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Compensatory ovarian changes, mast cell distribution and luminal structure changes following unilateral ovariectomy in rats

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Summary

To follow-up the present study, 40 mature female rats were used. The animals were divided into test, control and sham groups. The rats in the test group were laparotomized and unilateral ovariectomy was done. On the 1st and 2nd met-oestrous after operation, the tissue samples were dissected out and processed for histological study. The intact ovary in the test group underwent a significant ($P<0.05$) hypertrophy and compensatory changes including double angiogenesis, high cytoplasmic vacuolation in granulosa cell types of corpus luteum, compensatory follicular development, decreasing of follicular atresia, approximately double ovulation rate and increase in the width of cortex and medulla. The uterine horn on the intact side was approximately similar to the control and sham, while morphometric analyses showed that the horn on the ovariectomized side had significant decrease in the thickness of different layers. Scattering of endometrial glands on the uterine horn of the intact side was normal, showing a dense basophilic appearance in haematoxyline and eosin staining sections. The horn on the ovariectomized side showed low gland scattering. Histomorphometric analyses showed no significant differences between the vaginas in the three different groups. Distribution of mast cells (MCs) as essential cells participating in angiogenesis was investigated. In the test group, mast cells were considerably increased in number around the blood vessels in the medulla of the intact ovary and intact uterine horn. Mast cells were very low in number on the horn on the ovariectomized side.

Key words: Female rats, Compensatory response, Ovariectomy, Mast cells

Introduction

As same as total ovariectomy, unilateral ovariectomy results in significant decline in corpus luteum size in sow (Hunter, 1987). In pre-pubertal (170-day-old), cyclic and pregnant gilts, removal of one ovary results in compensatory growth of the other ovary as measured by the increased number or growth of follicles and an increased volume of follicular fluid (Short *et al.*, 1968; Dailey *et al.*, 1970; Rexroad and Casida, 1976). Ovarian follicular population changes with the advance of the breeding season in unilaterally ovariectomized ewes (Dufour and Guilbault, 1984). Compensatory ovarian dry and wet weight increased in gilts as an effect of unilateral ovariectomy. Following unilateral ovariectomy (ULO) in rats, the contralateral ovary underwent hypertrophy and the number of ovulations remained unchanged. In the operated rats, follicle recovery varied depending on the size of the follicle. Compared with the control rats, approximately twice as many large healthy follicles were saved from atresia at the "critical point", producing a compensatory increase in the ovulation number (Kagabu and Umezu, 2005).

According to many authors, mast cells (MCs) are one of the most important cells participating in angiogenesis in the reproductive system (Burd *et al.*, 1989; Selvan *et al.*, 1994; Özen *et al.*, 2002). The

MCs count in the oestrous phase was reported to be higher than that in the luteal phase in the cow uterus (Likar and Likar, 1964). Studies conducted on the oviduct of cows showed that the MCs count in the oestrous and luteal phase was higher in isthmus than in the ampulla region, while in the luteal phase it was higher than that in the oestrual phase in both regions (DuBois *et al*., 1980; Özen *et al*., 2002). The present study was designed to investigate further histological study of the ovaries after ULO in mature female rats, and also to investigate the effect of ULO on the uterine horn's histological structure in the met-oestrous phase of the oestrual cycle. We have hypothesized that MCs promote new double vessel formation in the ovary, uterine, and cervix of the ULO rats by a degranulation process. Because of mast cells participation in probable hyper-vasculature occurrence in ULO, the numerical distribution of mast cells in the different regions of the ovary, uterine horns and vagina was investigated after ULO.

Materials and Methods

Forty rats (*Rattus norvegicus*), 3–5-month-old, weighing 218 ± 4.0 g (mean ± SD) were used. The rats were obtained from the Animal Resources Center of the Faculty of Medicine, Urmia University of Medical Sciences, Iran and were acclimatized in an environmentally controlled room (temperature, 17-23°C; relative humidity, 50-70%; 12 h light/12 h dark). Food and water were given *ad libitum*. The rats were divided into three groups: test group (n = 20), sham group (n = 10) and control group (n = 10). The test group was subdivided into first (n = 10) and second (n = 10) met-oestrous phase after ULO. Vaginal smear was taken to diagnose the phase of oestrual cycle. Surgery was done on met-oestrous, thus there were no differences in the phase of menstrual cycle in the test group. In this study, all experiments which conducted on animals were in accordance with the guidance of ethical committee for research on laboratory animals of Urmia University.

Surgical methods

The animals in the test and sham groups were anesthetized intraperitoneally with ketamin HCl 5% (Iran, Iran-razak), 40 mg/kg and xylazine 2% (Holland, Woerden), 5 mg/kg, then the rats were laparotomized through a midline incision from 1/3 middle line, 2 cm caudally. Care was taken to avoid injury to the uterus and to the uterine circulation. The ovary adjacent to the horn containing the greater number of implantation sites was removed in the ULO group (test group) to minimize the number of corpus luteum (CL) remaining (Rahima and Bruce, 1987). So the rats in this colony revealed no significant differences in the number of CL on the intact ovaries after ULO (Folkman, 1982).

Histomorphological analysis

During the 1st and 2nd met-oestrous after operation, the ovaries were removed and fixed in isotonic formaldehyde acetic solution (IFAA) for 2 to 4 weeks. They were then dissected free from periovarian tissues. The samples were processed by paraffin embedding and cut with rotary microtome and then, stained with haematoxyline and eosine and toluidine-blue for MCs distribution serially. Tissue samples from the rat intestines were used as the control for the mucosal mast cells (MMC), while tissue from the skin of rats was used as the control for the connective tissue mast cells (CTMC) (Özen *et al*., 2002). The early antrum formation and some changes in granulosa cells including dissociation, floating, and luteinization, and also oocyte pyknosis and oocyte deformations were considered as the main characteristics for atresia of the follicles (Najati *et al*., 2006).

Mast cell counting

To determine MC distribution in the preparations stained with toluidine-blue, hundred-square ocular micrometer (Germany, Olympus) was used for the cell count. MCs within the ocular micrometer were counted in high power field (×400). The cells were counted from 18 different regions of the ovary (cortex, medulla) and endometrium, myometrium and perimetrium of the uterine horn of the test, control and sham groups. Thus, the average MCs numbers within the area covered with 100-square ocular micrometers were determined.

An area of 100-square ocular micrometers was calculated by means of micrometrical lam by 40 objective enlargement, and the mast cell density in each site was then found and recorded as mast cell numbers/mm^2 (Özen *et al*., 2007).

Statistical analysis

The results are presented as means ± SD. Differences between mast cell numbers and histomorphometric analyses in the 1st and 2nd met-oestrous of treatment were analyzed with a two-way ANOVA followed by a Bonferroni test, using Graph Pad Prism 4.00, Graph Pad Software, and P<0.05 was considered as significant difference.

Results

Ovaries in three groups (test, sham and control) were measured in size in the met-oestrous phase (1st and 2nd met-oestrous after surgery) of menstrual cycle by light microscopic analysis. In the test group, following ULO, compensatory hypertrophy processes were distinctly manifested in the remaining ovary for two consequent met-oestrous. Histological investigations showed that the cortex region of the ovaries had increased significantly in width. There was an obvious thickness in the tunica albuginea in the test group. Advanced vascular dilation anterior to the tunica albuginea was seen on the intact ovary (Figs. 1A and B). Surface epithelium of the ovaries in the test group increased in height and these cells showed hypertrophy with a stratified appearance and dense basophilic nuclei. Light microscopic analysis showed an obvious hypervasculature between the granulosa type cells of the CL in the ULO group. This hypervasculation was the same in proportion on the second met-oestrous of the menstrual cycle after ULO in rats (Figs. 2A and B). Further observations revealed some vacuolation in the cytoplasm of the granulosa type cells in one cross section from CL with haematoxyline and eosin staining technique, while few cells showed these vacuoles in the cytoplasm of granulosa type cells of CL in the sham and control groups (Figs. 3A, B and C).

There was a considerable increase in the number of CL per ovary in the ULO group (P<0.05). In comparing the number of atretic follicles (primary, secondary and growth) in the test and control groups, approximately twice as many large healthy follicles were saved from atresia (Table 1). Blood vessels in the medulla of the intact ovary increased considerably in the intravascular diameter, in comparison to the control and sham groups. There was edema in the intervascular connective tissue of the medulla on the intact ovary. New capillaries and vessels (double angiogenesis) were seen in the medulla. This new vascularization was clearly enhanced on the intact ovaries.

The comparative data of the ovaries are presented in Table 2. In the macroscopic observation, uterine horns had a normal view. The horn on the ovariectomized side had little decrease in diameter in the ULO cases. In the sham group, microscopic analysis showed normal endometrium, myometrium and perimetrium as measured histomporphometrically. The horn on the intact ovary had a normal histological structure. In contrast, the ovariectomized horn showed a significantly weaker appearance in the histological structures of the three different layers. Histomorphometry showed significant differences in the width of the uterine horn layers between the ovariectomized horns and the intact sides (P<0.05). Endometrial epithelium of the horn in the ovariectomized side decreased in height, but in the intact side was approximately the same as the control and sham. Some of the cases showed hypertrophy with cytoplasmic vacuolation. Scattering of the endometrial gland significantly decreased in the ovariectomized side (P<0.05), while glands had approximately normal scattering on the endometrium on the intact side. Endometrial glands were PAS positive and in haematoxyline and eosin staining had dense basophilic cytoplasm. High mononuclear cells infiltration was seen in the uterine horn in the intact side of the test group, but the horn in the ovariectomized side showed lower cell infiltration. Data for histomorphometry of uterine horns is depicted in Table 3.

Distribution of MCs in the ovaries of the three groups was investigated. Observations demonstrated that MCs were located close to

the blood vessels in the medulla in all groups, while they were presented densely near new vessels and capillaries generated (new double angiogenesis) in the medulla of the remaining ovary in the ULO cases. Total distribution and density of MCs in the medulla of the intact ovary was higher than the sham and control rats (Fig. 4). No mast cell was demonstrated in the cortex of any of the groups. There were no significant differences between total MCs numbers per ovary in the first and second met-oestrous after ovariectomy. Distribution and density of MCs/mm^2 in uterine horns were shown in Fig. 5. MCs distribution in ULO, sham and control rats showed that these cells were abundantly located in the perimetrium of the uterine horns. MCs distribution was higher in total mass than the sham and control group on the horn in the intact side. Because of high angiogenesis and double new vascularization of the perimetrium of the horns on the intact ovary side, MCs were indicated by the large population around the vessels in the perimetrial region of the ULO rat. A significant difference was revealed in the density of MCs distributions between the ovariectomized side and the intact normal side of the uterine horns in the operated rats (P<0.05). Histological and histomorphological analysis of the vagina in the three groups showed that there were no significant differences in tunica mucosa, tunica submucosa, tunica muscularis and

Table 1: Unilateral ovariectomy (ULO), control and sham ovarian follicular function and corpus luteum number (values are mean ± SD)

Groups	No. Corpus luteum/ovary	Atretic follicle (No.)	
		Small antral	Large antral
ULO (1st met-oestrous)	10.42 ± 1.72	21 ± 1.15	14.16 ± 4.58
ULO (2nd met-oestrous)	10.14 ± 1.07	17 ± 1.41	7.66 ± 1.03
Control (1st met-oestrou)	5.71 ± 1.11	13 ± 1.41	9.14 ± 1.68
Control (2nd met-oestrous)	5.42 ± 0.98	12.42 ± 1.72	8.28 ± 1.38
Sham (1st met-oestrous)	6 ± 0.82	13.28 ± 1.11	8.28 ± 1.49
Sham (2nd met-oestrous)	6.42 ± 0.98	12.85 ± 1.21	7.00 ± 1.00

There are significant (P<0.05) differences in all values between control and sham groups with ULO cases

Table 2: Average of the 1st and 2nd met-oestrous ovarian tissue morphometry in unilaterally ovariectomized, control and sham rats (values are mean ± SD)

Groups	Tissue morphometry (µm)			
	Cortex	Medulla	Tunica albuginea	blood vessels (diameter)
ULO	1379.12 ± 250.75	941.57 ± 330.27	36.12 ± 10.16	85.16 ± 23.44
Control	952.84 ± 341.97	787.91 ± 304.99	19.09 ± 3.91	43.87 ± 19.57
Sham	918.57 ± 303.76	786.37 ± 305.12	18.06 ± 4.08	48.51 ± 14.29

There are significant (P<0.05) differences in all values between control and sham groups with ovariectomized (ULO) cases

Table 3: Average uterine horn of the ovariectomized and intact side in the test, control and sham groups in the 1st and 2nd met-oestrous (values are mean ± SD)

	Tissue morphometry (µm)		
	Control-sham	Intact side	Ovariectomized side
Endometrial epithelium	23.80 ± 1.43	24.08 ± 2.11	15.35 ± 1.74
Endometrium	822.02 ± 123.96	813.69 ± 125.28	419.37 ± 164.78
Myometrium	180.88 ± 41.62	182.12 ± 41.29	118.62 ± 31.33
Perimetrium	24.83 ± 1.47	26.33 ± 1.21	11.5 ± 1.05
Lumen	216.37 ± 75.41	551.37 ± 145.50	195.48 ± 55.98
Endometrial glands Extending/100 µm	267.93 ± 66.62	325.81 ± 129.75	152.06 ± 176.02
Glands epithelium	9.63 ± 1.18	9.20 ± 1.81	5.37 ± 1.31

There are significant (P<0.05) differences in all values between control – sham and intact side groups with ovariectomized cases

Table 4: Average of the 1st and 2nd met-oestrous vagina morphometry in ULO, control and sham rats (values are mean ± SD)

	Tissue morphometry (µm)		
	ULO	Control	Sham
Epithelium	32.70 ± 18.66	33.58 ± 19.23	32.32 ± 18.40
Tunica submucosa	646.60 ± 222.18	630.69 ± 239.72	636.31 ± 232.04
Tunica muscularis	270.67 ± 57.15	267.04 ± 57.03	271.02 ± 55.32
Tunica serosa	276.68 ± 56.08	288.24 ± 54.39	284.85 ± 58.08

There are no significant differences between values of the morphometry of different layers of vagina

tunica serosa. In the numerical study of the mast cells (Fig. 6) and histomorphometric analyses of the vagina (Table 4), no differences were found between the three groups (test, sham and control).

Discussion

The results of present study revealed that the ovaries in the test group underwent a significant compensatory hypertrophy after ULO in comparison to the control and sham groups. These findings are similar to those reported previously by other authors. It was shown that, the 60-day-old and 170-day-old gilts are capable of compensatory ovarian hypertrophy in response to ULO (Dailey *et al.*, 1970; Redmer *et al.*, 1984). Also, compensatory ovarian hypertrophy has been reported following ULO in rabbits (Étingen, 1963) and rats (Kagabu and Umezu, 2005).

In the corroboration of those authors, the ovaries remaining in the test group showed an obvious compensative property. Over time this property advanced so that in the second met-oestrous it was distinctly higher. Also, increasing the width of the cortex region was manifested continuously in the second met-oestrous on the intact ovaries in the test group. Naturally, because of compensatory ovarian hypertrophy we saw a developmental thickness in the tunica albuginea of the intact ovaries.

The network of blood vessels and lymphatic capillaries developing along the periphery of Graafian atretic follicles testified to an intensified hormonal activity of the organ in ULO rabbits (Étingen, 1963). In corroboration to Étingen in the sub-capsular region, both around the Graafian follicles and CLs, and also in the side of the medulla of the ovaries, advanced vasodilatation was seen. High scattering of vessels between granulosa type cells of CL was demonstrated in the intact ovary in ULO cases (Figs. 2A and B). This strongly suggests that in ULO, morphological and evidently functional changes happen on the intact ovary.

According to Rahima and Bruce (1987), the number of CL per ovary is similar in the control and ULO rats. In contrast, in the current study our results showed that the CL values in the test group were approximately double in comparison to the control and sham groups. In the histological study of the test group, the results demonstrated cytoplasmic vacuolation in granulosa cell types of CL that was preeminent to the value of granulosa cell type per mm^2 in the control and sham groups. Also, it was interesting that cytoplasmic vacuoles in granulosa type cells in the test group were larger in size in comparison to the same cells of CL in the control and sham group.

In ewes at the 1st, 2nd and 4th oestrous cycles after ULO, the ovulation rate remained constant at 1.5 in the control (sham-operated) ewes, but increased from 1.3 to 2.0 after ULO in the test group (Dufour and Guilbault, 1984). ULO before mating results in compensatory follicular development in the remaining ovary (Peppler and Greenwald, 1970). According to Kagabu and Umezo (2005), following ULO in rats large follicles soon recover after surgery, but it took 5 weeks for small and medium sized follicles to recover. In corroboration with Kagabu and Umezo (2005), in the test group of the present study over the first met-oestrous, the proportion of large atretic follicles was lower than the control and sham groups (Table 1). It is strongly suggested that in ULO cases the rate of atresia decrease significantly and more Graafian follicles ovulate during the oestrous phase. Thus ovulation is not constant in ULOs. In cyclic (day 2 to 14 of

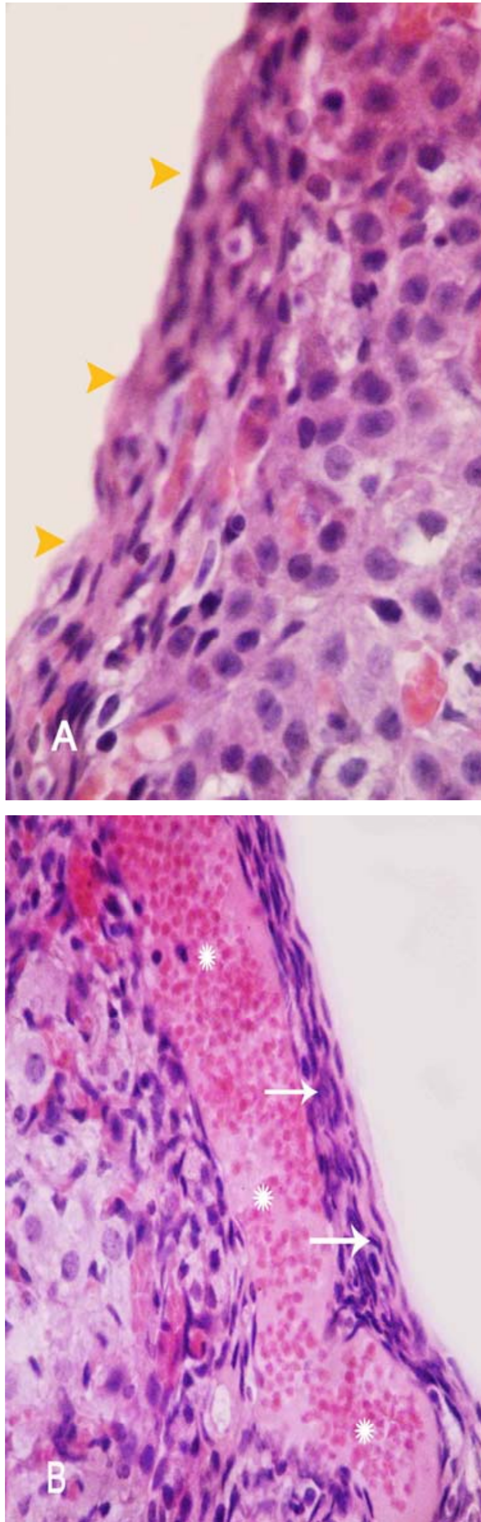


Fig. 1: (A) cross section from ovary in the control group and (B) in the ULO group, note the arrows in Fig. B, showing thickness of tunica albuginea, indicating hypertrophy of the ovary. Stars define the sub-capsular vasodilatation and thrombosis in ULO cases. In contrast, normal appearance of the tunica albuginea with no vasodilatation is shown with arrows in Fig. A, (H&E, A: ×500 and B: ×400)

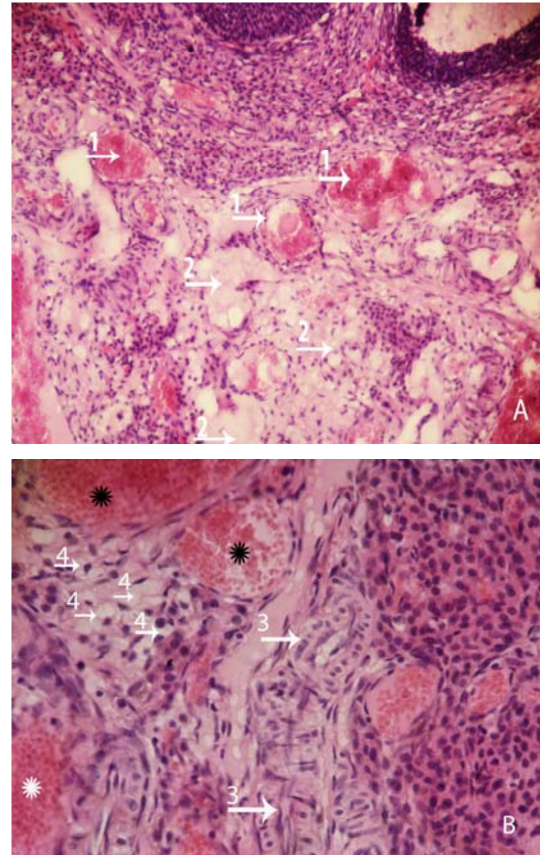
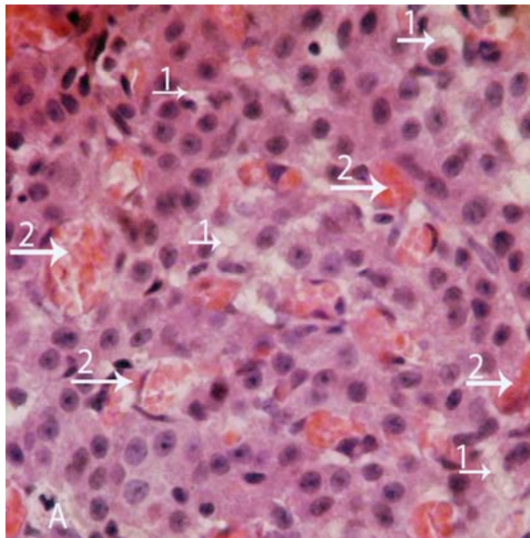
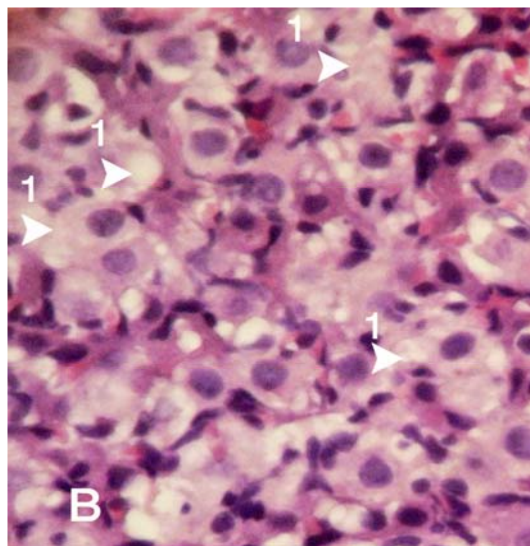


Fig. 2: (A) and (B), cross sections from medulla region of the intact ovary, arrows (1) vasodilatation with obvious thrombosis, (2) edema in the medulla region, (3) new vessel generation (angiogenesis) with considerable epithelial cells, (4) inter-cellular edema in the medulla region and stars indicating vasodilatation and hyperemia in high magnification in Fig. B. Note high infiltration of mono-nuclear immune cells around the vessels, (H&E, A: ×100 and B: ×400)

the oestrous cycle) and pregnant gilts, the number of large follicles (>6 mm) increase while the number of small follicles (1 and 2 mm) decrease following ULO (Staigmiller *et al.*, 1974; Rexroad and Casida, 1976; Redmer *et al.*, 1984). In the present study, small antral atretic follicles were higher in number during the first and second met-oestrous in comparison to the control and sham groups. These findings, in contrast to the gilt, showed that in operated rats small follicles were high in population, so in met-oestrous, the intact ovary had more countable small antral atretic follicles (Redmer *et al.*, 1984). Thus, the recoveries of follicles varied depending on the size of the follicles. As mentioned above, large





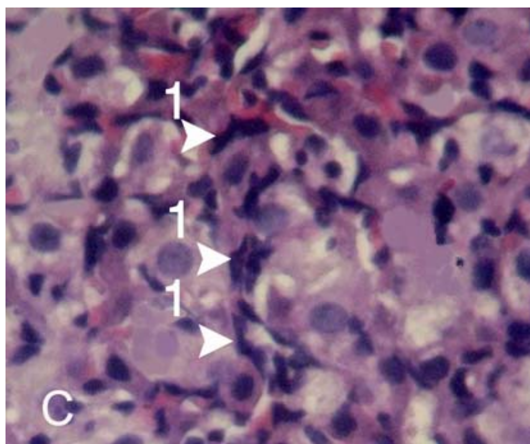


Fig. 3: (A) and (B), cross section of corpus luteom in ULO cases and (C), cross section from corpus luteom in control group, arrows (1) vacuolation in the cytoplasm of granulosa type cells that are in very high value in Fig. B, and (2) indicating vascular scattering with hyperemia in inter-cellular region of corpus luteum. Note the granulosa type cells in Fig. B, more cells are vacuolated. Note Fig. C, the ratio of the cytoplasmic vacuolation is low and no vasodilatation is detectable, (H&E, A: ×400, B: ×100 and C: ×100)

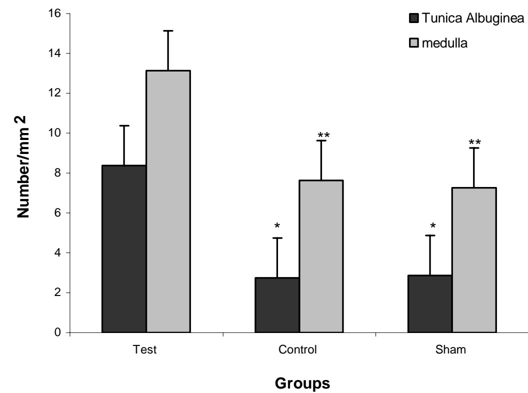


Fig. 4: Average of mast cells distribution in the 1st and 2nd met-oestrous in tunica albuginea and medulla of the ovaries, values are mean ± SD. ** and *: ULO values are significantly different from control and sham groups (P≤0.05)

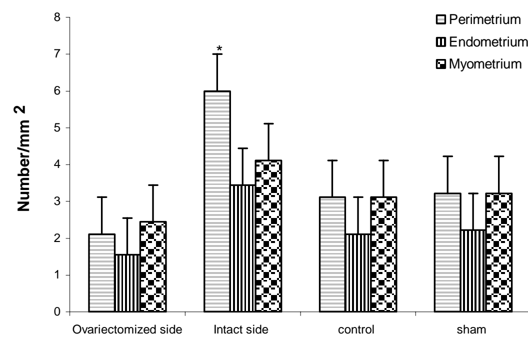


Fig. 5: Average of MCs distribution in the 1st and 2nd met-oestrous in endometrium, myometrium and perimetrium of uterine horns in three different groups. Values are mean ± SD. Star indicate significant differences with ULO, intact side (P≤0.05)

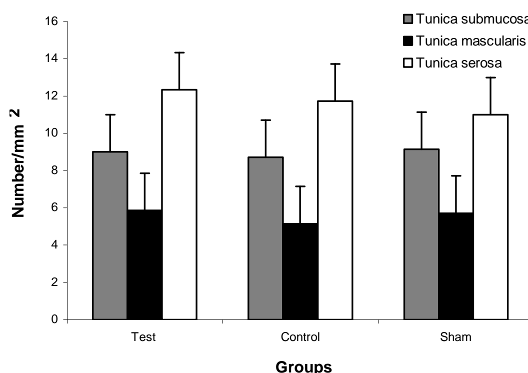


Fig. 6: Average of MCs distribution in the 1st and 2nd met-oestrous in tunica mucosa, tunica submucosa, tinica muscularis and tunica serosa of vagina in three different groups, values are mean ± SD. There were no significant differences between MCs distributions in vagina between different groups

atretic follicles were low in number on the intact ovary.

Angiogenesis refers to the growth of new blood vessels from pre-existing microcirculation and requires endothelial migration, proliferation and stabilization (Levi-Schaffer and Pe'er, 2001). Although it is substantially an endothelial cell event, there are other cell types and many kinds of mediators involved in this process (Griffioen and Molema, 2000; Hiromatsu and Toda, 2003). For almost 20 years, *in vitro* and *in vivo* studies have linked MCs degranulation and activation with angiogenesis and neovascularization (Levi-Schaffer and Pe'er, 2001; Norrby, 2002; Varayoud *et al.*, 2004). This assumption is partially supported by the close anatomical association between MCs and the vasculature, also the recruitment of these cells during tumor growth, wound healing and inflammation (Benítez-Bribiesca *et al.*, 2001). Moreover, degranulation of MCs by a variety of secretagues causes the release of potent angiogenic factors, e.g. vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and several interleukins (IL) such as IL-1 and IL-6 (Burd *et al.*, 1989; Selvan *et al.*, 1994). Also, MCs, basophiles, and platelets are well-known sources of histamine in the rat ovary (Krishna *et al.*, 1989; Jones *et al.*, 1994), histamine has been reported to regulate blood flow and vascular permeability in ovarian tissue, with a role in follicular development and ovulation (Nakamura *et al.*, 1987; Gaytan *et al.*, 1991). The complex phenomenon of angiogenesis begins with the degradation of the basement membrane by cellular proteases, allowing the endothelial cells to penetrate and migrate into the extra cellular matrix and then proliferate (Griffioen and Molema, 2000). MCs, which have been shown to accumulate around vessels and new capillary sprouting sites, have been implicated in angiogenesis (Fukushima *et al.*, 2001; Hiromatsu and Toda, 2003). In this study, as MCs are the main cells located closely to the blood vessels in the ovarian medulla, especially near the new vascularization that doubly generated in the compensatory ovary in ULO cases, we considered MCs as a remarkable biomarker for angiogenesis. It should be reported that scattering of MCs around the new vessel generation in the test group was significantly higher than those in the control and sham group ($P<0.05$). In rodents, MCs are found only in the medulla of the ovary (Krishna *et al.*, 1989; Norrby, 2002). In the present study, observations demonstrated no MCs in the cortex of the intact ovary in ULO, control or sham groups. In rats, MCs are absent from the theca externa of the Graafian follicle and the CL, while the MCs count in the medulla has been reported to change with the phase of the estrous cycle from a maximum during estrous, moderate numbers in met-oestrous to a minimum in pro-oestrous (Kathpalia and Parshad, 1990; Batth and Parshad, 2000; Özen *et al.*, 2007). Further, to considerable vasodilatation in the sub-capsular region and increasing the blood vessels diameter of sub-capsular and high vasculature, no MCs were seen in the cortex region or around the vessels in/or periphery of CL in the intact ovary in the test group. Also, no MC was demonstrated in the cortex of the ovaries in either the control or sham groups.

Many authors have described clusters of MCs in the myometrium and endometrium of non-pregnant women, proposing active roles for these cells in the control of implantation and in extra-cellular matrix remodeling during the menstrual cycle (Mori *et al.*, 1997; Vincent *et al.*, 2000). In the present study, in the result of ULO in the test group, we saw high MCs density around the double generated vessels in intact uterine horns, from which it could be understood that MCs are associated very well in the angiogenesis in the ULO cases, while in the control and sham groups, MCs were low in density around the vessels. In the other study that was carried out on the cow uterus by Özen *et al.* (2007), the results showed that MCs count in the estrous phase was higher than that in the luteal phase. In contrast to cows, in ULO rat MCs distribution and anatomical association were more critical to associate in angiogenesis in the met-oestrous phase of the menstrual cycle. Observations showed low MCs scattering in different regions of ovariectomized uterine horn (endometrium, myometrium and perimetrium). Histological analyses revealed that MCs distribution in different layers of the vagina was approximately similar to the control and sham groups. The vagina did not

differ from a histological point of view. According to MCs distribution, it could be concluded that the vagina may be affected by peripheral hormones, thus no significant differences were demonstrated relating to the vagina.

ULO can cause a compensatory ovarian hypertrophy in the intact ovary. This can naturally lead to an increase in numeric follicular growing, thus ovulation on the intact ovary will increase over time in ULO cases. Because of compensatory ovarian hypertrophy in ULO rats, intervascular edema in medulla, hyperemia, vasodilatation, double angiogenesis and thickness of tunica albuginea with hypertrophy of surface epithelium are considerable. This condition suggests that MCs may be a factor in the angiogenesis pathway and by secreting serotonin, which has a vasoconstrictor effect like histamine, can increase permeability of vessels, which in turn can cause edema. Also, the rate of follicular atresia depends on the size of the follicles in ULO cases. According to histomorphometric analyses the uterine horn in the intact side had an approximately normal condition, while the horn contra-lateral in the ovariectomized side showed significant decrease in all parameters analyzed in the current study. Furthermore, MCs (as an important biomarker for angiogenesis) numerical distribution differed (depending on ULO condition) between different groups.

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