Kafkas Univ Vet Fak Derg
22 (6): 877-882, 2016
DOI: 10.9775/kvfd.2016.15557

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Localisation of Estrogen Receptor Alpha and Progesterone Receptor B in Goat Ovaries During Breeding and Non-Breeding Season [1] [2]

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[1] *This study was supported by the Scientific Research Project Coordinator's Office at Yuzuncu Yıl University (2013-VF-B018)*

[2] *Summary of this study has been presented in "48th Annual Conference Physiology and Pathology of Reproduction and simultaneously 40th Joint Congress of Veterinary and Human Medicine February Conference, Zurich, Switzerland, 11th - 13th February 2015"*

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Article Code: KVFD-2016-15557 Received: 02.03.2016 Accepted: 26.04.2016 Published Online: 26.04.2016

Abstract

The main objective of this study was to investigate the localisation of estrogen receptor alpha (ER-α) and progesterone receptor B (PR-B) by immunohistochemistry in goat ovaries during in- and out of breeding season. The ovaries obtained from goats slaughtered in breeding season (n=10) and non-breeding season (n=10) were used. From the same animals, blood samples were taken to determine the levels of serum steroid hormones (E_2, P_4). The ER-α and PR-B immunohistochemical distributions within the ovaries were determined by the ABC method. In breeding season, the ER-α was detected in the germinal epithelium and follicular granulosa cells. The PR-B was determined to concentrate on the corpus luteum (CL) cells. The same receptors were also found to be weak in the theca externa cells of preovulatory follicles. In non-breeding season, the ER-α germinal epithelium and smooth muscle cells of certain blood vessels showed a weak positive reaction. The PR-B was positively stained in the germinal epithelium and few stroma cells. It was suggested that the average steroid hormone profiles in breeding (E_2: 11.83±1.70 pg/mL, P_4: 10.08±1.58 ng/ml) and non-breeding season (E_2 2.33±0.85 pg/ml, P_4 0.21±0.04 ng/ml) correlated well with the localisation intensity of receptors in goat ovaries.

Keywords: *Goat, Ovary, Immunohistochemistry, Estrogen receptor α, Progesterone receptor B*

Keçi Ovaryumunda Üreme Sezonu İçinde ve Dışında Östrojen Reseptör alfa ve Progesteron Reseptör B'nin Lokalizasyonu

Özet

Sunulan bu çalışmada, üreme sezonu içinde ve dışındaki keçi ovaryumlarında, östrojen reseptör alfa (ER-α) ve progesteron reseptör B'nin (PR-B) yerleşim yerlerinin immünohistokimyasal olarak araştırılması amaçlandı. Mezbaha şartlarında kesilen dişi keçilerden, üreme sezonu içinde (n=10) ve dışında (n=10) elde edilen ovaryumlar kullanıldı. Aynı hayvanların, serum steroid hormon düzeylerini (E_2, P_4) belirlemek için kan örnekleri alındı. ER-α ve PR-B'nin ovaryumlardaki yerleşimleri immünohistokimyasal olarak ABC metodu ile belirlendi. Üreme sezonu içinde, ER-α reseptörleri germinatif epitelyum ve foliküllerin granüloza hücrelerinde saptandı. PR-B reseptörlerinin ise corpus luteum (CL) hücrelerinde yoğunlaştığı belirlendi. Aynı reseptörlerin, preovulator foliküllerin teka eksterna hücrelerinde de zayıf olarak bulundukları gözlendi. Üreme sezonu dışında, ER-α reseptörleri germinatif epitelyum ve bazı kan damarlarının düz kas hücrelerinde zayıf pozitif reaksiyon gösterdi. PR-B ise germinatif epitelyum ve bazı stroma hücrelerinde pozitif boyandı. Keçilerde, üreme sezonu içi- (E_2: 11.83±1.70 pg/mL, P_4: 10.08±1.58 ng/mL) ve dışındaki (E_2: 2.33±0.85 pg/ml, P_4: 0.21±0.04 ng/ml) ortalama steroid hormon profillerinin bu reseptörlerin ovaryumlardaki lokalizasyon yoğunlukları ile uyumlu olduğu kanısına varıldı.

Anahtar sözcükler: *Keçi, Ovaryum, Immünohistokimya, Östrojen reseptör α, Progesteron reseptör B*

INTRODUCTION

Essential steroid hormones for reproduction are secreted from the ovary; such that progesterone is secreted from the corpus luteum (CL) while estrogen is secreted from ovarian follicles in different developmental stages [1]. These hormones play a crucial role in morphologic and functional changes in the reproduction organs in females [2].

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Estrogen is a well-known regulator in steroidogenesis and folliculogenesis. It regulates follicular development by stimulating the ovarian granulosa cell proliferation. Follicle stimulation hormone (FSH) secretion is also very important in the regulation of gap junctions between the granulosa cells [3,4]. There are two specific estrogen receptors, so called as the estrogen receptor alpha (ER-α) and estrogen receptor β (ER-β), that are localised dispersedly within the ovarian tissue and responsible for the intraovarian movements [5,6]. Localisation and distribution of these two receptors given have been defined in immunohistochemical studies conducted in various animal species [7-10]. The ER-α and ER-β are localised within the germinal epithelium, interstitial tissue and follicles in different developmental levels. Localisation places of two receptors could change according to the species of the animal and hormonal periods [5].

Progesterone hormone, secreted from the CL, is also critical in regulating reproductive cycles in mammals. It has primary function in implantation; supporting uterus with uterine milk and sustaining pregnancy following the embryo enters into the uterus [11]. The progesterone (P_4) gets activated after binding to the intracellular progesterone receptors (PR) in target tissues. There exist two isoforms of progesterone receptors A - B, (PR-A, PR-B) [12,13]. Further, there is a progesterone receptor C (PR-C), but it is seen in pathologic situations such as breast cancer [14]. Progesterone receptors have been studied in women [6], cows and bitches in different stages of their estrous cycles [15,16].

Researchers have long been working on reproduction to increase the productivity in goat breeding, as in other farm animals. In the literature, no data could be found on the immunohistochemical investigation and comparison of ER-α and PR-B in goat ovaries during breeding or non-breeding seasons. Therefore, the aim this study was to determine the localisation areas of ER-α and PR-B immunohistochemically in the ovaries of goats during in- and non-breeding season.

MATERIAL and METHODS

Animals and Tissues

An official approval from the YYU Animal Experiments and Ethical Council was received (dated on 16.05.2013, with decision number 05) prior to this study. In breeding- (n=10) and non-breeding season (n=10), goat ovaries were acquired from Van City slaughterhouse. Prior to slaughtering animals, 5 ml of blood samples were collected and their sera were extracted to determine estrogen (E_2) and P_4 hormone levels. The concentrations were measured with appropriate kits with ELISA system [17,18].

The ovaries collected were fixed with 10% formaldehyde for 24 h after morphologic evaluation; tissues were blocked with paraplast after routine histologic processes [19]. Serial sections of 5 µm obtained from each tissue were stained immunohistochemically to determine the ER-α and PR-B receptors.

Hormonal Procedures

Following samples of 5 ml blood were taken and serum extracted, estrogen and progesterone hormone levels were measured by Goat Estradiol (E_2)/Progesterone (P_4) ELISA Kits (Cusabio) respectively, according to the manufacturer's recommendations.

Immunohistochemical Procedures

Tissues were stained immunohistochemically with ABC method [20]. Tissue sections (4 µm) were taken on polysine-coated slide (Thermo scientific, Menzel -Glaser, Germany). Antigen retrieval (citrate buffer 10%, pH: 6) was applied for 40 min boiling after rehydration of deparaffinised sections. Peroxidase blockage was applied in H_2O_2 for 20 min to prevent non-specific bindings after cooling to room temperature (in 3% methanol). Protein blockage was performed by 10 min incubation with 1/5 concentrated rabbit serum after washing in phosphate buffer solution (PBS). They were incubated at room temperature for 2 h with 1/100 anti-estrogen receptor α (Abcam, ab75635), 1/50 anti-progesterone receptor B (Abcam, ab2765) primary antibodies. Then, PBS washing was performed. Conjugated secondary antibody with biotin was applied for 20 min. Streptavidin peroxidase was applied for 20 min after PBS washing for 20 min. Afterwards, firstly staining with AEC for 10 min (Zymed, 3- Amino-9-ethylcarbazole) and then counterstaining with Mayer's haematoxylin, enclosed with an aqua-based glue, were performed. In the immunohistochemistry for ER-α and PR-B, the staining intensity was graded semi-quantitatively as; no immunostaining (-), weak staining (+), moderate staining (++), and strong staining (+++).

Statistical Analysis

Data from the estrogen and progesterone concentrations of goats in breeding- and non-breeding seasons were analysed by regression analysis using MINITAB. Differences of means (±SEM) between the experimental groups were considered significant when $P<0.05$ [21].

RESULTS

Hormonal Results

Average levels of E_2 and P_4 hormones in goats were 11.83±1.70 pg/ml and 10.08±1.58 ng/ml during breeding season, and 2.33±0.85 pg/ml and 0.21±0.04 ng/ml in non-breeding season, respectively *(Fig. 1, Fig. 2)*.

Immunohistochemical Results

Stained sections were evaluated according to the frequency of reaction (-), (+), (++), and (+++) in the ER-α

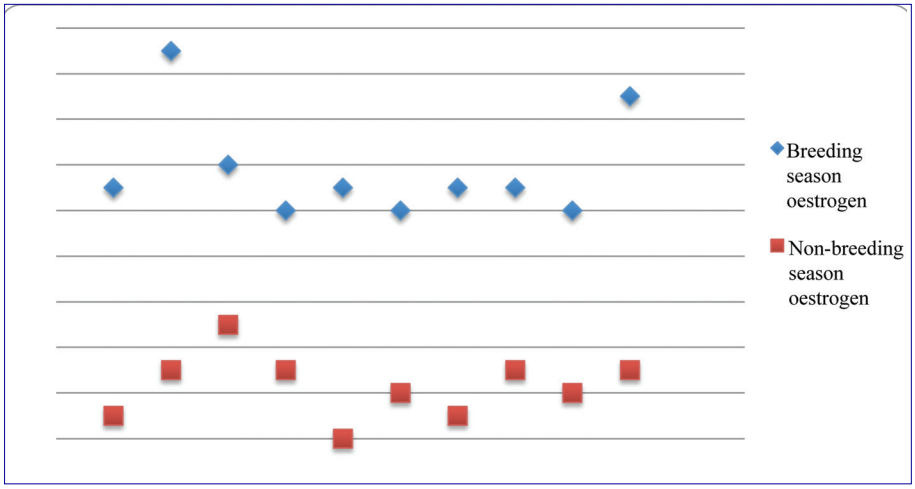


Fig 1. Seasonal distribution of serum estrogen levels (pg/ml)

Şekil 1. Sezonal serum östrojen düzeyi dağılımı (pg/ml)

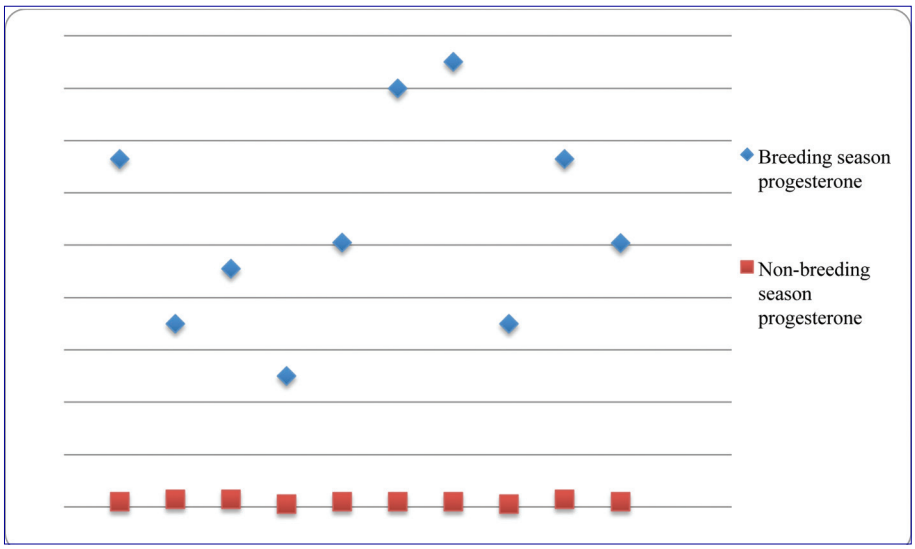


Fig 2. Seasonal distribution of serum progesterone levels (ng/ml)

Şekil 2. Sezonal serum progesteron düzeyi dağılımı (ng/ml)

Table 1. *ER-α and PR-B receptor reaction intensity*
Tablo 1. *ER-α ve PR-B reseptör reaksiyon yoğunluğu*

Receptor	Breeding	Non-Breeding
ER-α	Primary, secondary, tertiary follicle, preovulatory follicle granulosa cells (+++) Germinative epithelium (++) Wall of some vessels (+)	Germinative epithel (++) Wall of some vessels (+)
PR-B	In CL luteal cells (+++) Corpus albicans (++) epithelium, Theca int/ext (+)	Corpus albicans (++) Germinative epithelium (+) Stromal cells (+)

and PR-B receptor staining, and photos were taken (Leica ICC50) *(Table 1)*.

In breeding season, the ER-α reacted as (+) in ovarian primary, secondary, tertiary, and preovulatory follicles, as well as germinative epithelium and some smooth muscles in the walls of some blood vessels *(Fig. 3A-B)*. Reactions were observed for the PR-B as (+) in ovarian CL, corpus albicans, germinative epithelium, theca interna and externa, and stroma *(Fig. 4A-B)*.

In non-breeding season, the ER-α was observed (+) in ovarian germinative epithelium and the wall of small-shaped vessels *(Fig. 3D)*. The PR-B was observed (+) in ovarian germinative epithelium, corpus albicans cells, and stroma cells *(Fig. 4D)*.

Negative control was photographed in- and out of breeding season *(Fig. 3C, Fig. 4C)*.

DISCUSSION

There appears to be no data available on the ER-α and PR-B in goat ovaries in the literature survey, thus making the present study scientifically unique and valuable. So,

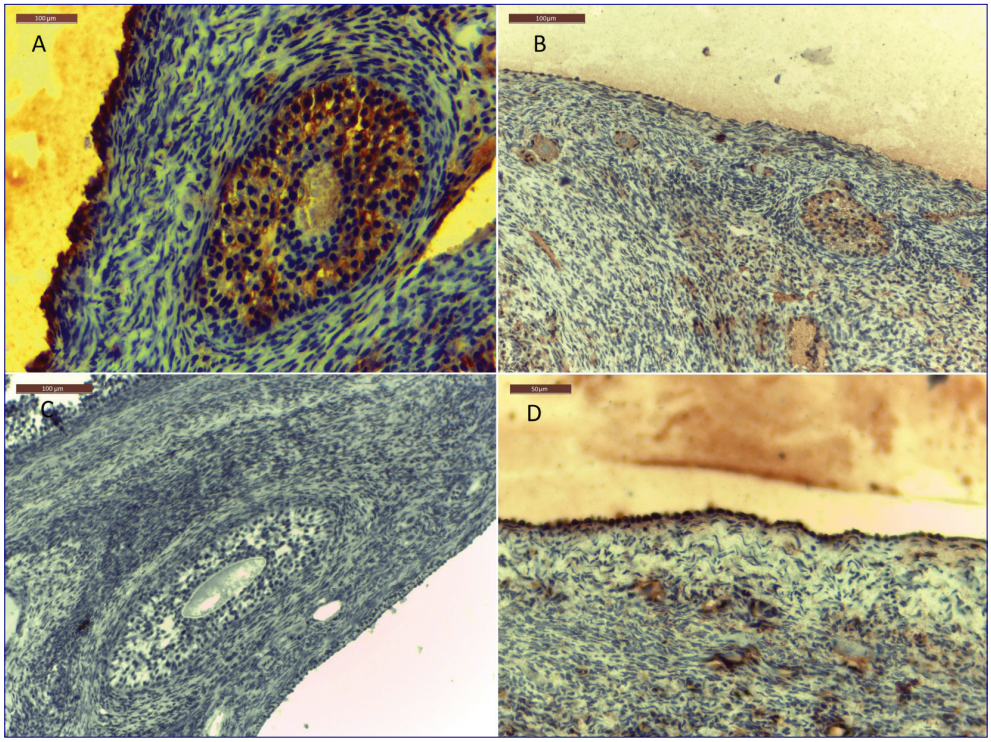
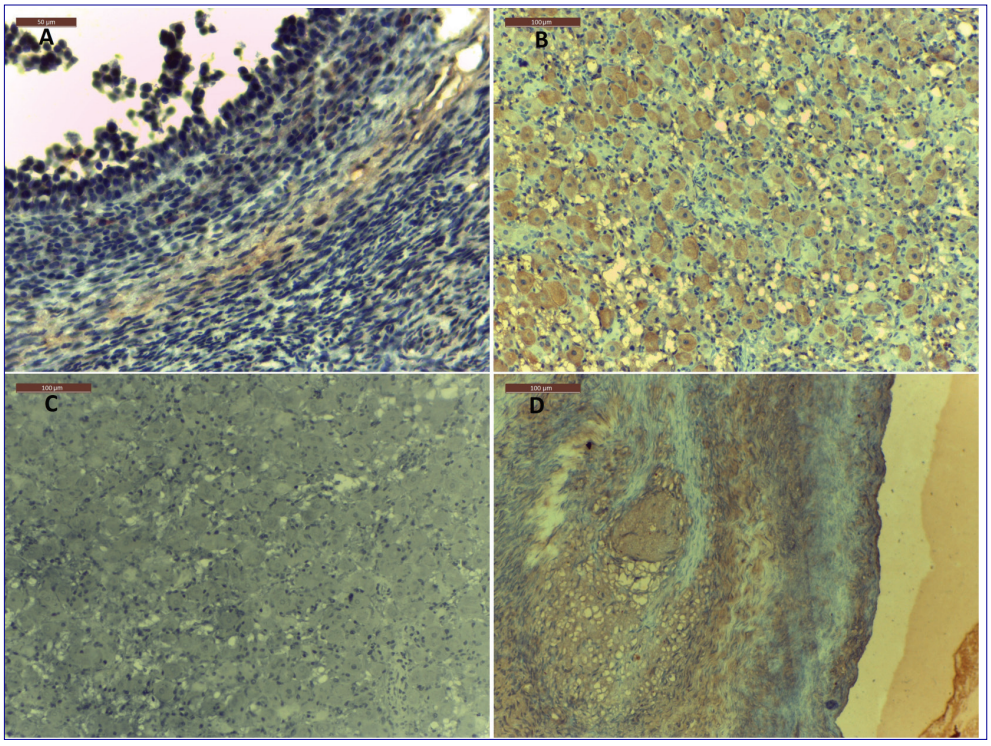


Fig 3. A- The ER-α in breeding season as (+) in ovarian, secondary follicles, germinative epithelium and some smooth muscles within the wall of some blood vessels, **B-** The ER-α in breeding season as (+) in ovarian primary and secondary follicles, germinative epithelium and some smooth muscles within the wall of some blood vessels, **C-** The ER-α as negative control in breeding season, **D-** In non-breeding season, the ER-α as (+) in ovarian germinative epithelium and wall of small-shaped vessels

Şekil 3. A- Ovaryumda üreme sezonunda, sekonder foliküller, germinatif epitelyum ve kan damarları duvarındaki düz kaslarda ER-α (+), **B-** Ovaryumda üreme sezonunda primer, sekonder foliküller, germinatif epitelyum ve kan damarları duvarındaki düz kaslarda ER-α (+), **C-** Üreme sezonunda ER-α negatif kontrol, **D-** Ovaryumda üreme sezonu dışında germinatif epitelyum ve bazı kan damarları duvarında ER-α (+)

Fig 4. A- The PR-B as (+) in ovarian theca externa, **B-** The PR-B as (+) in ovarian CL, **C-** The PR-B as negative control in breeding season, **D-** The PR-B (+) in ovarian germinative epithelium, corpus albicans cells, and stroma cells during out of breeding season

Şekil 4. A- Üreme sezonunda ovaryumda teka eksterna PR-B (+), **B-** Üreme sezonunda corpus luteumda PR-B (+), **C-** Üreme sezonunda PR-B negatif kontrol, **D-** Üreme sezonu dışında germinatif epitelyum, corpus albicans ve stroma hücrelerinde PR-B (+)



our results obtained herein will be compared with those in other animal species studied rather scarcely.

In cattle, the PR localised in secondary and tertiary follicles, granulosa cells, theca interna cells, and theca externa cells. It was concluded that progesterone production is regulated not only by granulosa cells but also by theca interna and theca externa. Also, we claimed herein that progesterone has negative impact on the PR immunohistochemical staining, as reported earlier in cattle [22]. Further, we observed that there was a positive reaction of PR-B with theca interna and theca externa cells in breeding season. Indeed, positive reactions were also observed in the cells of CL, germinal epithelium and stroma.

Further, The PR in cows was investigated and progesterone

membrane component 1 stained immunohistochemically in estrous cycle. The cells of granulosa, theca, stroma, endothelium, germinal epithelium and the CL showed (+) reactions in both follicular and luteal phases. In this study, the localisation of receptors was determined in cytoplasm and nucleus of the cells of granulosa, theca, endothelium, stroma, germinal epithelium, and the CL. We concluded that the PR showed weak reaction in follicular and luteal cells. The present study in goats had similarities with studies like immunohistochemically staining (+) of PR in germinative epithelium, theca and CL luteal cells [23].

To prove the relationship between steroid hormones and progesterone, Vermeirsch et al.[16] determined in dog ovaries that although the PR stained (+) in surface epithelium, and the cells of follicle, theca, and the CL, but PRs stained (-) in vessel wall, stroma cells and oocyte. Herein, we observed that the PR showed (+) reactions immunohistochemically with surface epithelium, theca, and luteal cells. Unlike the findings in dog ovaries, we found that there was (+) staining in ovarium stroma. In dogs, progesterone level in estrus was 3.33±2.49 ng/ml while the E_2 was 12.42±8.05 pg/ml. In this study, progesterone hormone level in anestrous period was 0.61±0.56 ng/ml, while E_2 hormone level was 4.26±3.49 pg/ml. Differences in both P_4 and E_2 levels arise mainly the stage of sexual cycle and species of the animals (estrus in dogs *vs.* anoestrus in goats).

In mouse, the relationship between the age and reproduction cycles of animals was investigated based on androgen and PR localisation and distribution in the ovary. It was concluded that the PRs are more connected with the cycle phase rather than the animal's age [24]. In the present study however, no such comparison was made since animals used herein were all mature individuals being slaughtered.

In another study conducted in cows, localisations and densities of E_2 and progesterone receptors of ovaries during the luteal and follicular phases were compared [20]. It was reported that the ER-α was immunohistochemically stained (+) in all developmental phases in the cells of granulosa, germinal epithelium, stroma, theca, the CL and corpus albicans from primary follicle to mature follicle, while the PR-B gave immunohistochemically positive results with germinal epithelium, stroma cells, corpus albicans and the CL. According to the localisation of receptors given, similar results were obtained in goats studied herein, as compared to those in cow ovaries.

Hulas-Stasiak and Gawron [5] studied the ER-α and ER-β receptors immunohistochemically in spiny mouse ovaries and found the localisation of ER-α. Granulosa cells carried α receptors in all stages of developing follicle. Nucleus contained the ER-α in the cells of prenatal follicles, theca, and pre-CL. However, the atretic follicles did not show such staining. Similar to their study, the receptors in granulosa cells of developing follicles showed staining in goat ovaries. Further, the ER-α were lacking in any phase of the CL cells herein. Also, Li et al.[25], studying distribution of ER-α and PR in mouse ovaries, uterus and oviduct, reported that ovarian ER localised in granulosa cell nuclei and interstitial cells, while the PR localised in prenatal follicle nuclei. They obtained some receptor localisations similar to the ovarian findings herein. Additionally, Sar and Welsch [26], studying about separating the ER-α and ER-β in rats, determined that beta-receptors are localised in the middle of some follicle nuclei. The ER-α was detected in uterus and oviducts of adolescent and non-adolescent rats. In our study however, the receptors were observed in nucleus.

Korte et al.[27] determined that the more distributed the receptors the lesser the hormone (P_4) concentration in rabbit ovaries. In the ovaries of canine [16], primate [7] and porcine [23], a profound negative correlation was observed between hormone levels and receptor distributions. In the present study, we found similar results (negative correlation between hormone level and receptor localisation) in goats.

It was concluded that, as expected, the localisation intensity of estrogen and progesterone receptors within the ovaries correlated well with their respective steroid hormone profiles in goats during breeding and non-breeding seasons. Determining the estrogen and progesterone profiles (including their intraovarian receptor sites), thus understanding the ovarian functions in more detail would provide crucial data for future studies on goat reproduction in different seasons. Present results might also be helpful (with receptor sites and their localisation density) for greater understanding the relationship of endocrinological and receptor cascades of reproduction in other animals showing seasonal breeding (such as ewe, mare, bitch, etc.).

Acknowledgements

We greatly acknowledge funding from the Yuzuncu Yil University Scientific Research Projects Directorate for conducting this project (No: 2013-VF-B018).

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