mounted

on slides, deparaffinised and hydrated then stained with modified azan to demonstrate collagen fibres. For histochemical studies, the sections were stained by alcian blue at pH 2.5 to visualize carbohydrates [15].

Immunohistochemistry

Immunohistochemical detection of Ki-67, active caspase-3, P450-Arom and PR was performed on paraffin-embedded sections of ovaries using avidin-biotin complex method (ABC) (vectastain Elite ABC kit, Vector Laboratories, Burlingame). Before proceeding with the immunodetection of active caspace-3, sections were deparaffinised, hydrated and put in PBS (0.1 M; pH=7.2) then permeabilized at room temperature in 0.2 mg/mL saponin (Fischer Scientific UK) and proteinase K (Eurobio) mixture [3]. For the immunolocalisation of P450-Arom, PR and Ki-67 [15], the antigen retrieval step was realised by immersing of the section in 10 mM of sodium citrate solution (pH=6.0) in a water bath during 40 min at 95°C. The endogenous peroxidases are blocked by H_2O_2 (3% in PBS) during 5 min then rinsed in PBS. The nonspecific background was blocked by a normal horse serum for 30 min at room temperature. Sections were incubated with different primary antibodies at room temperature: rabbit monoclonal active caspase-3 antibody (1:50, ab32042 Abcam, Cambridge, UK Cambridge, UK) for 1h, mouse monoclonal Ki-67 antibody (1:50, RM-9106-S, Thermo Fisher Scientific, USA) for 1h, rabbit polyclonal CYP19A1 antibody (1:100, H-300, SC-30086 Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal PR antibody (1:50, AB-52, sc-810, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After rinsing for PBS, all sections were incubated with a biotinylated anti-mouse/rabbit IgG secondary antibody (CA 94010, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame) for 1 h at room temperature. They were incubated for 1h at room temperature with Avidin-Horse Radish Peroxidase complex and then rinsed in PBS. For the visualisation of the immunolabeling, the DAB (3,3′-diaminobenzidine) chromogen (Dako) was added as a chromogen staining substrate. Sections were counterstained in Harris haematoxylin (Hematoxylin QS, H-3404; Vector lab, Burlingame, A, USA). After rinsing in water, the sections were dehydrated and cover slipped with Eukitt. For negative controls, a similar protocol was performed for each antibody, except for incubation of the primary antibody which is replaced by the normal horse serum.

Morphometric Analysis

The histological slides were stained with modified azan coloration and observed with light microscope (Optika B-350) using a computer program Ts View connected to a digital eye-camera (Hirocam MA88-500). The parameters evaluated for CL were: CL, large luteal cell (LC), small luteal cell (SC), large luteal cell nuclei (LCN), small luteal cell nuclei (SCN) diameters and cytoplasm-to-nucleus ratio (CNR) of LCN and SCN. The area (A) of these parameters was measured using Axiovision software. The shape of the CL cells and nuclei was assumed to be spherical; their diameter (D) was deduced by the mathematical relationship applied for the calculation of the area.

$$D = \sqrt{\left(\frac{4A}{\pi}\right)}$$

with $A = \pi \times r^2$ and $r = D/2$

Statistical Analysis

The results were represented by means ± SEM. Statistical analyses were performed using the SPSS for Windows v.26.0 (IBM Corp., NY, USA). Data which were not normally distributed were submitted to logarithmic transformations. The data were analysed with independent *t*-test or with Mann-Whitney test as appropriate. These tests were used to compare the differences in parameters between the ovaries with and without CL also between pregnant and non-pregnant CL groups. We considered that $P<0.05$ was statistically significant.

Results

Morphological Study

The morphological study of the ovaries *(Fig. 2-A)* revealed an ovoid shape with an irregular surface *(Fig. 2-B)* containing some follicles and CL *(Fig. 2-C)*. Both of ovaries from pregnant and non-pregnant females were exhibited the same colour and did not show apparent differences in their morphology.

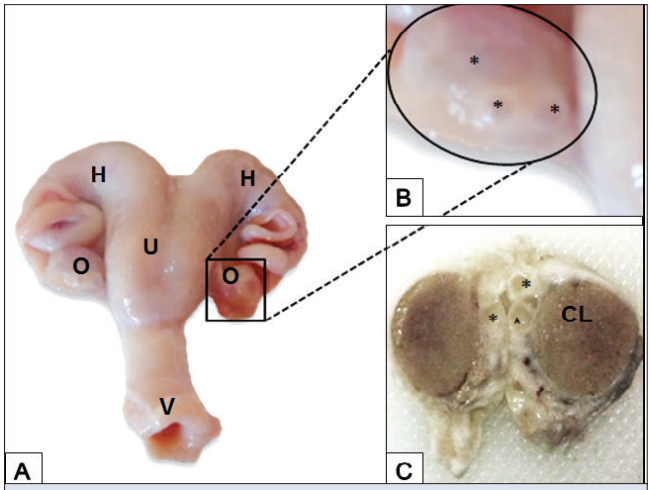

Fig 2. Genital tract of Bedouin goats reared in arid environment. A- Localisation of the ovary in the genital tract. B- External view of ovary. C- Sectioned ovary. CL: corpus luteum, H: horn, O: ovaries, U: uterus, V: vulva, *: Ovarian follicles

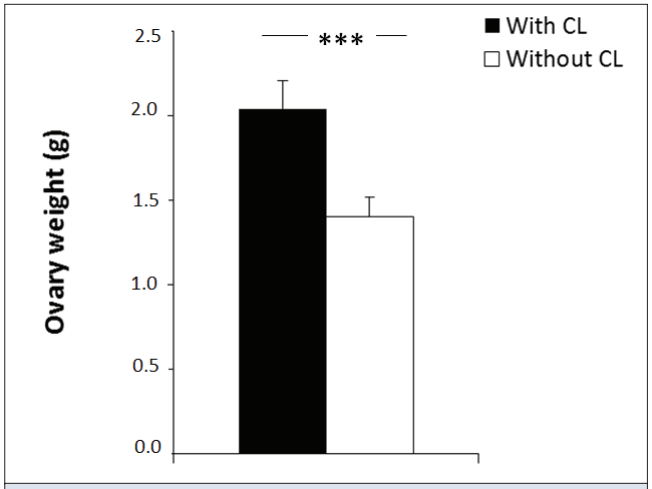

Fig 3. Weight of the ovaries with and without corpus luteum of Bedouin goats reared in Arid environment. Data are presented as mean ± SEM. ***P<0.001

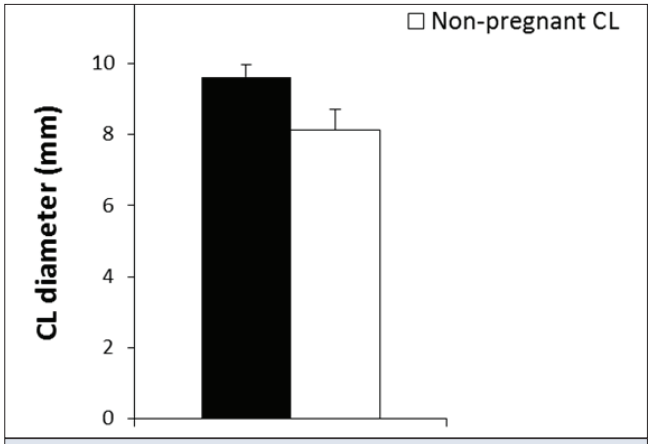

Fig 5. Diameter of corpus luteum from pregnant and non-pregnant Bedouin goats reared in arid environment. Data are presented as mean ± SEM

Ovarian Morphometric Study

The morphometric analysis of the ovaries parameters were reported in the *Fig. 3* and *Fig. 4.* The weight of ovaries with and without CL (2.04±0.16 g *vs* 1.4±0.12 g respectively) showed a highly significant difference (P<0.001) (*Fig. 3*).

Ovaries with and without CL showed a non-significant difference (P>0.05) in length (19.09±0.74 *vs* 18.05±0.63 mm), width (15.55±0.45 *vs* 12.33±0.54 mm) and thickness (11.00±0.38 *vs* 10.53±0.52 mm) *(Fig. 4).*

The diameter of pregnant CL was higher (P>0.05) than the diameter of non-pregnant CL (9.63±0.34 mm *vs* 8.12±0.59 mm respectively) *(Fig. 5).*

Histomorphometry of Corpus Luteum

Histologically, the CL was delimited by a capsule which was composed of fibroblasts, mainly comprised of collagen fibbers and blood vessels surrounded the CL

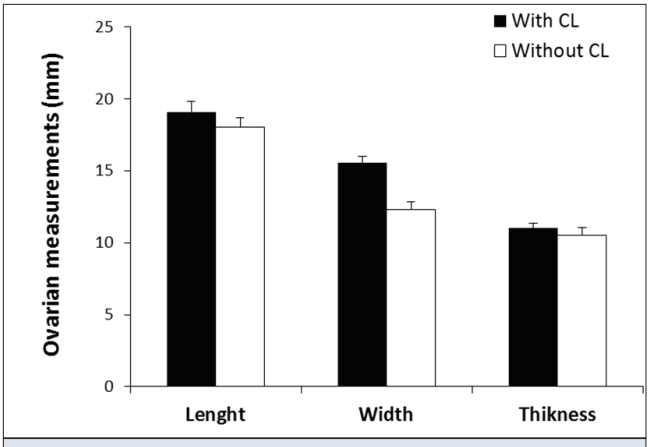

Fig 4. Measurements of the ovaries with and without the corpus luteum of Bedouin goats reared in arid environment. Data are presented as mean ± SEM

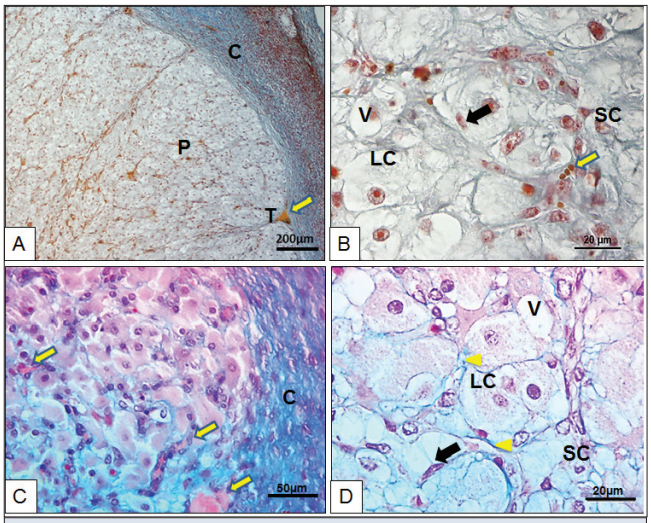

Fig 6. Histology of corpus luteum of Bedouin goats reared in arid environment. A: Photomicrograph showing capsule and trabeculae richly vascularised *(yellow arrow)* surrounding the parenchyma which is crossed by trabeculae, B: Photomicrograph showing the composition of CL parenchyma, LC are ovoid and enclose vacuole and eccentric nuclei with a prominent nucleolus, small luteal cell (SC), blood cells *(yellow arrow)* and fibroblast cells *(black arrow),* C-D: Photomicrographs showing the carbohydrates in the CL, the capsule were highly reactive to the alcian blue and were richly vascularized *(yellow arrow),* the alcian blue reactivity was also observed surrounding the luteal cells *(yellow arrow ahead)* in (D). C: capsule; LC: Large luteal cells; SC: small luteal cells; P: parenchyma; T: trabeculae; V: vacuole. Stained with modified azan (A and B) and alcian blue (C and D)

parenchyma *(Fig. 6-A).* Histochemically, this capsule exhibited a positive reaction to the alcian blue *(Fig. 6-C).* The CL parenchyma was crossed by trabeculae containing connective tissue and constituted by endothelial, blood, small and large luteal cells *(Fig. 6-B,C).* Both of LC and SC were spherical cells centred by spherical nucleus. However, the LC is characterised by its large size and the presence of vacuoles in its cytoplasm *(Fig. 6-B).*

The morphometric measurements of the diameter of luteal cells and nuclei between pregnant and non-pregnant CL

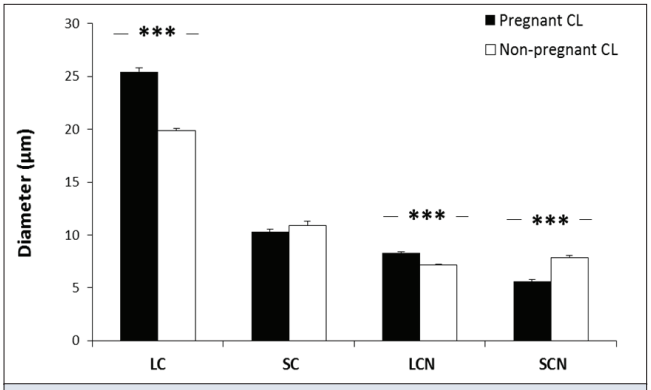

Fig 7. Diameter of luteal cells and nuclei from pregnant and non-pregnant corpus luteum of Bedouin goats reared in arid environment. LC: large luteal cells; LCN: large luteal cells nuclei; SC: small luteal cells; SCN: small cells nuclei. Data are presented as mean ± SEM. ***P<0.001

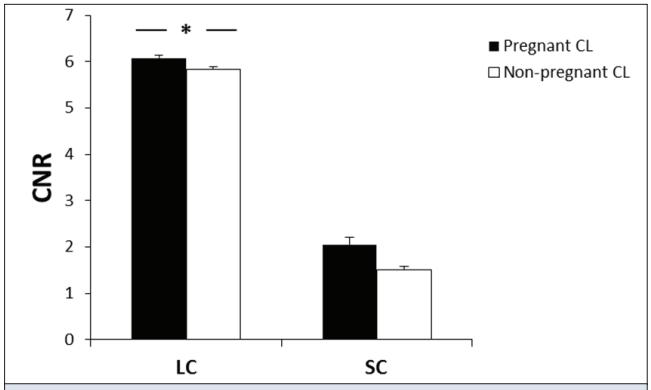

Fig 8. The cytoplasme-to-nucleus ratio of luteal cells from pregnant and non-pregnant corpus luteum of Bedouin goats reared in arid environment. LC: large luteal cells; SC: small luteal cells. Data are presented as mean ± SEM. *P<0.05

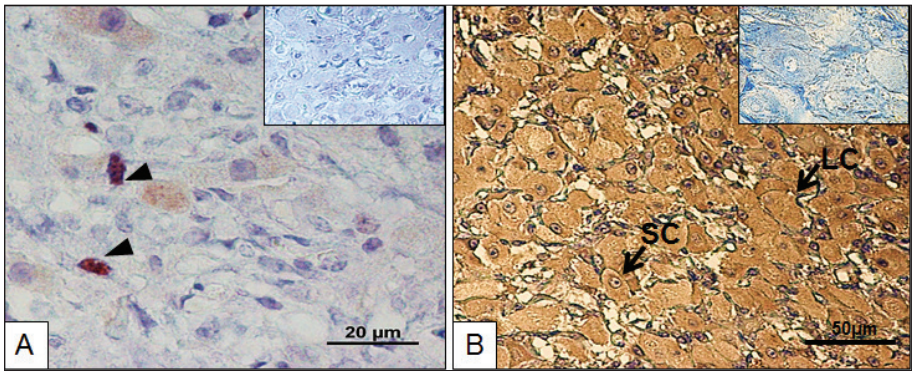

Fig 9. Immunohistochemistry of Ki-67 and active caspase-3 in non-pregnant corpus luteum of Bedouin goats reared in arid environment. A - Immunolocalization of Ki-67 in nuclei of SC *(black arrow)*. B- Immunolocalisation of active caspase-3 in both LC and SC cytoplasm, DAB visualization system. Negative controls insert in A and B

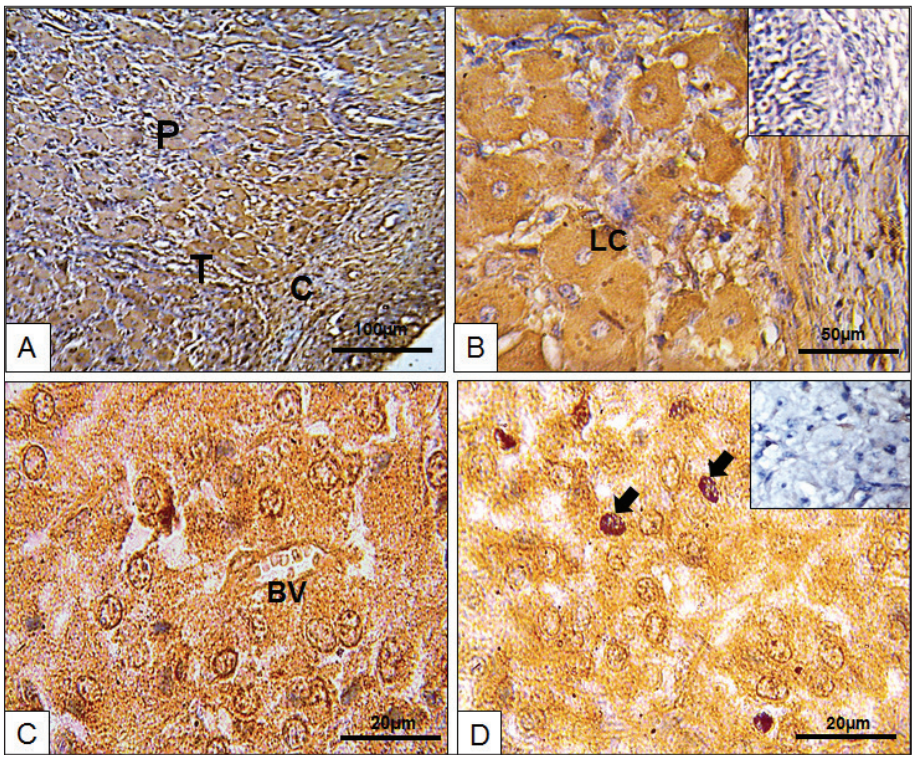

Fig 10. Immunohistochemistry of aromatase and progesterone receptor in non-pregnant corpus luteum of Bedouin goats reared in arid environment. A and B- Immunolocalization of P450-Arom in non-pregnant CL showed cytoplasmic staining in the luteal cells and capsule cells. C and D- immunolocalization of PR in the cytoplasm of CL cells predominantly and in some nuclei *(black arrow)*, DAB visualization system. Negative controls insert in B and D

were reported in *Fig. 7*. The LC diameter from pregnant CL (25.44±0.34 µm) was significantly higher (P<0.001) than LC diameter from the non-pregnant CL (19.85±0.21 µm). The LCN diameter was significantly higher (P<0.001) in the pregnant CL (8.33±0.08 µm) than that of non-pregnant CL (7.14±0.1 µm). Moreover, the SC diameter

from pregnant CL (10.27±0.29 µm) was lower compared to that to the SC from the non-pregnant CL (10.89±0.44 µm) (P>0.05). A significant difference has been observed between the SCN from pregnant CL and that from non-pregnant CL (P<0.001). Additionally, the CNR of LC (6.078±0.06 *vs* 5.85±0.06) (P<0.01) and SC (2.05±0.16 *vs* 1.50±0.09) (P<0.001) was significantly higher in pregnant CL than that of non-pregnant and *(Fig. 8)*.

Immunohistochemistry of Corpus Luteum

The immunohistochemistry of Ki-67 and active caspase-3 in non-pregnant CL was reported in *Fig. 9*. The immuno-localization of Ki-67 was observed only in nuclei of SC *(Fig. 9-A)*; the other CL cells did not exhibit any immunostaining. However, the active caspase-3 was immunolocalized in the cytoplasm of both LC and SC of non-pregnant CL *(Fig. 9-B)*.

The immunohistochemistry of P450-Arom and PR in non-pregnant CL was reported in *Fig. 10.* The CL expressed P450-Arom in all of the CL cells cytoplasm and capsule fibroblasts *(Fig. 10-A)*. The PR was observed in SC and LC cytoplasm with a variable intensity of immunostaining at the level of the nucleus of these cells *(Fig. 10-B)*.

Discussion

In this study, the morphological aspects of the goat ovaries collected from local slaughterhouses have been described; we reported that all of the ovaries were similar in shape and colour. Macroscopically, the description of ovaries was similar to those reported in other goats [16]. Regarding the presence or absence of CL, these ovaries were recorded as ovaries with and without CL. On the other hand, the mean weight of ovaries was significantly higher (P<0.01) and length, width and thickness were comparatively higher in the ovaries with CL than that without CL. It has been revealed that the presence of CL in ovaries increases ovarian biometric measurements. The same observations have been reported in other goat breeds [17-19]. Shathi et al.[20] and Mervat et al.[21] demonstrated the effect of CL presence on the morphometry of goat and cow ovaries respectively which affects the weight and dimensions. Miranda-Moura et al.[22] showed the existence of positive correlation between the dimensions of the ovary and the CL and between the weight of the ovary and the CL. Jablonka-Shariff et al.[23] explained that the higher value of biometric measurements of ovaries were due to the hypertrophy of luteinized granulosa cells, hyperplasty of fibroblasts of the connective tissues and vascularity of the CL.

The diameter of the non-pregnant CL found in this study was lower than that of pregnant CL. The non-pregnant CL of Bedouin goat was similar of that of the Alpine goat in dioestrus phase [24]. This increase in CL diameter, during pregnancy, suggest that the growth of luteal tissue is positively correlated with CL functionality which is represented by synthesis and production of progesterone [24].

Histologically, it was observed that the CL in the Bedouin goat is formed by heterogeneous population cells (small luteal, large luteal, fibroblastic and endothelial cells) in accordance with the composition of the CL observed in the Alpin goat [19], Nelore sheep [25] and cows [10].

Our study demonstrates that the morphometric diameters of the large and small luteal cells was higher compared to the luteal cells from Angora goat which varied as CL aged [26]. The CNR of both large and small luteal cells was significantly higher in the pregnant CL than in the non-pregnant CL; it is admitted that in growing cells, the cytoplasm is continuously expanding from amino-acid and nutrient import, and also protein synthesis [27]. Moreover, the CNR was affected by numerous factors: protein and ribosome synthesis, transport across both cell surface and the nuclear envelop and protein degradation and ribosome disassembly [28]. This may explain why the luteal cells of the pregnant CL were more active than the luteal cells of the cyclic CL , this activity seems to be related to steroidogenic synthesis to maintain the pregnancy which is essentially dependent on the presence of CL in goat [9] .

In this study, we have highlighted some factors such as Ki-67 as a cell proliferation marker, active caspase¬-3 a luteolytic factor, P450-Arom and PR. Indeed, the activity of CL was influenced by the balance between luteotropic and luteolytic factors which affected the structural and morphological appearance of the CL [29]. Investigating the CL activity, we found that the Ki-67 was localized in the nucleus of steridogenic cells, more precisely in the SC in the non-pregnant CL. The same observation was reported by Yoshioka et al.[30] in cattle CL which demonstrated that only SC proliferate during luteal development. Indeed, during the ruminant CL life, granulosa-derived luteal cells were predominantly non-proliferative while theca-derived luteal cells were proliferative during the early luteal phase and become non-proliferative by the late luteal phase [31]. In addition, it was reported that in the early luteal phase the development of CL is supported by robust angiogenesis which is accompanied by dynamic extracellular matrix remodelling that affects deeply the CL development and maturation [32]. As a result, the mature CL is a highly vascular gland and luteal endothelial cells comprise the larger part of its cells [32].

In the other hand, we reported that the luteal cells in some of CL were positive to active caspase-3 suggesting that these luteal cells initiated the cell death pathway via a caspase dependent mechanism. In this study, caspase-3 activity (marker of apoptosis) was detected in the large and small

luteal cells cytoplasm. It was reported in a previous study in the Bedouin goat, that morphological changes reach atretic follicles wall by apoptosis via caspase-3 signalling pathway in breeding and non-breeding seasons [3]. The active caspase-3 being expressed in the granulosa and theca cells [3] suggested that these cells retain the same cell death mechanism after their differentiation into luteal cells in CL. Caspase-3 has been also shown to be involved in luteal regression in cows [33], sheep [34], rabbits [35], women [36] and mice [37]. However, morphologically we did not observe any structural changes indicating the luteolysis, it seems probably a functional luteolysis as reported in the study of Hiti et al.[38] yet, the transition from functional luteolysis to structural luteolysis has not been precisely presumed. In another study, It was reported that the activity of caspase-3 was involved in initial process of cell death and observed before morphological changes [32]. In addition, It seems that the expression of the active caspase-3 interfered with functional luteolysis including cessation [39]. Accumulating evidence indicates that luteolysis was divided into two phases, namely functional luteolysis characterized by a decline in progesterone concentration and structural luteolysis characterized by the degradation of luteal tissues from the ovary [40].

The present study shows that both large and small luteal cells exhibited positive cells immunostaining to P450-Arom, this finding agreed with the study done in Japanese Shiba goat [41], Criollo goat, sheep [42] and pigs [43]. Previous studies demonstrated the expression of P450-Arom in both luteal cell type which increased as the luteal phase progresses [44]. Gregoraszczuk reported a few positive cells to P450-Arom in the early porcine CL cells but no reactivity was detected in mid luteal CL [44].The presence of positive signal for P450-Arom in the non-pregnant CL of the Bedouin goat suggested that this tissue has the capacity to produce oestrogen.

As a result of our study, we observed strong immunostaining for PR in goat luteal cells in non-pregnant CL; the immunolabeling was essentially cytoplasmic with few occasional immunostaining nuclei which suggest specific genomic response. It is well known that the primary function of the CL is the secretion of progesterone (P4), which is required for maintenance of normal pregnancy in mammals [42]. The P4 exerts its main function by binding with progesterone receptors PR to induce cellular responses through genomic or non-genomic signalling cascades[45]. The increase of the diameter of large luteal cell in our study seems due to its steroidogenic activity to produce more progesterone during CL progression to prepare and maintain the gestation. It is reported in the previous data that both SC and LC, are capable of producing this steroid, however, LC are more secretory-active [46]. In the Nelore cow, it is suggested that there is a stimulatory effect of progesterone in a paracrine/autocrine manner on the formation and the initial secretory activity of the CL. In addition, in the pseudo pregnant rabbit, the atretic large lutein cells of the regressed corpus luteum showed negative immunostaining for PR [47]. Indeed, While the majority of the existing research on Progesterone focuses on classic P4/PR paired actions such as nuclear transcriptional factors, there is new evidence suggesting that P4 also induces a wide variety of P4 actions through non-classic membrane PR receptors [45].

In conclusion, the presence of CL caused a significant increase in the ovarian wight and also increased various ovarian biometric parameters. The large luteal cells increased in diameter in the pregnant CL compared to the non-pregnant CL suggesting that the steroidogenic activity may be provided by the large luteal cells more than the small ones which are characterised by proliferative ability. The functioning of the non-pregnant CL is represented by the oestrogen production via the aromatase activity and P4/PR regulation. The luteolysis in CL of the Bedouin goat undergoes the apoptotic mechanism via caspase-3 pathway.

Availability of Data and Materials

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Author Contributions

S.K.M.: Conceptualization, Methodology, Validation, Formal analysis, investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. N.B.F.: Methodology, Formal analysis, Visualization, Review & Editing. L.A.A.A.: Visualization and Review. E.M.: Conceptualization, Methodology, J.M.E.: Visualisation and Revision. F.K.: Resources, Visualization & Supervision.

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