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RESEARCH ARTICLE

Localization of Estrogen Receptor Alpha and Progesterone Receptor B in the Bovine Ovary During the Follicular and Luteal Phase of the Sexual Cycle

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Summary

Ovarian steroid hormones, mainly estrogen and progesterone, play a central role in the regulation of ovarian functions. The biological effects of progesterone (PR) and estrogen (ER) are dependent on the activation of both PR and ER isoforms. In the present study, we studied the cellular distribution and localization of progesterone receptor B (PR-B) and estrogen receptor alpha (ERα) in the bovine ovaries during the follicular and luteal phases of the sexual cycle using immunohistochemical methods. The estrous cycle stage of 23 Holstein bovines was evaluated by gross and histological appearance of ovaries and blood steroid hormone values. Tissue samples from ovaries were fixed in 10% formaldehyde for routine histological processing. ERα immunoreactivity was observed in nuclei of granulosa cells of growing follicles at all stages from primary to mature follicles, epithelial cells of the germinal epithelium, stroma cells, theca cells, corpora lutea cells and cells of the corpora albicantia. Nuclear staining for PR-B was not detected in the granulosa cells in all sizes of follicles, but was positively stained in germinal epithelium cells, stroma cells, corpora albicantia cells and corpora lutea cells. In conclusion, ERα and PR-B immunoreactivity in bovine ovary were not indicate too much difference between the phases of the sexual cycle but have important differences in cell-spesific localization.

Keywords: *Bovine ovary, Estrogen receptor, Progesterone receptor, Immunohistochemistry*

İnek Ovaryumunda Seksüel Siklusun Foliküler ve Luteal Fazı Boyunca Östrojen Reseptör Alfa ve Progesteron Reseptör B'nin Lokalizasyonu

Özet

Ovaryum steroid hormonları, özellikle östrojen ve progesteron, ovaryum fonksiyonlarının düzenlenmesinde merkezi bir rol oynar. Progesteron (PR) ve östrojen (ER)'in biyolojik etkileri hem PR hem de ER izoformlarının aktivasyonuna bağlıdır. Bu çalışmada, seksüel siklusun foliküler ve luteal fazı boyunca inek ovaryumunda progesteron reseptör B (PR-B) ve östrojen reseptör alfa (ERα)'nın lokalizasyonunu ve hücresel dağılımını immunohistokimyasal yöntemler kullanarak çalıştık. Yirmiüç Holstein ineğin östrus siklus dönemi, ovaryumun bütünü ve histolojik görünümü ve kan steroid hormon değerleri ile değerlendirildi. Ovaryumdan elde edilen doku örnekleri rutin histolojik işlemler için %10'luk formaldehit içinde tespit edildi. ERα immunoreaktivitesi, korpus albikans hücrelerinde, korpus luteum hücrelerinde, teka hücrelerinde, bağ doku hücrelerinde, germinal epitel hücrelerinde ve primer folikülden olgun foliküle her aşamada gelişen foliküllerin granuloza hücre çekirdeklerinde gözlendi. PR-B için çekirdek boyanma, tüm büyüklükteki foliküllerin granuloza hücrelerinde saptanamadı, ancak korpus luteum hücrelerinde, korpus albikans hücrelerinde, bağ doku hücrelerinde ve germinal epitel hücrelerinde pozitif boyanma görüldü. Sonuç olarak, inek ovaryumunda ERα ve PR-B immunoreaktivitesi açısından seksüel siklusun fazları arasında çok fazla fark olmadığı ancak hücre-spesifik lokalizasyonda önemli farklılıkların olduğu gösterildi.

Anahtar sözcükler: *İnek ovaryumu, Östrojen reseptör, Progesteron reseptör, İmmunohistokimya*

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INTRODUCTION

The best-known and best-characterized secretory products of the ovary are the steroid hormones. They produced by follicles and corpus luteum cells and act locally with in the follicles or corpus lutea as paracrine/autocrine agents [1]. Ovarian steroid hormones, mainly estrogen and progesterone, diffuse from the blood and binds to a specific receptor located in the nuclei. These hormones interplay the role of controlling the morphological and the functions of female reproductive organs [2,3].

Estrogen stimulates the development and maturation of ovarian follicles, increases expression of FSH receptor and LH receptor by granulosa cells, modulates steroid production by granulosa and theca cells and induces the development of gap junctions between granulosa cells [4]. Estrogen shows its effect by binding receptors that are members of the nuclear receptor family called estrogen receptors (ERs). These receptors are expressed as two related subtyped ER- alpha (α) and ER- beta (β), which are encoded by two genes. ERβ was mainly detected in granulosa cells, ERα was detected in theca cells, intersititial glands, stroma cells and germinal epithelium [5,6]. In the ovary of domestic animals, ERα expression was demonstrated in monkeys [7], humans [8,9], rats [10], cattle [4], sheep [11,12], and pigs [13].

Progesterone has a crucial role on the growth and differentiation of ovarian structures and makes the endometrium ready for the implantation of the embryo [14]. Progesterone features its effects on target tissues by binding a receptor called progesterone receptors (PRs). Two different receptors have been identified (PR-A and PR-B) [15]. The presence of PR has been demonstrated in the ovary of a number of species, e.g. human [16], monkey [7], bovine [17], pig [18], rabbit [19], dog [20] and mouse [21] but not PR-B in bovine ovary.

Although the presence of estrogen and progesterone receptors have been studied in the bovine ovary [4,17], we have seen a different pattern of ERα distribution and cellular localization among ovarian tissues, for this reason we wanted to demonstrate the differences for ERα and the first time PR-B immunoreactivity in the bovine ovary.

MATERIAL and METHODS

Animals and Samples

Bovine ovaries from 23 different animals were obtained from a local slaughterhouse where the tissues were removed immediately after sacrificing the animal. Blood samples from these animals also were taken before death to measure estrogen and progesterone levels. The blood serum concentrations of estradiol-17β (DRG Intl. Inc., DRG Aurica Elisa Estradiol Kit, cat. no. EIA-2693, Marburg/Germany) and progesterone (DRG Intl Inc. Company, DRG Aurica Elisa Progesterone Kit, cat. no. EIA-1561, Marburg/Germany) were measured using Enzyme Immun Assay (EIA**)** to determine the sexual cycles.

The stage of the sexual cycle was determined by the morphological appearance of the ovarian follicles and the blood serum hormone levels. The animals were classified into two groups as follicular (n = 10) and luteal (n = 13) phases as reported by Saruhan et al.[22]. For the immunohistochemical determination of ERα and PR-B, samples of ovaries were fixed in 10% neutral formaldehyde solution for 24 h at room temperature, washed in water, dehydrated, cleared, and embedded in Paraplast. Two slides were prepared from each sample and each slide contained a minimum of three serial sections cut at 5 µm thickness at least 100 µm apart. One slide was immunostained for ERα and the following adjacent slide was immunostained PR-B, employing primary antibodies.

Immunohistochemical Procedure

Immunostaining was performed at room temperature according to the protocol reported by Thermo Scientific Lab Vision (Fremont, CA). Tissue sections (5 µm) were mounted on a coated glass slide (Superfrost plus, Menzel-Glaser, Germany). Briefly, sections were deparaffinized in xylene and rehydrated through a graded series of ethanols. Sections were heated for 20 min at 95°C in 0.01 M antigen retrieval citrate solution, (0.2 M $NaH_2PO_4.2H_2O$; 0.1 M citric acid) pH 6.0. After cooling at room temperature for 20 min, the sections were washed in phosphate buffered saline (PBS; pH 7.4) four times [23]. To block endogenous peroxidase activity or nonspecific staining, the sections were treated with 3% (v/v) H_2O_2 in methanol for 15 min. Slides were washed in PBS and incubated in a blocking reagent (Ultra V Block; Thermo Fisher Scientific, Waltham, MA) for 10 min at room temperature to prevent nonspecific binding. The sections then were incubated with a rabbit polyclonal anti-ERα antibody (Thermo Fisher Scientific; cat. no. RB-1521-P) and a mouse monoclonal anti-PR-B (Thermo Fisher Scientific; cat. no. MS-192-P) for 30 min at 1:200 dilution. Sections then were washed in PBS, incubated with biotinylated anti-mouse antiserum (primary antibody recognized PR-B) or biotinylated anti-rabbit antiserum (primary antibody recognized ERα) (Thermo Fisher Scientific) for 20 min at room temperature and washed in PBS. Sections were incubated with streptavidin peroxidase for 20 min at room temperature and washed with PBS. The peroxidase activity was visualized with 3,3-diaminobenzidine (DAB) or 3-Amino-9-ethylcarbazole (AEC) (Thermo Fisher Scientific) for ERα and PR-B, respectively. Sections were counterstained with Gill's hematoxylin and mounted in Entellan or aqueous mounting under a coverslip.

The intensity of staining was evaluated three times indepently by an experienced microscopist. The intensity of positive staining was described as being + weak, ++

moderate, +++ strong, +/++ weak to moderate, ++/+++ moderate to strong.

The stained slides were examined under microscope and photographed using a digital camera attached to the microscope (Nikon Eclipse e-400, Coolpix-4500, Tokyo/ Japan).

RESULTS

The intensity of the reaction for ERα and PR-B were indicated by the intensity of the brown-positive and red-positive staining in the cell nuclei, respectively. Cytoplasmic staining was considered to be non-specific and no staining was determined in the negative controls *(Fig. 1)*.

Localization of ERα in the Bovine Ovary

No significant differences were found for intensity staining during the follicular and luteal phases. Nuclear ERα immunoreactivity was observed in granulosa cells of the primary, secondary, tertiary and preovulatory follicles during follicular and luteal phases *(Fig. 2A-C)*. ERα immunostaining was determined in germinal epithelium and ovarian stroma cells (not all cells); endothelial and smooth muscle cells of the blood vessels *(Fig. 2D-E)*. Furthermore corpora lutea cells had a moderate to strong intensity staining during the follicular and luteal phases. The immunopresence for ERα was obvious in cells of the corpora albicantia during the luteal phase *(Fig. 2F) (Table 1)*.

ERα immunoreaction of some theca cells of secondary follicles were observed and theca interna cells had a lower staining intensity than theca externa cells of tertiary follicles during the follicular and luteal phases *(Fig. 2A-C) (Table 1)*.

Localization of PR-B in the Bovine Ovary

Positive staining of germinal epithelial cells had a significantly lower intensity in follicular phase than

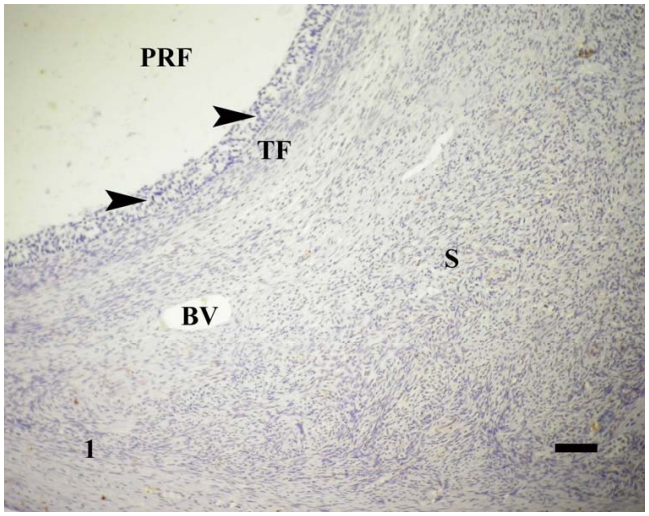

Fig 1. Negative control in which non-immune serum was used instead of primary antibody. Preovulatory follicle **(PRF)**, theca folliculi **(TF)**, granulosa cells *(arrowheads)*, stroma **(S)**, blood vessel **(BV)**, *Bar: 50 µm*

Şekil 1. Primer antikorun yerine non-immun serumun konduğu negatif kontrol. Preovulatuar folikül **(PRF)**, teka folikülü **(TF)**, granuloza hücreleri *(ok başları)*, bağ doku **(S)**, kan damarı **(BV)**, *Bar: 50 µm*

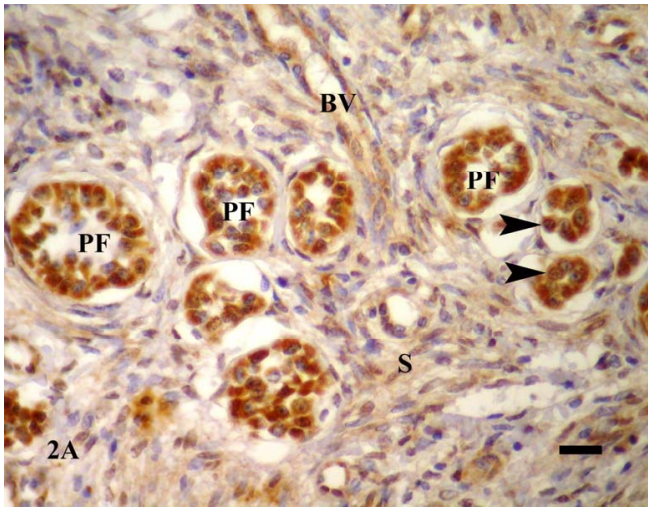

Fig 2A. Immunohistochemical localization of ERα in bovine ovary. Primary follicle **(PF)**, granulosa cells (arrowheads), stroma **(S)**, blood vessel **(BV)**, *Bar: 25 µm*

Şekil 2A. İnek ovaryumunda ERα'nın immunohistokimyasal lokalizasyonu. Primer folikül **(PF)**, granuloza hücreleri *(ok başları)*, bağ doku **(S)**, kan damarı **(BV)**, *Bar: 25 µm*

Table 1. *Average staining intensity of ERα and PR-B in the ovary at different phase of the sexual cycle*

Tablo 1. *Seksüel siklusun farklı fazlarındaki ovaryumda ERα ve PR-B'nin ortalama boyanma yoğunluğu*

Sexual Cycle		Primary Follicle	Secondary Follicle	Tertiary Follicle	Preovulatory Follicle	Germinal Epithelium	Stroma	Corpus Luteum	Corpus Albicans
			Granulosa cells/ Theca follicularis	Granulosa cells/ Theca interna/ Theca externa	Granulosa cells/ Theca interna/ Theca externa				
Follicular Phase	ERα	++	(++/+++)/(+)	(++/+++)/(+)/(+++)	(++/+++)/(+)/(+++)	++/+++	++	++/+++	++/+++
	PR-B	No staining	No staining	No staining	No staining	+	+/++	No staining	No staining
Luteal Phase	ERα	++	(++/+++)/(+)	(++/+++)/(+)/(+++)	(++/+++)/(+)/(+++)	++/+++	++	++/+++	++/+++
	PR-B	No staining	No staining	No staining	No staining	++	+/++	+++	++

Staining intensity: *+ weak, ++ moderate, +++ strong, +/++ weak to moderate, ++/+++ moderate to strong*

the luteal phase *(Fig. 3A-B)*. PR-B immunostaining was determined ovarian stroma cells (not all cells) with similar intensity during the follicular and luteal phases *(Fig. 3C)*. Besides a higher intensity staining was observed in corpora lutea cells during the luteal phase *(Fig. 3D)*. No staining for PR-B was observed in cells of the corpora albicantia in follicular phase, but detected in luteal phase *(Fig. 4A-B) (Table 1)*.

No staining was observed in the ovarian follicles, endothelial and smooth muscle cells of the blood vessels during the follicular and luteal phases *(Fig. 3C- Fig. 5) (Table 1)*.

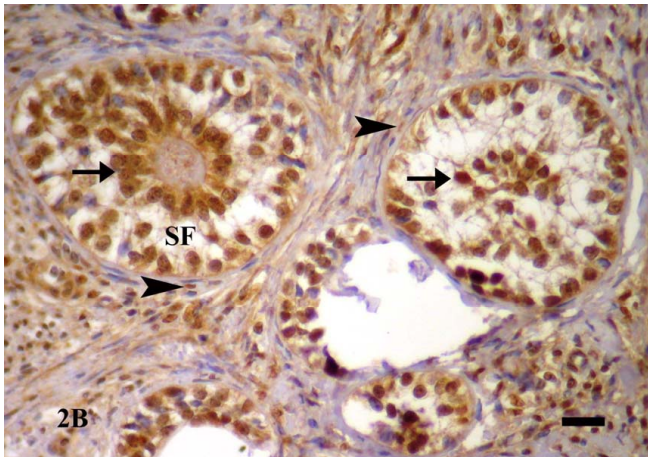

Fig 2B. Immunohistochemical localization of ERα in bovine ovary. Secondary follicle **(SF)**, theca folliculari*s (arrowheads)*, granulosa cells *(arrows), Bar: 25 µm*

Şekil 2B. İnek ovaryumunda ERα'nın immunohistokimyasal lokalizasyonu. Sekonder folikül **(SF)**, teka folikülaris *(ok başları)*, granuloza hücreleri *(oklar), Bar: 25 µm*

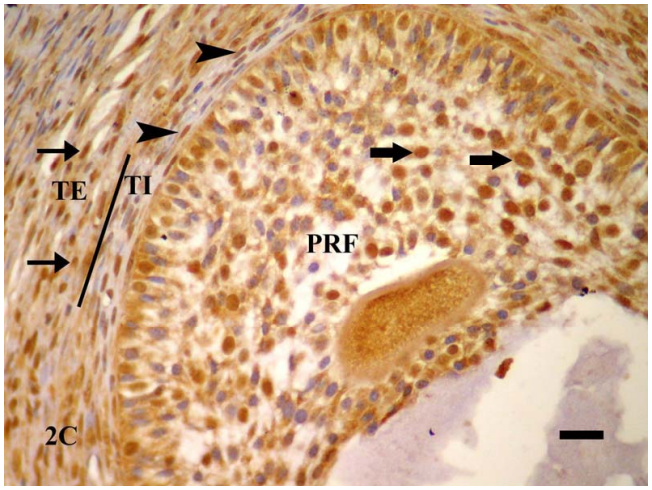

Fig 2C. Immunohistochemical localization of ERα in bovine ovary. Preovulatory follicle **(PRF)**, theca externa **(TE)**, theca interna **(TI)**, theca interna cells *(arrowheads)*, theca externa cells *(thin arrows)*, granulosa cells *(thick arrows), Bar: 25 µm*

Şekil 2C. İnek ovaryumunda ERα'nın immunohistokimyasal lokalizasyonu. Preovulatuar folikül **(PRF)**, teka eksterna **(TE)**, teka interna **(TI)**, teka interna hücreleri *(ok başları)*, teka eksterna hücreleri *(ince oklar)*, granuloza hücreleri *(kalın oklar), Bar: 25 µm*

DISCUSSION

We demonstrated a nuclear immunoreactive expression of ERα in germinal epithelium with the follicular and luteal phases of the sexual cycle. This is in agree with previous studies for cat [24] and rat [10]. It is supported the concept that epithelial in growth of the ovary was mediated by estrogen via ERα [25].

We observed ERα immunoreactivity in granulosa cells of the primary, secondary, tertiary and preovulatory follicles during follicular and luteal phases. But the demonstration of ERα in granulosa cells is still not clear. Data obtained

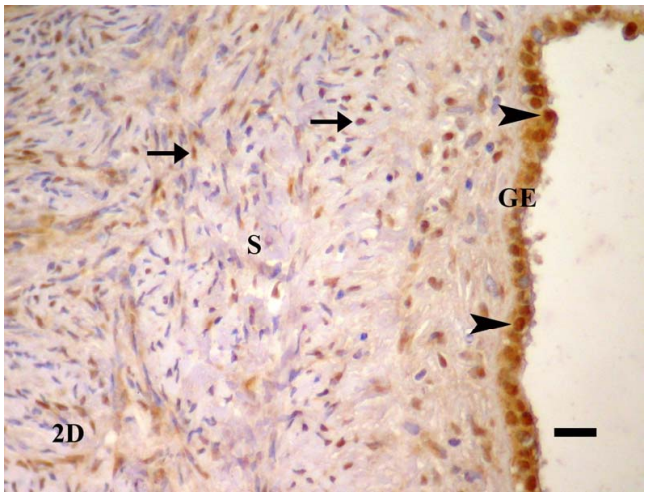

Fig 2D. Immunohistochemical localization of ERα in bovine ovary. Germinal epithelium **(GE)**, stroma **(S)**, germinal epithelium cells *(arrowheads)*, stroma cells *(arrows), Bar: 50 µm*

Şekil 2D. İnek ovaryumunda ERα'nın immunohistokimyasal lokalizasyonu. Germinal epitel **(GE)**, bağ doku **(S)**, germinal epitel hücreleri *(ok başları)*, bağ doku hücreleri *(oklar), Bar: 50 µm*

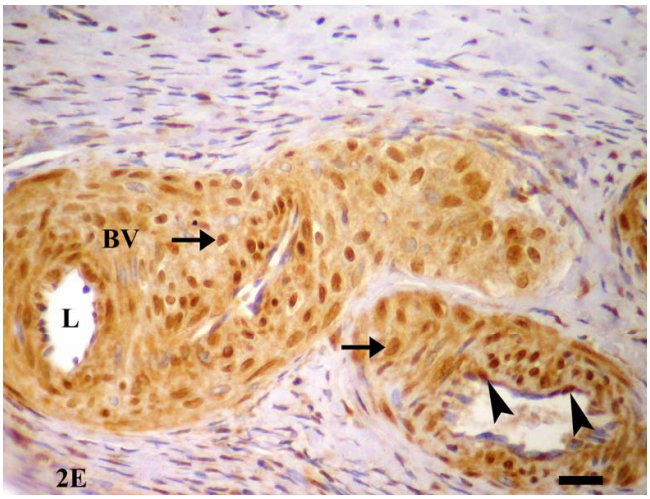

Fig 2E. The endothelial *(arrowheads)* and smooth muscle cells *(arrows)* of the blood vessels showed positive ERα staining. Blood vessels **(BV)**, lumen **(L)**, *Bar: 25 µm*

Şekil 2E. Kan damarlarının endotel *(ok başları)* ve düz kas hücrelerininde *(oklar)* ERα boyanması pozitif. Kan damarları **(BV)**, lumen **(L)**, *Bar: 25 µm*

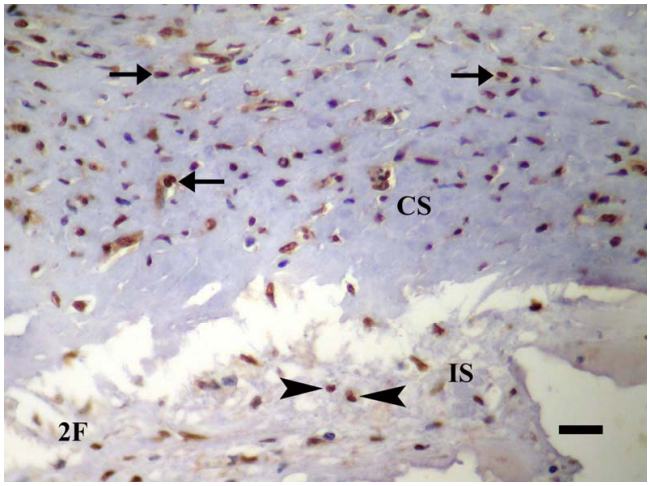

Fig 2F. Nuclei of internal stroma cells *(arrowheads)* and capsular stroma cells *(arrows)* of a corpus albicans is stained. Capsular stroma **(CS)**, internal stroma **(IS)**, *Bar: 25 µm*

Şekil 2F. Korpus albikans'ın internal bağ doku hücreleri *(ok başları)* ve kapsular bağ doku hücreleri (oklar) boyalı. Kapsular bağ doku **(CS)**, internal bağ doku **(IS)**, *Bar: 25 µm*

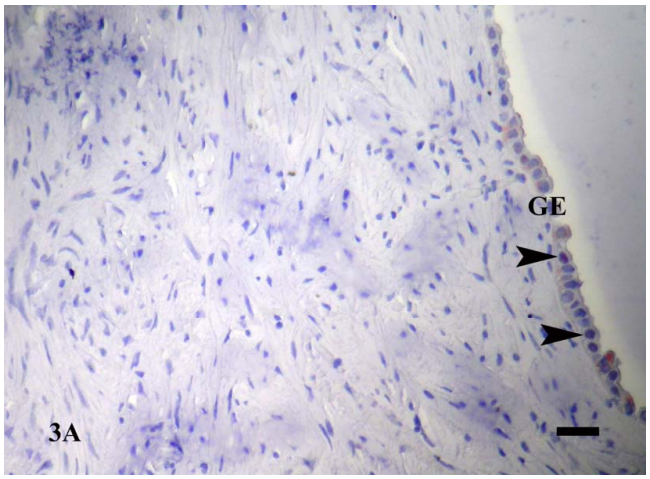

Fig 3A. Immunohistochemical localization of PR-B in bovine ovary during follicular phase. Germinal epithelium **(GE)**, germinal epithelium cells *(arrowheads), Bar: 50 µm*

Şekil 3A. İnek ovaryumunda PR-B'nin foliküler fazdaki immunohistokimyasal lokalizasyonu. Germinal epitel **(GE)**, germinal epitel hücreleri *(ok başları), Bar: 50 µm*

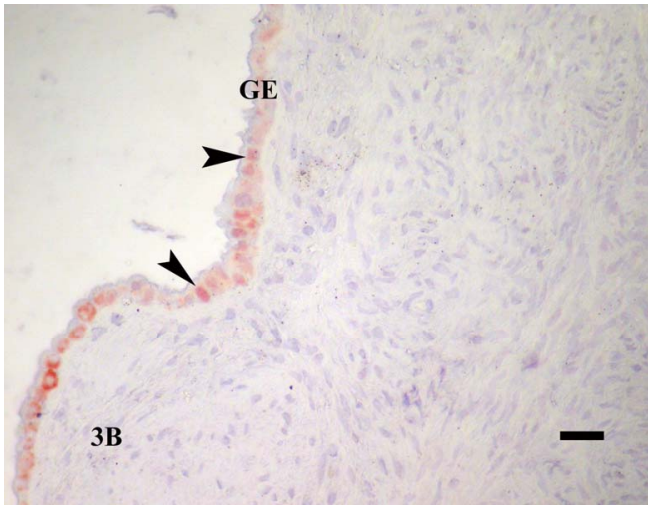

Fig 3B. Immunohistochemical localization of PR-B in bovine ovary during luteal phase. Germinal epithelium **(GE)**, germinal epithelium cells *(arrowheads), Bar: 50 µm*

Şekil 3B. İnek ovaryumunda PR-B'nin luteal fazdaki immunohistokimyasal lokalizasyonu. Germinal epitel **(GE)**, germinal epitel hücreleri *(ok başları), Bar: 50 µm*

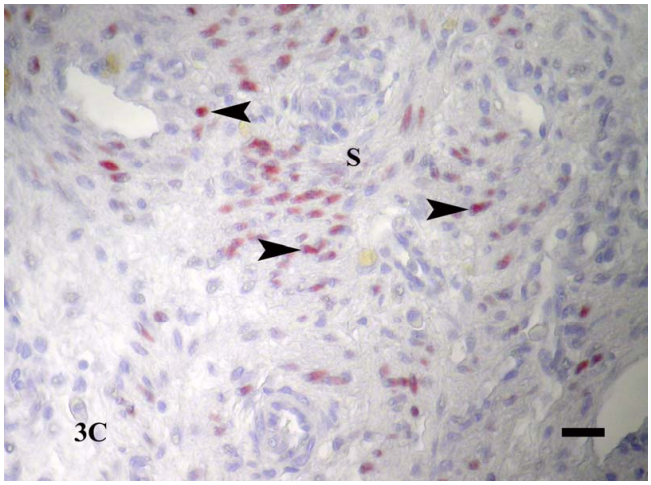

Fig 3C. Immunohistochemical localization of PR-B in bovine ovary. Stroma **(S)**, stroma cells *(arrowheads), Bar: 25 µm*

Şekil 3C. İnek ovaryumunda PR-B'nin immunohistokimyasal lokalizasyonu. Bağ doku **(S)**, bağ doku hücreleri *(ok başları), Bar: 25 µm*

from several immunohistochemistry laboratories have shown that the expression of ERα is localized only in the granulosa cells of tertiary follicles such as cattle [26,27], ice [28], baboons [29], rabbits [19]. However, in agreement with our reports, in different animal studies such as cattle [6], bitch [30] and mice [31] demonstrated that ERα observed granulosa cells, different staining intensities, in growing follicles from primary to tertiary ones. Furthermore in human [32] and monkey [7], ERα were also expressed in granulosa cells of preovulatory follicles. In contrast Jefferson et al.[33], Sar and Welsch [10] did not observe ERα immunoreactivity in mouse and rat granulosa cells, respectively. This shows that the amount of this proteinin ovary might be too different to detect using immunohistochemistry.

In present study, contrary to previous studies including intensity staining for cattle [5,26,27] and rats [10], the expression of ERα was low in some theca cells of secondary follicles, strong in theca externa cells of tertiary follicles and in agreement with report concerning cattle [27], the expression of ERα was low in theca interna cells of tertiary follicles. On the other hand we found that the expression of ERα was localized in stroma cells. This finding agrees with the report for cattle [26]. These indicates that high expression of ERα in theca and stroma cells in comparison with that in follicle cells suggests an indirect effect of estrogen on

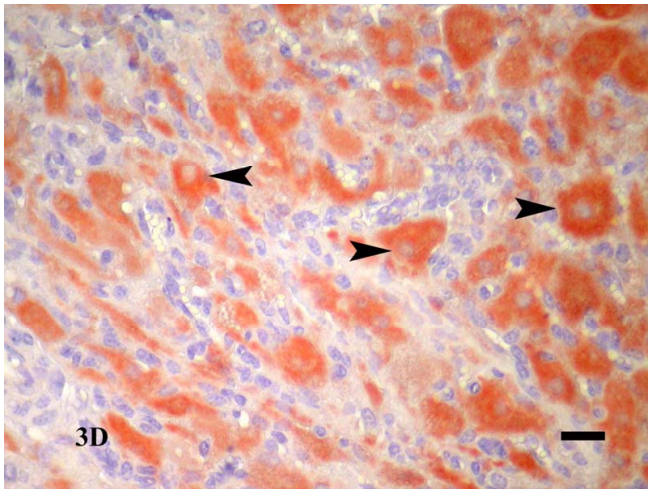

Fig 3D. Immunohistochemical localization of PR-B in bovine ovary during luteal phase. Nuclei of corpora lutea cells *(arrow-heads)* of a corpus luteum is stained, *Bar: 25 µm*

Şekil 3D. İnek ovaryumunda PR-B'nin luteal fazdaki immunohistokimyasal lokalizasyonu. Korpus luteum hücrelerinin çekirdekleri *(ok başları)* boyalı, *Bar: 25 µm*

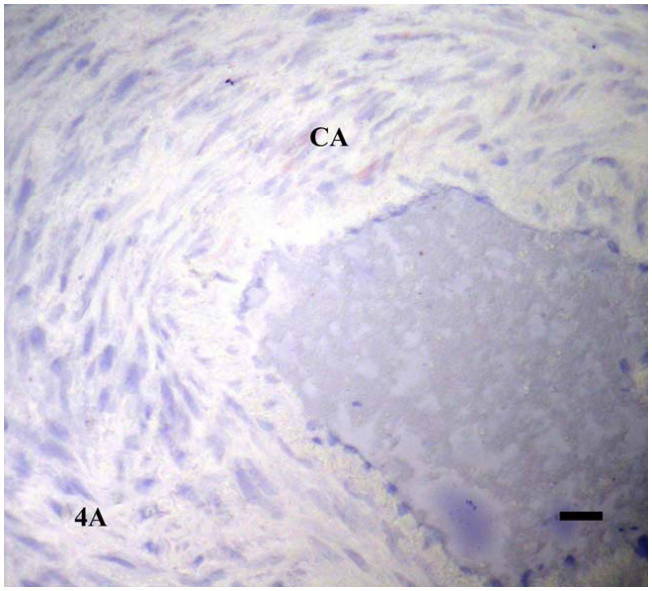

Fig 4A. In the corpus albicans **(CA)** no positive staining for PR-B during the follicular phase, *Bar: 50 µm*

Şekil 4A. Foliküler fazda korpus albikans'ın **(CA)** PR-B boyanması negatif, *Bar: 50 µm*

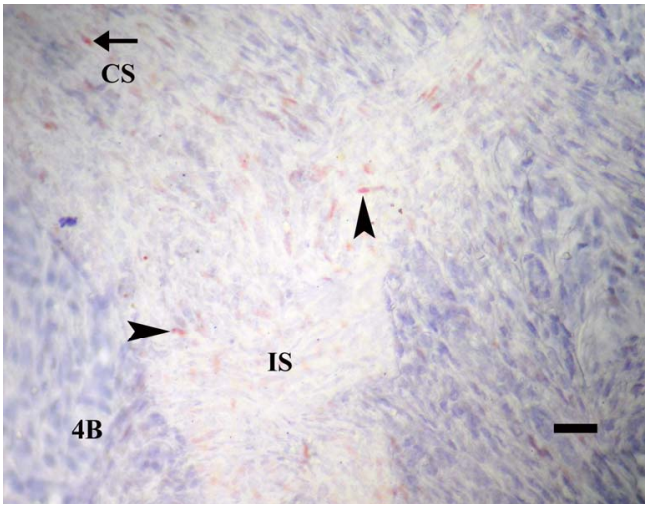

Fig 4B. Immunohistochemical localization of PR-B in bovine ovary during luteal phase. Nuclei of internal stroma cells *(arrowheads)* and capsular stroma cells *(arrows)* of a corpus albicans is stained. Capsular stroma **(CS)**, internal stroma **(IS)**, *Bar: 50 µm*

Şekil 4B. İnek ovaryumunda PR-B'nin luteal fazdaki immuno-histokimyasal lokalizasyonu. Korpus albikans'ın internal bağ doku hücreleri *(ok başları)* ve kapsular bağ doku hücreleri *(oklar)* boyalı. Kapsular bağ doku **(CS)**, internal bağ doku **(IS)**, *Bar: 50 µm*

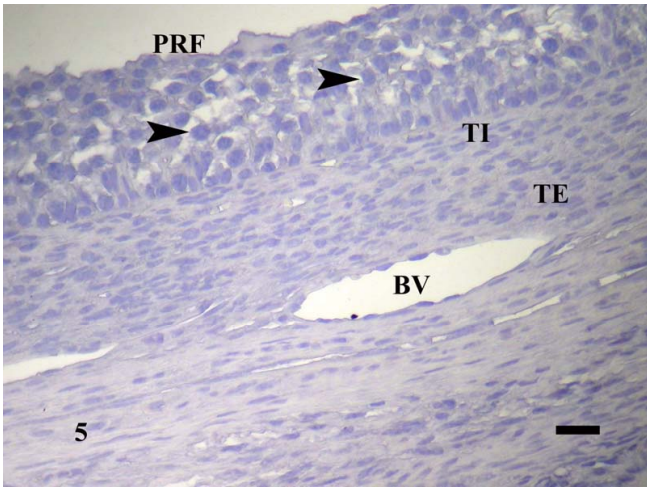

Fig 5. In the ovarian follicles no positive immunostaining for PR-B. Preovulatory follicle (PRF), theca externa (TE), theca interna (TI), blood vessel (BV), granulosa cells (arrowheads), *Bar: 50 µm*

Şekil 5. Ovaryum folikülleri'nin PR-B immun boyanması negatif. Preovulatuar folikül **(PRF)**, teka eksterna **(TE)**, teka interna **(TI)**, kan damarı **(BV)**, granuloza hücreleri *(ok başları), Bar: 50 µm*

the follicular development [27].

Our results revealed that the endothelial cells and smooth muscle cells of blood vessels in the bovine ovary reacted positively for ERα during follicular and luteal phases of the sexual cycle. This finding may be interpreted as indicating that ERα is involved in the proliferation of the endothelial and smooth muscle cells of blood vessels [34].

D'Haeseleer et al.[26] reported that immunoreactivity of ERα was observed in low amounts in corpora albicantia and stronger in the capsular stroma than in the internal stroma. Author noted that the high scores for ERα in the superficial stroma cells which are in direct contact with the follicles and in the deep stroma cells which surround the corpora albicantia are suggestive of possible stroma-epithelial interactions [35].

Research conducted on various animal species has demonstrated that the number and staining intensity of ERα positive cells in the ovary vary with the animal species and different phases of the sexual cycle. But in most studies [26,27,30] corpora lutea cells has high ERα immuno-

staining as well as present study. This shows us the presence of ERα in corpus luteum is suggestive of their involvement in the process of maintenance and luteinization of the corpus luteum [36].

In the current study PR-B immunoreactivity was detected in the cells of germinal epithelium with a low intensity. This finding agrees with the report for human [37] and porcine [38]. It has been suggested that ovarian surface epithelial cells are enzymatically involved in the ovulation under the influence of progesterone and its receptors [39]. In contrast Gava et al.[40] did not observe PR-B immunoreactivity in mouse epithelial cells. This finding that might be of interest is the localization of PR-B in the germinal epithelium among different species.

We demonstrated that the expression of PR-B was detected in the stroma cells both follicular and luteal phases of the sexual cycle. This data indicate that the possibility of such a paracrine action of ovarian steroids on follicles mediated through stroma steroid receptors has been suggested by Revelli et al.[41].

Expression of progesterone receptor in granulosa and theca cells are highly variable. In primates, follicles that developed beyond the primary stage no longer expressed progesterone receptors until after the surge of LH, when the luteinizing preovulatory follicles again stained positive for progesterone receptors [7]. Similarly PR protein being observed granulosa cells in cattle [17], humans [41] and mice [42]. However staining for PR-B protein was limited to granulosa layer of large antral follicles, where cells showed considerably weak intensity of immunohistochemical reaction [38]. In contrast, in agreement with our reports, Juengel et al.[43] and Suzuki et al.[32] did not observe PR-B immunoreactivity in ovine and human granulosa cells, respectively.

It has been reported that expression of PR has been observed in the theca cells of pre-antral and antral follicles [17,32]. On the contrary, similar to our reports, theca layer of pig follicles of both sizes was devoid of the immunoreactivity for PR-B [38]. Furthermore ovulation is unaffected in PRBKO (ablation of PR-B protein) mice indicating that PR-A expression is both necessary and sufficient to mediate the ovulatory response to progesterone [44]. In addition within the porcine ovary predominantly PR-A was observed both in follicular and luteal cells [38]. Taken together, the results of our study postulated that in bovine ovary the other PR isoform may be the regulatory of follicle development rather than PR-B.

Progesterone acting through its receptors regulates development and functions of the corpus luteum [45]. In our study we observed strong staining for PR-B in corpora lutea cells during the luteal phase. This finding agrees with the report for porcine [38]. This finding might be explained by locally produced progesterone.

In conclusion, we have demonstrated that ERα and PR-B in the bovine ovary between the follicular and luteal phases of the sexual cycle have not a significant differences, whereas it has differences in cell-spesific localization. ERα proteins have been immunostained in granulosa cells. This suggest that ERα may play an important role in granulosa cells proliferation and differentiation. In addition to this, PR-B is not localized both in granulosa and theca cells of the bovine ovary and we postulate that PR-A isoform may be of critical importance to provide suitable responses to progesterone. Therefore, alternative studies are needed and it should be investigated at the molecular study.

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