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Immunohistochemical Expression of Tryptase-chymase and Mast Cell Heterogeneity in Capsaicin-treated Rat Ovaries

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Abstract
Red hot pepper, in the science of botany is a plant belonging to the Solanaceae family and known as Capsicum annuum. Capsaicin is the active ingredient in cayenne pepper. Mast cells are cells with intracytoplasmic granules in the connective tissue, showing metachromasia under appropriate conditions. The aim of the study is to observe mast cell localization and tryptase and chymase expression in ovaries of rats administered subcutaneous capsaicin at 1 mg / kg dose during postnatal development periods. Sixty female Spraque-Dawley rats (21 d old) were used. The rats were randomly divided into 3 groups (n=20 each) as pubertal, post pubertal and adult. Each group was subdivided into two groups. The first subgroup (control) was given no injections. The second subgroup (experiment) received subcutaneous injection of equal volume of *cap- saicin* (1 mg/kg/d) for 7 weeks. Mast cells were observed in the cortex and medulla regions of the ovary during three different developmental periods, giving rise to tryptase and chymase positive reactions. In conclusion, low dose long-term capsaicin administration does not inactivate the presence of mast cells in the ovarian tissue, and the observation of fewer tryptase and chymase immunoreactive cells in the capsaicin-treated experimental groups led us to the conclusion that capsaicin positively affected mast cell heterogeneity in gonads.

Keywords: Capsaicin, Mast cell, Ovary, Immunohistochemistry, Histochemistry, Rat

INTRODUCTION

Capsaicin is a substance in the alkaloid structure [N-vanillyl-8-methyl-alphanonenamide-C18H27NO3-] which gives the red hot pepper bitterness (Erdost 2004). Vanilloids are divided into two groups as endogenous and exogenous; and capsaicin which is the active ingredient of red hot pepper is in the exogenous vanilloid group (Szallasi and Bumberg 2009; Szallasi 2001). The effects of capsaicin change on the dose, application and the target organ (Pyan et al. 1984, Kress et al. 1999). Rat ovaries take neural stimuli through sympathetic, cholinergic, peptidergic, and sensory nerve fibers. It is generally accepted that the sensory extrinsic nerves are related to follicular growth, interstitial tissue and vascularization in the ovaries (Burden 1978). There are studies showing that tachykinins such as Substance P (SP), Neurokinin A (NKA) and Neurokinin B (NKB) have roles in the regulation of reproductive functions (Traurig et al. 1988; Patak et al. 2000). It was showed that immune reaction of SP, Calsitonin Gene Related Peptid (CGRP), NKA and somatostatin in female genital organs was markedly reduced after capsaicin injection in newborn rats (Cotton et al. 1988). Mast cells are connective tissue cells that contain numerous basophilic granules in the cytoplasm and show strong metachromasia because of heparin and sulfated proteoglycans present in their granules (Kürüm et al. 2014; Uslu and Yörük 2015). Histochemical heterogeneities of mast cells divided into two subgroups, mucosal mast cells (MMC) and connective tissue mast cells (CTMC) can be determi- ned using different fixing and staining methods (Enerback 1966). Two types of mast cell granules which are pre-synthesized and stored in granules and synthesized after stimulation, were reported (Garthner and Hiatt 2007; Erekli and Cınar 2015; Ismael and Çınar 2018). Cytoplasmic granules of mast cells contain histamine, heparin and neutral proteases such as tryptase and chymase (Garthner and Hiatt 2007; Krystel-Whittemore and Dileepan 2016; Yıldız et al. 2016). Mast cells are also classified according to the protease content in their granules. The first contains only tryptase, similar to MMC. The second type contains carboxypeptidase and cathepsin, as well as tryptase. The third type contains chymase and carboxypeptidase (Kurum et al.., 2014; Ismael and Çınar, 2018; Krystel-Whittemore and Dileepan, 2016).

Mast cells are heterogeneous cell populations that play significant roles in many organs and systems and involve various physiological processes (Kürüm et al., 2014). Mast cells are shown in mice, rats, hamsters, avians and cow ovaries including many organs and tissues (Uslu and Yoruk 2015; Yıldız et al. 2016; Jones et al. 1980; Nakamura et al. 1987; Uslu et al. 2016; Ertugrul et al. 2018; Ertuğrul and Kurtdede 2017). As a result of studies conducted in rats, it has been reported that mast cells have many important functions such as regulating blood supply in genital organs (Karaca et al. 2007a). Recent studies have shown that mast cells number in ovaries differs according to cycle period (K*ürüm* et al. 2014; Hayıroglu et al. 2016) and cycstic ovaries (Razi et al. 2010).

As a result of the literature review; capsaicin-mast cell-ovarian triangle did not provide sufficient information *in vivo*. The aim of this study is to investigate the possible changes in the mast cell heterogeneity, immunohistochemical expression of tryptase and chymase in the ovaries of capsaicin treated rats during different development periods.

MATERIALS AND METHODS

A total of 60 Sprague Dawley female rats (21-day-old) were used. Rats were divided into three groups as pubertal (42-day-old), postpubertal (56-day-old) and adult (70-day-old), and each group was divided into two treatment groups. Rats were fed *ad libitum* with standard rat food pellets, consuming drinking water freely, and were left in an environment with a 12-h light, 12-h dark cycle at 21–23 °C temperature and 50–60% humidity. Before each capsaicin injection of rats, they were weighed and the amount of capsaicin was determined. In the experimental group, 1 mg / kg subcutaneous capsaicin administration was performed every day until 70 days of age from 21 days of age and the untreated group was not subjected to any application. (Experimental protocol number No: 25.04.2006/1)

In the respective development periods, cervical dislocation was performed on the rats in the treated and untreated groups under dietylether anesthesia, and rat ovaries were removed and fixed in 10% formaldehyde solution. There after passed through routine histological tissue processes and blocked in paraffin.

Immunohistochemical staining

The presence of chymase and tryptase was demonstrated in 5 µm thickness ovarian sections from paraffin blocks by using streptavi-dinbiotin-complex method (True 1990). Mouse monoclonal tryptase (Abcam, ab2378) and rabbit polyclonal chymase (Biorbyt, orb11030) primary antibodies were used for immunohistochemical staining. Histostain Plus (Zymed kit: 85-6743) kit was used as secondary antibody. Serial sec- tions were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase activity was blocked with H_2O_2 3% in absolute methanol for 15 min. The sections were rinsed with phosphate buffered saline (PBS, pH 7.2) and subsequently heated in citrate buffer (pH 6.0) in a microwave oven (700 W) for 10 min for antigen retrieval. After washing with phosphate buffer solution (PBS), sections were incubated (1/100) with primary antibody at +4ºC for one night. After brief rinsing, the biotinylated secondary antibody was applied to the sections and incubated in the streptavidin-HRP complex. In the final stage, 3,3' diamino-benzidine (DAB) was used as chromogen and sections were counterstained with haematoxylin for 1 min, rinsed with tap water, and mounted with entellan mounting medium. Primary antibodies were omitted from negative control sections, which were incubated with PBS.

Following immunohistochemical staining, chymase positive and tryptase positive mast cell distribution was evaluated semiquantitatively. In semiquantitative evaluation following criteria was used; no positive cell in the scanned area (-), 1-2 cells (±), 3-4 cells (+), 5-6 cells (++), 7 and more cells (+++) (Hayıroglu et al. 2016).

Mast Cell Histochemical staining

From the prepared blocks, 10 serial cross-sections of 5 µm thickness were taken in 30 µm intervals, and they were stained with 0.5% toluidine blue prepared in McIlvaine's citric acid disodium phosphate buffer to count the mast cells. In order to determine the subtypes of the mast cells and their distributions in the tissues, the combined staining method of alcian blue/safranin O (AB/SO) was used (Enerbaeck 1966). For the control of this staining method, tissue samples were taken from the intestines of the rats for the MMC and from the skin of the rats for the CTMC.

To determine the numerical distribution of the mast cells in the prepared serial cross-sections, cell counts were performed with a 100 square ocular micrometer (eyepiece graticule). The mast cells in eyepiece graticule were counted in per unit at the 40× objective. The cells were counted in 10 randomly selected areas for each section of rats ovaries. (Bock 1989). Then, all the numerical data were converted into the number of mast cells in 1 mm^2 area. One-way analysis of variance (ANOVA) was used to compare the numbers of mast cells among the groups (Freld 2009). The results were interpreted as with the minimum error rate of 5%.

RESULTS

Histochemical Findings

In the sections stained with toluidine blue, it was determined that mast cells showing metachromasia were markedly rounded or oval (Fig.1,2). Most of the cells were found in the core or eccentric core which was covered by granules. Mast cells were observed in the ovarian tissue in the medullary region, especially around the blood vessels. In the cortical region, mast cells were detected in the connective tissue beneath the germinative epithelium and they were also located near the follicles of various developmental stages, and at the periphery of the corpus luteum and between the follicles. There were fewer mast cells in the cortical region than the medullary region. In the ovarian tissue outside the medulla and cortex, there were more mast cells in the hilus region of mesovarium where the vessels and nerves enter and exit the tissue.

Mast cells in the ovarium were observed in blue colour AB (+) (MMC), in a pink-red colour SO (+) (CTMC) cells and red-blue colour AB/SO (+) (mixed) cells. In our study, it was observed that SO (+) stained mast cells were numerically higher than AB (+) and AB/SO (+) stained mast cells. When the ovarian tissues, pubertal (42 days), postpubertal (56 days) and adult (70 days) period of the rats were examined in the control and experimental groups, in the experimental groups, mast cells showed a significant increase in both toluidine blue and alcian blue/safranine O in the cortical, medullar and hilus regions ($P<0.05$) (Table1, Fig.1,2). The increase was especially observed in the vessels. In addition, mast cells were degranulated in experimental groups. There was a statistically significant difference between mast cell numbers and average number of mast cells compared to the control and experimental groups rats (Table 1).

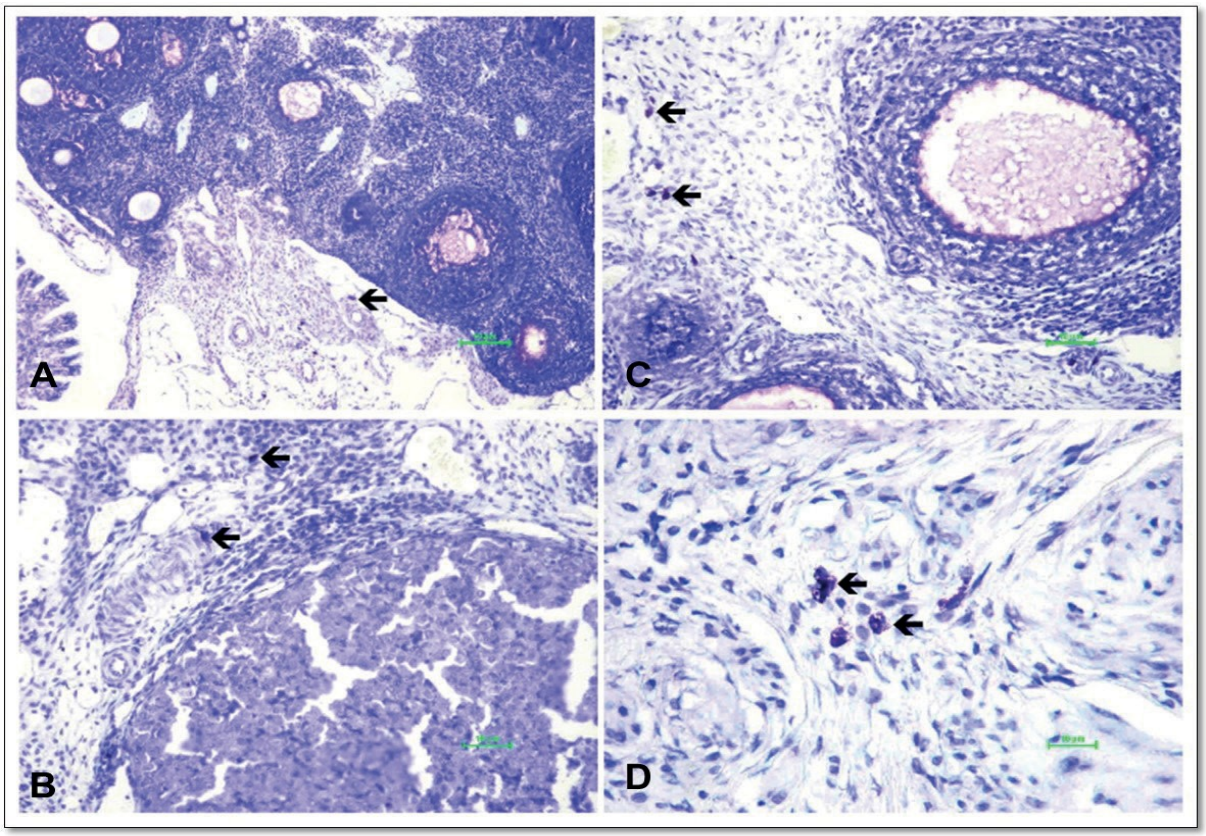

Figure 1. Distribution of mast cells of ovary in all groups. → mast cells. Toluidine Blue Stain. 56d: A (control), C (capsaicin treated); 70d: B (control), D (capsaicin treated).

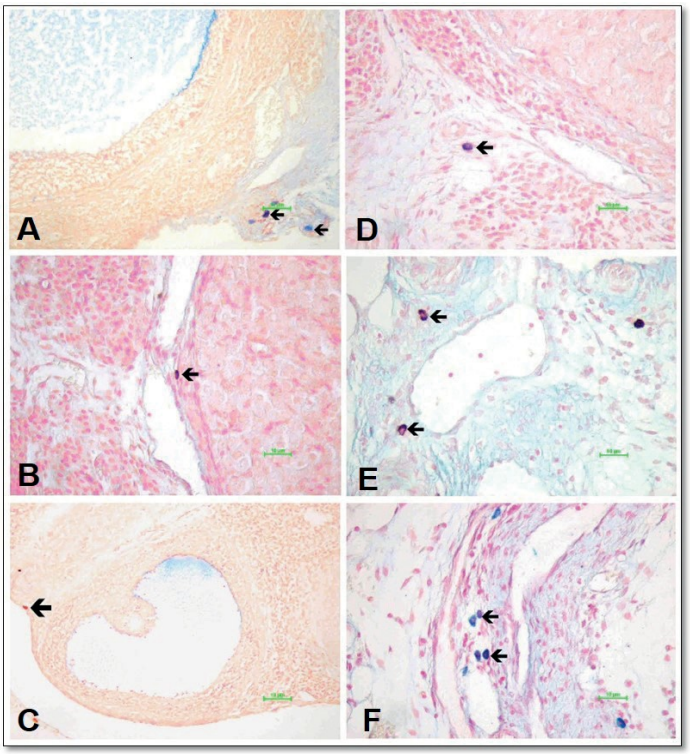

Figure 2. Distribution of mast cells of ovary in all groups. → mast cells. AB/SO (+) (mixed) Stain Method. 42d: A (control), D (capsaicin treated); 56d: B (control), E (capsaicin treated); 70d: C (control), F (capsaicin treated).

Table 1. Distribution of mast cells between groups, (X □Sx),*: P<0.05

Group		n		Minimum	Maximum
42d	Control	10	66.90±1.42	60.80	75.40
	Experimental	10	75.90±3.20	64.00	91.20
	P	*			
56d	Control	10	67.84±5.85	60.80	76.80
	Experimental		75.52±6.73	65.60	84.80
	P	*			
70d	Control	10	66.24±1.19	60.80	72.00
	Experimental	10	71.80±1.68	64.00	80.00
	P	*			

Immunohistochemical Findings

Immunohistochemical evaluation of chymase and tryptase expressions were performed by looking at the cortex and medullary regions of the control and experimental groups of the ovaries. Follicular and interstitial areas were evaluated in the cortex region. In immunohistochemical staining, chymase positive and tryptase positive mast cell distribution was evaluated semiquantitatively (Table 2, 3). In all three main groups (42, 56 and 70 days), different staining reactions were observed in the cortex and medulla regions of the follicles at different developmental periods.

Tryptase Positive Mast Cell Distribution

In pubertal (42 days), postpubertal (56 days) and adult (70 days) rats, tryptase positive stained mast cell distribution in ovarian tissues is shown in Table 2. The ovaries of the rats in the 42 day group were evaluated. In the control and groups, ovaries of rats were evaluated in general. The number of reacting cells in the experimental and control groups was similar (Fig. 3), (Table 2). When 56 and 70 day groups were examined, the number of cells that reacted in the experimental group increased compared to the control group (Fig.3), (Table 2).

Chymase Positive Mast Cell Distribution. In pubertal (42 days), postpubertal (56 days) and adult (70 days) rats, chymase positive stained mast cell distribution in ovarian tissues is shown in Table 3. 42 and 70 days in groups of chemically positive reaction to the number of cells in the experimental groups were decreased compared to the control group (Fig.4), (Table 3). The 56-day group did not show any difference between the control and experimental groups (Fig.4), (Table 3).

Table 2. Tryptase positive cell reaction in ovaries of group 42d, 46d and 70d

Tryptase	42 day	56 day	70 day
Control	+	++	±
Experimental	+	+++	+

No positive cell (-), 1-2 cells (±), 3-4 cells (+), 5-6 cells (++), 7 and more cells (+++)

Table 3. Chymase positive cell reaction in ovaries of group 42d, 46d and 70d old

Chymase	42 day	56 day	70 day
Control	+++	+++	+++
Experimental	+	+++	++

No positive cell (-), 1-2 cells (±), 3-4 cells (+), 5-6 cells (++), 7 and more cells (+++)

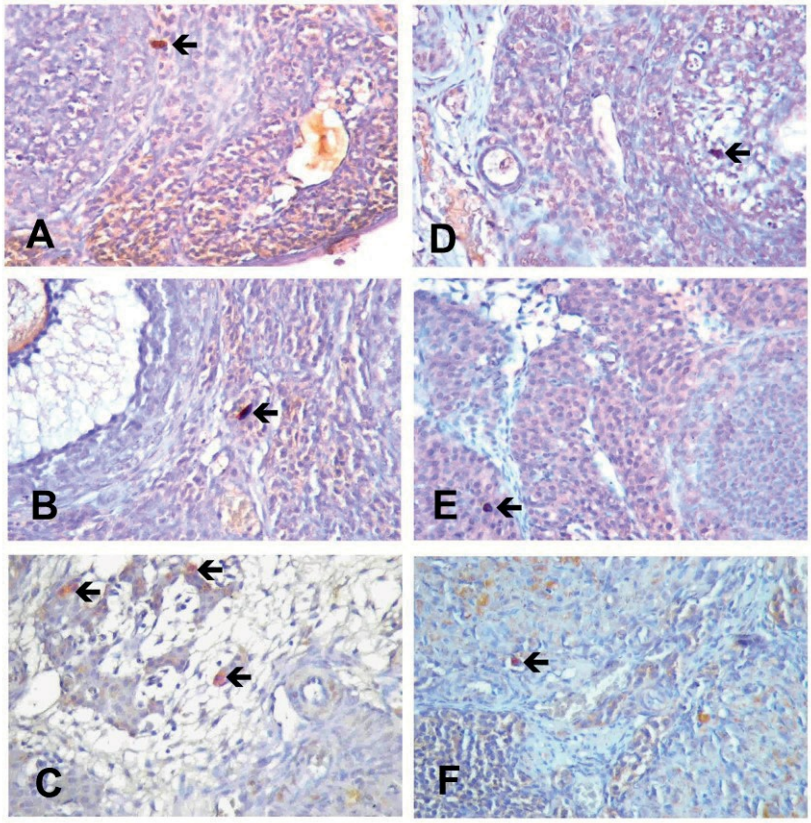

Figure 3. Tryptase immunostaining in the ovarian cells from experimental group during the puberty period (42 d) (D), post- puberty (56 d) (E) and in adult rat (70 d) (F). Control rats during the puberty period (42 d) (A), postpuberty (56 d) (B) and in adult rat (70 d) (C). → mast cells, (x40)

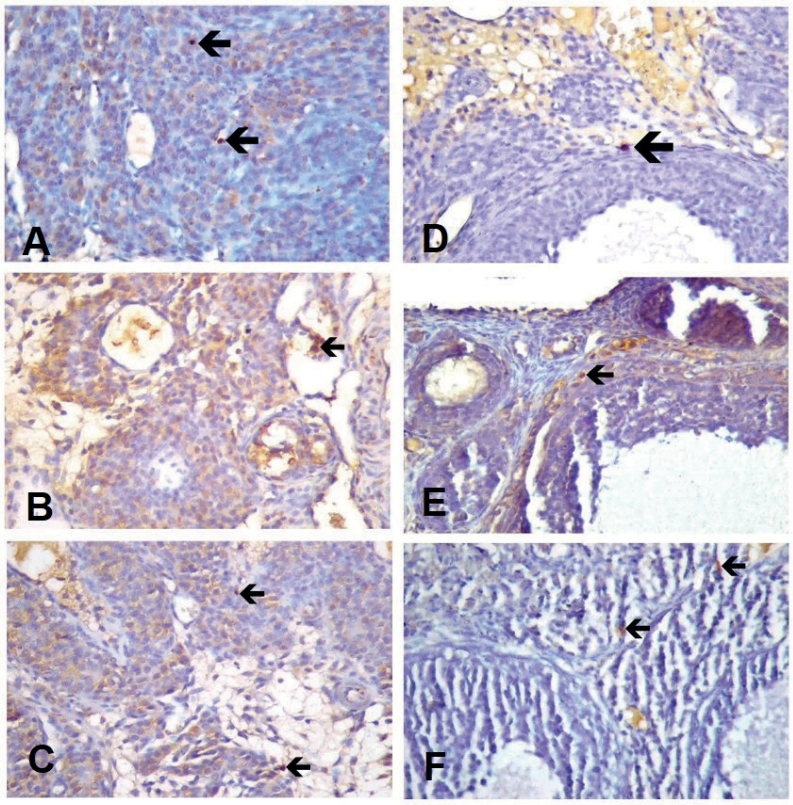

Figure 4. Chymase immunostaining in the ovarian cells from experimental group during the puberty period (42 d) (D), post- puberty (56 d) (E) and in adult rat (70 d) (F). Control rats during the puberty period (42 d) (A), postpuberty (56 d) (B) and in adult rat (70 d) (C). → mast cells, (x40).

DISCUSSION

Results showed that developing follicular density was higher in the pre-pubertal and pubertal period in the experimental sub-groups than in the control group. In the adult group, no difference was observed in the follicle density in the control and experimental groups. Moran et al..,2003 found that high dose capsaicin administration to newborn rats caused a decrease in the number of follicles and an increase in the number of atretic follicles in ovaries contrary to our findings. The difference between results is possibly due to the application time and dose of capsaicin. As a result of our histochemical and immunohistocheical staining, different numbers of mast cells were observed in varied regions of the ovary. In the studies on ovarian tissue revealed that mast cells were located around the blood vessels especially in the hilus region (Jones et al. 1980; Gaytan et al. 1991) and medullary region (Karaca et al. 2007a; Razi et al. 2010; Razi et al. 2010) in rats. In a study performed on cows, mast cells were found in the cortical region, around the graff follicle and the corpus luteum (Ozen 2007). Mast cells have been reported in rat's ovarium, follicles and near the corpus luteum (Gaytan et al. 1991) and located right under the germinative epithelium layer (Hayıroglu et al. 2016). In our study, the shape, metacromatic staining, distribution and location of the mast cells in ovarian tissue are similar to those of previous studies.

Researchers were reported that the number of mast cells increased in different stages of the oestrous cycle on rat ovaries (Gaytan et al. 1991). It has been reported that experimental diabetes (Hayıroglu et al. 2016) and toxic effects (Karaca and Simsek 2007b) causes an increase in the number of mast cells in rat ovaries. In unilateral ovariohisterectomy (Razi et al. 2010b) and experimentally formed cystic ovaries (Razi et al. 2010a), significant mast cell increase was detected in the ovarian tissue of the rats. The findings of this study that there were mast cells in the ovarium are in parallel to those of previous studies.

It was reported that capsaicin causes mast cell degranulation in the mouth region of the rats (Alheal et al. 2014) and on normal human skin (Bunker et al. 1991). Capsaicin increased mast cell degranulation in the central nervous system of rats (Xanthos et al. 2011) and in mouse ears (Inoue et al. 2011). Karaca and Şimşek, appointed that the effect of spirulina on ovaries of rats cause a significant degranulation in mast cells (Karaca and Simsek 2007b). In this study, degranulation of mast cells in the ovarian tissues of capsaicin treated rats showed compliance with previous studies.

Alcian blue / safranin combined dye method was used to determine the subtypes of mast cells (Enerback 1966). In recent studies, on rat ovary and uterus to determine mast cell distribution in different stages of oestrus, mast cell granules were stained in red with SO (+), red-blue colour AB/SO (+) (mixed) cells and blue colour (AB) (+) (14). AB (+) mast cells were detected in capsaicin-treated pig lung tissue (Alving et al. 1991) as well.

Our immunohistochemical staining results revealed that the number of chymase and tryptase positive mast cells decreased in the experimental groups compared to the control groups. Our study is in parallel with the study of the effect of capsaicin in the treatment of otitis media in rats (Basak et al. 2005). In another study conducted on the central nervous system with rats, it has been reported that capsaicin causes an increase in mast cell number (Xanthos et al. 2011). These differences may arise from the content of mast cell granules. Despite the literature reviews, capsaicin and tryptase-chymase mast cell studies were not observed.

CONCLUSION

In conclusion, the presence of mast cells was demonstrated by histochemical and immunohistochemical methods and this study demonstrates *in vivo*, the intereaction between capsaicin-mast cell in ovary. The observation of mast cells in all groups suggests that application of capsaicin for a long period does not adversely affect mast cells. However, low levels of follicular atresia and decreased number of chymase and tryptase immune positive cells in the experimental groups led us to conclude that capsaicin has positive effects on mast cells.

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