Study of the Mast Cells Distribution and Heterogeneity in Experimentally Induced Cystic Ovaries in Rats

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ABSTRACT

To determine the effect of high serum concentration of estradiol on mast cell distribution and heterogeneity in experimentallyinduced cystic ovary (CO), 56 mature female rats were subjected to study. Following CO induction by unilaterally ligation of the ovarian artery, all rats were euthanized on days 5, 10, 20, 30, 40, 50, and 60, and the ovaries were collected. The blood samples were collected and serum samples were prepared. The histological sections were stained with toluidine blue in order to determine the mast cell distribution. The observation demonstrated that in test group mast cells were found in theca externa, theca interna, and cortex of the cystic ovaries. Observations also showed that mast cells were extensively located in the helium of the treated ovaries. These cells were in the group form closed to the blood vessels in endometrium of the uterine and uterine horns. Mast cells were located in the perimetrium around the blood vessels in the test groups. However, no mast cell observed in both theca interna and theca externa of the follicles in control group. The mast cells distribution in the helium of the control group was significantly ($P \leq 0.01$) less than that test group. Moreover, no mast cell demonstrated in the cortex of the control group. Hormonal analysis showed that there are significant decline in the progesterone and FSH concentrations and increase in the estrogen and LH levels of the serum in CO group. This finding confirmed the hormonal changes in CO condition and may suggest that mast cells are involved in CO induction. It could be also concluded that there should be a sort of mutual effects on hormonal changes and the mast cells distribution in CO cases. Moreover, it might be suggested that mast cell number increasing in cortex of

the ovary could be counted as a biomarker indicating a cystic condition.

INTRODUCTION

Mast cells, basophiles, platelets, and endothelial cells are well-known source of histamine in the ovary.[1,2] Histamine has been reported to regulate blood flow and vascular permeability in the ovarian tissue, along with an important role in the follicular development.[2,4] Previous in vitro and in vivo studies have hypothesized that there is an association between the mast cells degranulation, and consequently, activation and angiogenesis, and neovascularization.[5,8] This hypothesis is partially supported by the close anatomical association between mast cells, the vasculature and the recruitment of these cells during tumor growth, wound healing, and inflammation processes.[9] It has been clearly established during the last years that mast cells of the female reproductive system are subjected to cyclical changes during the estrus cycle. For example, in the hamster ovary, mast cells mediate the vascular response to the gonadotropin surge in proestrus stage.[2] The Cyclic change in the number and the degranulation pattern of the cells during the estrus cycle have also been reported in the reproductive system.[10,11] It is generally accepted that estradiol influence mast cells density and more likely function, as well.[12,14]

The CO is a common problem in the reproduction, both in veterinary and medical fields. It is associated with insulin resistant, hyperinsulinaemia, glucose intolerance, obesity, and altered lipid profile.[15,17] High serum concentrations of androgenic hormones and increasing of estradiol may be encountered in these patients.[18] The CO could be resulted from abnormal hormonal reaching to the ovary, as well.[7,14,15] It is well known that reproductive hormones and in particular estradiol has influential effects on mast cells. There is, however, a lack of knowledge as to how CO condition can affect the mast cells histological distribution and hetrogenesity. Hence this study carried out to examine the distribution of mast cells on the reproductive system of female rats in both normal and CO condition to give a new approach in order to evaluate CO condition based on mast cell distribution, which in turn related closely to estrogen level in serum. Moreover, serum hormonal and glucose levels also were measured.

MATERIALS AND METHODS

Fifty six female 90-days-old rats (Sprague-Dawley) were used. The average weight of the rats was 180 ± 10.1 g. Animals were kept at 22 ± 2° C temperature and 12/12 hours light /dark conditions. They were fed from standard rat plate and tap water. The rats were assigned into two groups, ie, the treatment (n = 48) and control group (n = 8), which had menstrual cycle of normal length (4-5 days). The ethical concerns of current study were approved by the institutional animal care and use committee of Urmia University, which is in accordance with NRC Guide for the Care and Use of Laboratory Animals.

The main method to determine the ovulation day was based on vaginal smear that was conducted on 200 rats, in order to select 56 rats which that exhibited the characteristics of ovulation day in light microscopic analyses of smear slides, as well known method of the ovulation day diagnosis in rats or other rodents.19 The test group was anesthetized by using the combination of ketamin HCl 5% (Iran- Razak), 40 mg/kg and xylazine 2% (Germany) 5mg/kg intraperitoneally. Then, the rats were laparatomized from 1/3 caudal end of middle line and when the site of ligation was exposed, unilaterally the right ovarian artery was ligated by 0-2 silk and blocked completely.20 Also, the rats randomly selected to make a sham control group, which surgery processes were performed on them without ligation of any artery or arteriole. On days 5, 10, 20, 30, 40, 50, and 60, the rats were euthanized by using CO^2 gas in a special device and both ovaries were dissected out and cleaned by using chilled saline normal.

Histomorphological Analysis

The ovaries were fixed in the isotonic form-

aldehyde acetic solution (IFAA, pH 2.8). Samples were processed through paraffin embedding and cut with rotary microtome and stained with toluidine-blue technique. Distribution of the mast cells was studied in the different parts of the collected organs. From the above mentioned section series, 10 sections (each 6 µm thickness) were randomly selected as semi- serially. Tissue samples from rats' intestine 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).

Cell Count

A hundred – square ocular micrometer was used for cell count to determine the mast cell distribution in the preparations stained with toluidine-blue. Mast cells within ocular micrometer were counted in high power field (400X).[19] The cells were counted within 18 areas per tissue, selected from different regions of the ovary (cortex, medulla) and epithelium, tunica sub mucosa, tunica muscularis in the uterus and the uterine horns of the test group, and different regions from the ovaries of control group. Thus, average mast cell numbers within the area covered with 100 square ocular micrometers was determined. The area of 100 square ocular micrometers was calculated by means of micrometrical lam by 40 objective enlargements. Then, the mast cell density in each site (proposed tissues) was found and recorded as mast cell numbers /mm.^{2}

Serum Sampling and Hormonal Analysis

In order to avoid any effect of surgical procedures on hormonal situ, 5 days after surgery, the blood samples from corresponding animals were collected directly from the heart and the serum samples separated by centrifugation. The collected serum samples were subjected to hormonal analysis. The principle of estradiol, progesterone, LH, and FSH levels measuremed in serum on ELISA method was based on competition binding. The competition binding is between two non-classified antigen and conjugated enzyme antigen for binding of limited antibody position on micro-well plate. The limits of detection (LOD) were 0.25mIU/ml, 0.3 mIU/ml, 4.5 pg/ml and 0.05 ng/ml for FSH, LH, estradiol and progesterone in ELISA test, respectively. The intra-assay coefficients variance for FSH, LH, estradiol and progesterone were, 3.56 (for 10 times), 2.64 (for 10times), 5.9 (for 10 times) and 4.8 (for 10 times) respectively and inter-assay coefficientsvariances of 8.98 (for 10 times), 7.52 (for 10 times), 5.9 (for 10 times) and 9.9 (for 10times) were found for FSH, LH, estradiol and progesterone, respectively. Furthermore, the blood glucose was measured by an Oncull now set with test strioe glucose.[20] In order to measure the blood glucose, the blood samples were sampled from caudal artery and immediately put on set and measured.

Statistical Analyses

All results are presented as means ± SD. Differences between mast cell numbers, hormonal concentrations, and glucose level in various days of treatment were analyzed with a two-way ANOVA followed by a Bonferroni test, using GraphPad Prism 4.00, GraphPad Software. $P < 0.05$ was considered significant.

RESULTS

Mast cells in sections stained with toluidine blue had various size and appearance. They were oval, flat, or in the form of spindle shape. Cytoplasms of the mast cells taken almost from all samples and stained with metachromatic dye were homogenous. In the present, study mast cells were observed in the theca externa and theca interna (Figure 1) of the cystic follicles. Observations demonstrated that mast cells were abounded in the cortex of the ovary that had cystic follicle (Figure 2). Mast cells were also located around the blood vessels in the medulla of the cystic ovary (figure 3). In the control group, mast cells were absent in the cortex of the ovary and they were only observed in the medulla. The comparative data for the ovary various regions are presented in tables 1 and 2.

In the right uterine horn (the side that

Table 1: *Comparative distribution of mast cells in the left Ovary Cortex (OC), Medulla (OM) Capsule (LOCp), Teca Interna (TI) and Eexterna (TE) of the follicles (Mean ± SD).*

Parameter Days	TI	TE	LOM	LOC	LOCp
5	2.88±0.08^{a}	3.14±0.09^{a}	9.55±0.28^{a}	29.8±0.87^{a}	8.00±0.23^{a}
10	2.80±0.08^{a}	3.55±0.10^{b}	9.88±0.29^{a}	31.4±0.92^{a}	9.4±0.27^{b}
20	3.40±0.10^{bc}	4.17±0.12^{bc}	13.70±0.40^{bc}	31.90±0.94^{b}	10.2±0.30^{bc}
30	3.88±0.11^{bcd}	4.90±0.14^{bcd}	13.90±0.41^{bc}	33.0 ± 0.95^{b}	14.00±0.41^{bcd}
40	4.00±0.11^{bcd}	5.67±0.16^{bcde}	15.20±0.44^{bcd}	33.67±0.99^{b}	17.45±0.51^{bcde}
50	4.00±0.11^{bcd}	6.00±0.17^{bcde}	15.78±0.46^{bcd}	35.00±1.03^{b}	19.40±0.57^{bcdef}
60	4.51±0.13^{bcde}	6.20±0.18^{bcdeg}	16.1±0.47^{bcd}	36.00±1.06^{b}	19.20 ± 0.56^{bcdef}

^{abcdefg} *values in same column with different superscripts indicate differ significantly ($p < 0.05$).*

Table 2: *Comparative distribution of mast cells in the Right Ovary Cortex (ROC), medulla (M), Capsule (ROCp), Teca Interna (TI), and Externa (TE) of follicles on the right ovary (Mean ± SD)*

Parameter Days	TI	TE	ROM	ROC	ROCp
5	2.40±0.07^{a}	2.31±0.06^{a}	9.47±0.27^{a}	31.00±0.91^{a}	9.22±0.27^{a}
10	2.31±0.06^{a}	4.39±0.12^{b}	12.75±0.37^{b}	31.25±0.92^{a}	10.87±0.32^{b}
20	2.30±0.06^{a}	4.87±0.14^{bc}	13.88±0.40^{bc}	31.09±0.91^{a}	10.90±0.32^{b}
30	3.05±0.08^{b}	4.80±0.14^{bc}	14.00±0.41^{bc}	32.43±0.95^{a}	15.09±0.44^{bc}
40	3.90±011^{bc}	6.40±0.18^{bcd}	15.45±0.45^{bcd}	34.07±1.00^{b}	19.54±0.57^{bcd}
50	3.95±0.11^{bc}	6.47±0.19^{bcd}	15.72±0.46^{bcd}	34.88±1.02^{b}	19.70±0.58^{bcd}
60	4.02±0.11^{bc}	6.74±0.19^{bcd}	15.75±0.46^{bcd}	36.04±1.06^{b}	19.76 ± 0.58^{bcd}

^{abcd} *values in same column with different superscripts indicate differ significantly ($p < 0.05$).*

Figure 1*: Paraffin- embedded ovarian section from study group stained with toluidine blue; Metachromatic mast cells (arrows) are located in the theca intrna of the cystic follicle (400X).*

Figure 2: *Paraffin- embedded ovarian section from study group stained with toluidine blue. (A) Metachromatic mast cells (arrows) are located in the cortex (sub capsular region) of the cystic ovary; there is a high density of the mast cells than normal ovary (400X), (B) Mast cells are located in group form in the cortex near the blood vessels (arrows metachromatic mast cells), (1) blood vessels (1 000X)*

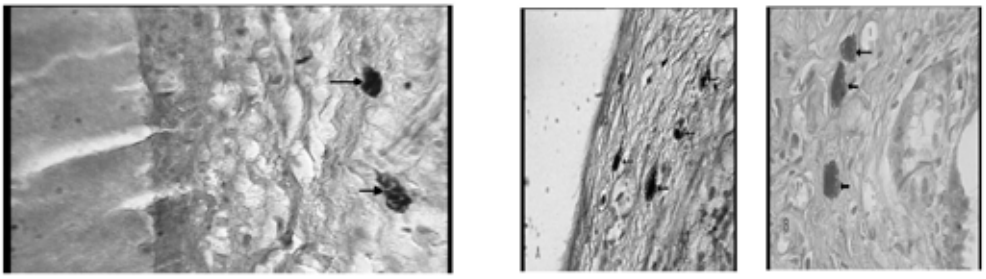
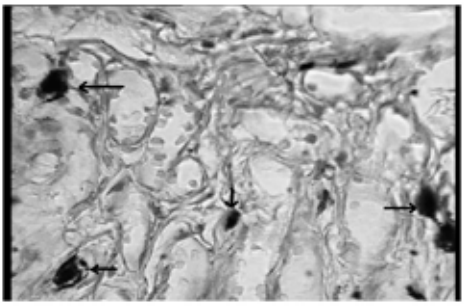


Figure 3: *Paraffin-embedded ovarian section from study group stained with toluidine blue. Mast cells (arrows) are located around the blood vessels in the medulla of the cystic ovary (1000X).*



had ligated ovary), the histological studies demonstrated that mast cells were extensively located close to the blood vessels in the endometrium. In the intact normal side of the uterine horn, mast cells had low density around the blood vessels than the ligated side. In some cases, they were observed close to the blood vessels in the endometrial region of the uterine horn.

There were significant ($P \leq 0.05$) differences in the density of mast cells distributions between treated side and intact normal side of the uterine horns in rats. Mast cells were very dense close to the perimetrium's blood vessels in the treated side, while the distribution of mast cells was normal in the control group. The average mast cells per mm2 in the mentioned organs are depicted in table 3. In the histological study, mast cells were located closely to the blood vessels in the cervix and time-dependently these cells showed more density and distribution. However, this increasing was not very remarkable in different layers of the cervix like the ovaries. As mentioned in the Table 4, they were in a big population in the tunica serosa of the cervix and this high density was very obvious time-dependently in some cases. The biochemistry analysis revealed that the blood glucose increased significantly in the treatment groups only after 40 days. This study also showed that the blood estradiol and LH levels increased while progesterone and FSH concentrations decreased time dependently in the treated group (Figure 4).

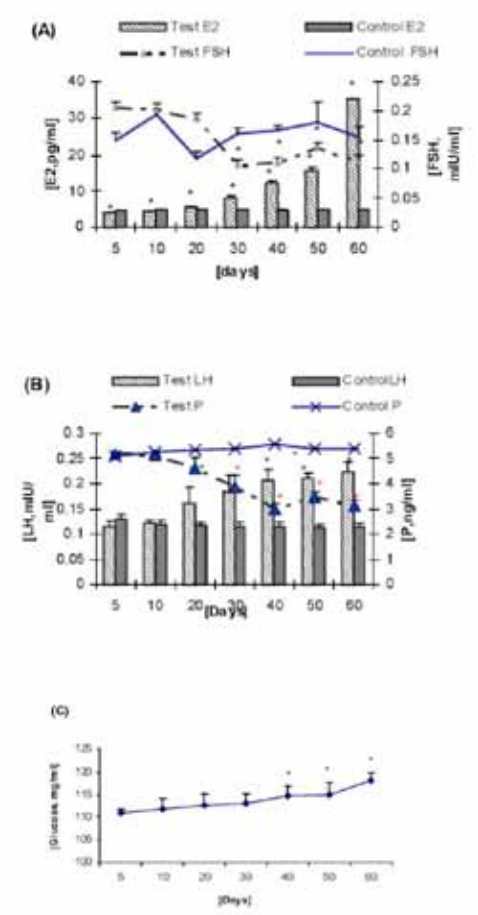
DISCUSSION

This study demonstrated remarkable differences in the mast cells distribution and density in various parts of the ovary between intact and experimentally-induced CO cases. These differences were accompanied by massive changes in reproductive hormone levels and slight alteration of the blood glucose.

It is well known that the degranulation of mast cells by a variety of secretageus causes the release of potent angiogenic factors, eg, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and several interleukins (IL) such as IL-1 and IL-[6,9,17] Additionally, mast cells along with basophiles and endothelial cells are recognized as an important source of histamine in the ovary.[1,2] playing a vital role in the regulation of the blood flow and vascular permeability in the ovarian tissue. Moreover, these cells function equally importantly in follicular development.[2,4] As reported by previous studies, there are an association between the mast cells degranulation and consequently activation and angiogenesis and neovascularization.[8] Thus, the very first finding of the current study could be in accordance with those early reports, where we demonstrated high density of mast cells around the blood vessels.

Early studies showed that mast cells in hamster's ovary are found exclusively around the blood vessels of the medulla, indicating that those cells participate in gonadotropin–induced preovulatory events.[2] In rodents, mast cells are found only in the medulla of the ovary and not in the corpus luteum, the interestitium or the follicles. In contrast, mast cells are found in all parts of the ovary in several other spices, including humans, cows, and monkeys.[2,19,21] In intact rats, mast cells are absent from the theca externa of the graafian follicles and the corpus luteum, while the mast cells count in the medulla has been reported to change with

Figure 4: *Effect of CO on hormonal and glucose concentrations in serum, values are presented as Mean ± SD, (A) E2 and FSH level, (B) Progesterone and LH level, and (C) Glucose level*



the phase of estrus cycle from a maximum during estrous, through moderate number in met-estrus to a minimum in pro- estrus.[15,22,19] Our finding showed that in CO cases mast cell are distributed on theca externa and theca interna. There also was extensive localization of mast cells in the cortex of the cystic ovaries. This localization of mast cells might suggest their role in different pathways of blood flow control in the ovary.

On the other hand, mast cells are main origin of histamine, heparin, chemotactic factors, β-glycoaminidase, β-glocoronidase, and serotonin secretion in rats.[23,24] Serotonin has vasso-constructor effect, and like histamine, can increase permeability of the vessels, which in turn can cause edema. This fact can suggest us that in the CO ovaries surgical ligation can cause lower blood flow and consequently mast cells may participate in physiological pathways to regulate blood flowing and to normalize the ischemic condition. In the light of this hypothesis in this study, we found that mast cell numbers increased time-dependently, which is possibly due to estradiol level increase in CO cases. Also, microscopic analysis showed that there is an obvious edema in the medulla of the CO that may be created by the serotonin secretion from mast cells which is special to the rat's mast cells. Estradiol which is increased in CO condition, is also necessary for this pathway to gather the mast cells close to vessels.

Reibiger and Spanel–Borowski[16] observed deposition of the mast cells in the adventitia of thick–walled muscular arteries in the ovary of cattle, leading to suggestion of an effect on smooth muscle. In this study, mast cells were found abundantly in the periphery of respectively small to medium blood vessel in the ovarian medulla in the cystic ovaries. From histological point of view, as medulla of the ovaries is region of high vassculated, thus mast cells are located in this region in normal cases. It is interesting to be noted that in CO cases, histological investigations showed that there were high density of mast cells located in the medulla that may suggest their role in blood flowing regulation.

Gaytan and co-workers[12] reported that estrogen treated rats presented increased numbers of mast cells in the testis of puberty and adult life. In the present study, mast cells were found in high density in the medulla, theca externa, theca interna, and cortex of the cystic ovaries. It is important to note that these two findings of high estradiol level in serum and in parallel high mast cells density are in good accordance with previous reports and might be influencing each

Table 3: *comparative distribution of mast cells in Endometrium(E) , Myometrium(M) and Perimetrium (P) of right and left uterine horns (Mean ± SD)*

Days parameters	5	10	20	30	40	50	60
LHE	4.2 ± 0.12^{a}	10.8 ± 0.31^{b}	11.3 ± 0.33^{b}	12.5 ± 0.36^{bc}	13.7 ± 0.40^{bcd}	13.4 ± 0.39^{bc}	14.1 ± 0.41^{bcd}
LHM	4.0 ± 0.11^{a}	7.8 ± 0.23^{b}	7.0 ± 0.20^{b}	8.4 ± 0.24^{bc}	8.2 ± 0.24^{bc}	8.7 ± 0.25^{bc}	9.2 ± 0.27^{bcd}
LHP	32.4 ± 0.95^{a}	40.4 ± 1.19^{b}	40.5 ± 1.19^{b}	44.3 ± 1.30^{bc}	43.53 ± 1.28^{bc}	45.6 ± 1.34^{bc}	45.8 ± 1.35^{bc}
RHE	3.8 ± 0.11^{a}	9.0 ± 0.26^{b}	9.8 ± 0.28^{bc}	10.8 ± 0.31^{bcd}	12.0 ± 0.35^{bcde}	12.9 ± 0.38^{bcdef}	13.6 ± 0.40^{bcdef}
RHM	4.1 ± 0.12^{a}	5.9 ± 0.17^{b}	7.2 ± 0.21^{bc}	7.5 ± 0.22^{bc}	7.8 ± 0.23^{bcd}	8.0 ± 0.23^{bcd}	8.8 ± 0.28^{bcde}
RHP	32.5 ± 0.95^{a}	37.8 ± 1.13^{b}	37.8 ± 1.11^{b}	39.9 ± 1.17^{b}	39.66 ± 1.16^{b}	42.7 ± 1.25^{bc}	43.0 ± 1.26^{bc}

^{abcdef} *Values in same raw with different superscripts indicate differ significantly ($p < 0.05$).*

other in CO cases. Varayound and co authors have examined the mast cell distribution on the uterus of rats during the pregnancy in the perivascular zone, and they reported that mast cells were located around the blood vessels, and they suggested that mast cells are very important cells in the regulation of the vascular permeability.[8]

According to the Hiromatsu and Toda,[25] mast cells are at a medium density around the vessels in the endometrium of the uterus. In current study indeed we observed the high separation of mast cells around the endometrium of the blood vessels in CO cases and they were denser near the myometrium of the uterine horns and the uterus. Mast cells had the extensive distribution around the vessels in perimetrium of the CO rats, while the distribution of mast cells on the intact side in both myometrium and perimetrium was normal. As mentioned in the results section, there are statistical differences between the mast cells population and the distribution in myometrium and perimetrium of the right and left uterine horn. Also they were grouped in the tunica serosa of the uterus closed to the blood vessels. The mast cells distribution, however, in the uterine and uterine horns was not time-dependentl in severely increased instances like the ovaries. As mentioned in Table 4, they were in a big population in the tunica serosa of the cervix, and this high density was very obviousy time-dependent in some cases. This situ suggest that the uterine horns and uterus are not severely affected from ischemia and consequently hypoxic condition of the ovarian artery ligation. This fact led us to conclude that the ovarian artery ligation can not completely influence the uterine horns and uterus function, because the uterine artery supplies 90 to 95% of the blood flow.[25] Thus, the mast cells distribution and anatomical association differs in both mentioned organs.

High serum concentration of estrogen and low progesterone level have been reported in CO cases.[26,27] Our results in hematological studies indeed confirmed and extended the previous reports and showed an increase in estrogen and decrease in progesterone levels time-dependently ($P \leq 0.05$) . As experimentally-induced CO could affect the normal levels of key hormones including FSH, LH, progesterone, and estradiol in the blood, it would be logical to hypothesize the same pattern of disturbance in hormonal concentrations in pathologic conditions, which could be observed in both veterinary and medical cases.[26,28]

Histological studies demonstrated that estradiol surging force affected the mast cell distribution in the ovary. With assumption of the fact that estradiol affects the mast cells distribution, we observed large population of mast cells in the cystic ovary. According to previous studies, in CO cases level of: 1) FSH is low or sometimes proximally normal, 2) LH increases, 3) 17- hydroxy progesterone increases, and 4) free estradiol increases.[29] Our results are in good agreement

with these findings except that we showed a decrease in progesterone level in serum. A good interpretation for this alteration during CO cases could be any changes in activity of the enzymes such as Cytochrome P450s, which are involved in biosynthesis and biotransformation of endogenous compounds including sex hormones.[17] Additionally, the hematological observations demonstrated that over the time, LH secretion increased and progesterone level was lowered, which at the same frame of changes a dramatic increase of E2 and remarkable decrease of FSH level may indicate a negative feedback of this hormones on concentrations of each other.

The CO is considered not only as a reproductive disorder but also as a metabolic deficiency consequence, which is associated with insulin resistant, hyperinsulinemia, glucose intolerance, obesity, and altered lipid profile.[4,15,16] In some studies, possible communication between the genes that helps body to use insulin and CO have been demonstrated.[31] Another finding of the current study which could be remarkable point in CO cases is increasing of blood glucose. Most reasonable explanation for this finding might be any disorder in insulin synthesis or release. In accordance to our finding some reports indicating a sort of resistance to insulin in CO cases which in turn could decrease the sensitivity to insulin and ultimately results with an increase in insulin synthesis.[30,31.32]

In conclusion, since the mast cells distribution and histologically localization is dependent on physiologic and pathologic condition., Thus in this study, we demonstrated that in experimentally-induced CO cases, the distribution and heterogeneity of mast cells differs in several parts of the reproductive system. Very notable finding of the current study may be suggested as a biomarker which mast cell number increasing in cortex of the ovary may show cystic condition. The hormonal alteration and in particular estrogen surging force and at the same time increasing in the mast cell population can be observed in CO cases, as well.

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Table 4: Comparative distribution of mast cells in Tunica mucosa(TM), Tunica Sub Mucosa (TSM), Tunica Mascularis (TM) and Ttunica Serosa (TS) of cervix (Mean ± SD)

Days parameters	5	10	20	30	40	50	60
TM	4.0 ± 0.11^{a}	4.3 ± 0.12^{b}	4.7 ± 0.13^{bc}	5.9 ± 0.17^{bcd}	9.7 ± 0.28^{bcde}	9.8 ± 0.28^{bcde}	9.8 ± 0.28^{bcde}
TSM	12.1 ± 0.35^{a}	15.4 ± 0.45^{b}	15.4 ± 0.45^{b}	15.9 ± 0.46^{b}	20.1 ± 0.59^{bc}	20.7 ± 0.59^{bc}	20.8 ± 0.61^{bc}
TM	14.8 ± 0.43^{a}	20.2 ± 0.59^{b}	20.1 ± 0.59^{b}	22.7 ± 0.66^{bc}	24.5 ± 0.72^{bcd}	24.9 ± 0.73^{bcd}	25.0 ± 0.73^{bcd}
TS	$25.4 \pm .73^{a}$	28.4 ± 0.83^{b}	28.5 ± 0.84^{b}	35.4 ± 1.04^{bc}	40.2 ± 1.18^{bcd}	40.4 ± 1.19^{bcd}	40.7 ± 1.20^{bcd}

^{abcde} *values in same row with different superscripts indicate differ significantly ($p < 0.05$).*

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