

MAST CELL DENSITY AND EXPRESSION OF TRYPTASE, IFN- γ , TNF- α IN CAPSAICIN-TREATED RAT UTERUS

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(Received 05th August 2021; accepted 11th September 2021)

ABSTRACT. Capsaicin is the active ingredient in cayenne pepper. Capsaicin is used in medicine and the pharmaceutical industry due to its physiological and pharmacological effect. Mast cell are scattered a long both external and internal surfaces of the body where they act as the first line defense. It is known that immune system cells and some cytokines secreted from these cells play a key role in the early stages of implantation. It is known that mast cells and many cytokines can indirectly affect hormonal mechanisms in the uterus. The aim of this study is to investigate the mast cell heterogeneity and numerical distribution in the uterus of capsaicin applied rats during different developmental periods. Forty Sprague Dawley female rats were used. Rats were divided into two groups as pubertal and adult, and each group was divided into two treatment groups. The first group remained without any treatment (control group), the second group (experimental-capsaicin treated group or CAP group) received daily subcutaneous injections of 1 mg/kg/day capsaicin and tissue samples were processed for conventional histology and for immunohistochemistry using the Streptavidin-Biotin Peroxidase method and a rabbit polyclonal anti-VR1 primary antibody. In the presented study the high expression of TNF- α and IFN- γ and mast cell number were observed in capsaicin group. In a conclusion, this study revealed the relationship between capsaicin, TNF- α , IFN- γ and mast cells in the uterus.

Keywords: *Capsaicin, uterus, mast cell, cytokines, puberty and adult rats*

INTRODUCTION

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is the active component of chili peppers, which gives the red hot pepper bitterness [1]. Capsaicin is used in medicine and the pharmaceutical industry due to its physiological and pharmacological effects [2]. The anti-inflammatory properties of capsaicin are thought to cause the release of pro-inflammatory mediators and hydrolytic enzymes. It is known that capsaicin-induced intracellular signaling in neuronal cells occurs via vanilloid receptors [3]. It has been reported that capsaicin may cause uterine contraction by affecting the sensitive nerve fibers in the female genital system [4].

Mast cells are scattered along both external and internal surfaces of the body where they act as the first line of defense [5]. Mast cells may be activated by discharging their granule ingredient when they are stimulated by physical, immunological, and neurogenic factors [6]. The steroid sex hormones, estradiol and androgen, are also thought to have an effect on mast cells [7]. It is also known that immune system cells and some cytokines secreted from these cells act indirectly on mast cells [8]. Tryptase is a potent angiogenic

factor [9]. It participates in the development of neurogenic inflammation in afferent neurons and prevents neurogenic inflammation by reducing the level of anti-inflammatory neuropeptides [10]. Tumor necrosis factor alpha (TNF- α) is known to be released as preliminary mediators that activate neutrophils, stimulate other effector cells, and increase chemokine synthesis [11]. Studies have shown that TNF- α plays an important role in blastocyst implantation [12], growth and development of the placenta, and survival of the embryo [13]. Interferon-gamma (IFN- γ), a proinflammatory cytokine, regulates hematopoietic cell maturation, differentiation, activation and apoptosis [14]. IFN- γ participates in the decidualization process of the endometrial stroma, helps maintain pregnancy [15] and increases the chances of the embryo survive [16].

It is known that mast cells and many cytokines can indirectly affect hormonal mechanisms in the uterus. Hormonal mechanisms organize the uterus for possible implantation. In this study, it was aimed to reveal the possible effects and heterogeneity of capsaicin on mast cells in pubertal and adult rat uterus. In addition, it is aimed to show immunohistochemical expressions of tryptase, TNF-alpha, IFN gamma, which are known to exist in mast cell granules, and to examine their possible effects on each other in this process.

MATERIALS AND METHODS

A total of forty Sprague Dawley female rats (21 day old) were used. Rats were divided into two groups as pubertal (42 day old), and adult (70 day old), and each group was divided into two treatment groups (control and capsaicin treated group). 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. The first group remained without any treatment (control group, n=20), the second group (capsaicin treated group or CAP group, n=20) received daily subcutaneous injections of 1 mg/kg/day capsaicin (Sigma, St. Louis, MO, USA). Before each capsaicin injection of rats, they were weighed and the amount of capsaicin was determined. In the experimental group, capsaicin administration was performed every day until 42 and 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)

At the end of the 42nd and 70th day of age, the animals were weighed and sacrificed under ether anaesthesia. The uterus were removed carefully and fixed in a 10% formaldehyde solution for histopathological examinations. Thereafter passed through routine histological tissue processes and blocked in paraffin.

Histochemical Staining

The tissue samples were fixed in 10% neutral buffered formalin , washed, and passed through routine histological tissue processes and blocked in paraffin. From the prepared blocks, 10 serial cross-sections of 5 μ m thickness were taken in 30 μ m 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 [17].

All the numerical data were converted into the number of mast cells in a 1 mm² area. One-way analysis of variance (ANOVA) was used to compare the numbers of mast cells

among the groups [18]. The results were interpreted as with the minimum error rate of 5%. 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. The mast cells in eyepiece graticule were counted in per unit at the 40x objective. The cells were counted in 10 randomly selected areas for each section of uterus tissue.

Immunohistochemical Staining

The presence of IFN- γ , TNF- α and tryptase was demonstrated in 5 μ m thickness uterus sections from paraffin blocks by using streptavidin-biotin complex method [19]. Rabbit monoclonal IFN- γ (1/500 dilution, Shanghai YL Biotech, YID2791), rabbit polyclonal TNF- α (1/200 dilution, Abcam, AB-9739) and mouse monoclonal tryptase (1/200 dilution, Abcam, ab2378) primary antibodies were used for immunohistochemical staining. Histostain Plus (Zymed kit: 85-6743) kit was used as a secondary antibody. The sections were deparaffinized, hydrated and processed for antigen retrieval using microwave a oven. The sections were incubated with buffered citrate (pH 6) for 5x3 minutes. In order to block endogenous peroxidase activity, the tissues were incubated in 3% hydrogen peroxide solution. Following washing with phosphate buffer solution (PBS), serum in the kit was instilled to prevent nonspecific protein binding in sections. After the serum blocking sections were incubated with primary antibody at +4 $^{\circ}$ C for one night. Then brief rinsing, the biotinylated secondary antibody was applied to the sections and incubated in the streptavidin-HRP complex. 3,3' diaminobenzidine was used as chromogen and sections were counterstained with hematoxylin for 1 min, rinsed with tap water, and mounted with mounting medium. Primary antibodies were omitted from negative control sections, which were incubated with PBS.

Following immunohistochemical staining, 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 (\pm), 3-4 cells (+), 5-6 cells (++) , 7 and more cells (+++) [20]. Intensity and distribution of positive staining in the immunohistochemical examination for IFN- γ and TNF- α were assessed using a standard four-point scoring scale for intensity, with slides being scored as negatively (-), mildly (+), moderately (++) , severely or strongly (+++) stained [21]. The resulting preparations were photographed under a Nikon 50i research microscope, with a Nikon digital-sight DS-Fi1 imaging system.

RESULTS AND DISCUSSION

Histochemical Results

Toluidine blue staining: In the uterus of each of the four groups stained with toluidin blue, mast cells were distinguished with metachromatically stained. Mast cells were seen close to the epithelial cells and distributed around uterine glands, in basal and deeper layers of endometrial stroma. They were observed to be located around capillaries inside the lamina propria and perimetrium connective tissue. In the myometrium, they were predominantly detected in close proximity to smooth muscle cells in both experimental and control group (Fig. 1). There was no significant change in the number of mast cells between the age groups. The number of mast cells in the capsaicin applied groups was highest. Significantly higher mast cell counts were observed in the capsaicin treated group compared to the control group (Table 1).

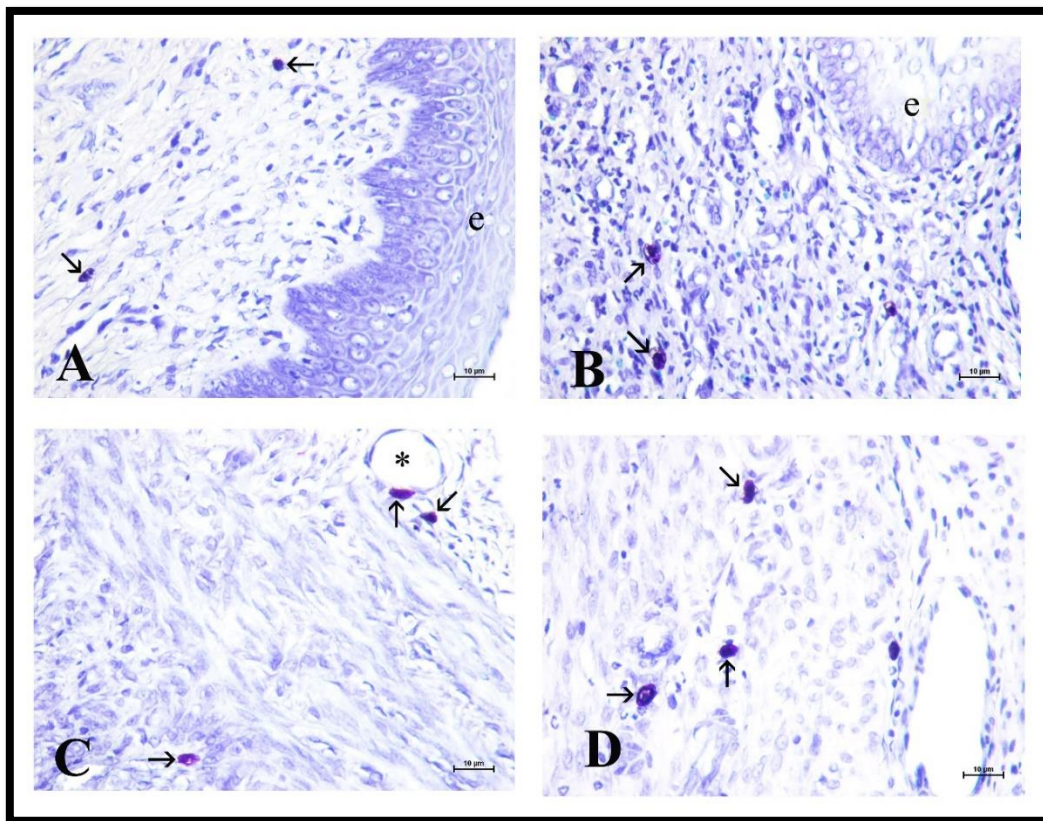


Fig 1. Control group (A), Capsaicin treated group (B) (42d old); Control group (C), Capsaicin treated group (D) (70d old). Toluidine blue staining; arrow: metachromatic mast cells, epithelium:e, blood vessel: asteriks, original magnification $\times 40$; range bar, $10 \mu\text{m}$.

Table 1. The number of mast cells after staining with toluidine blue in four groups. The statistical difference between both groups was found insignificant ($p > 0.05$).

Group	Mast cell count ($\times \pm Sx / \text{mm}^2$)	P
Control group (42d old)	40.96 \pm 1.64	*
Capsaicin treated group (42d old)	50.88 \pm 3.29	*
Control group (70d old)	42.88 \pm 1.85	*
Capsaicin treated group (70d old)	52.80 \pm 2.99	*

*: $p < 0.05$

AB/SO combine staining: AB (+), SO (+) and AB/SO (+) mast cells were detected in rat uterine tissues stained with alcian blue/safranin O combined staining technique. In the uterus, both control group and capsaicin treated group were observed that red SO (+) mast cells were more intense than AB (+) in blue color and AB/SO (+) (mixed) mast cells in red-blue color. In our study, at least (mixed) mast cells were detected in uterine tissue in

all groups. In this dyeing method, mast cells were observed more frequently in the lamina propria and submucous layer (Fig. 2).

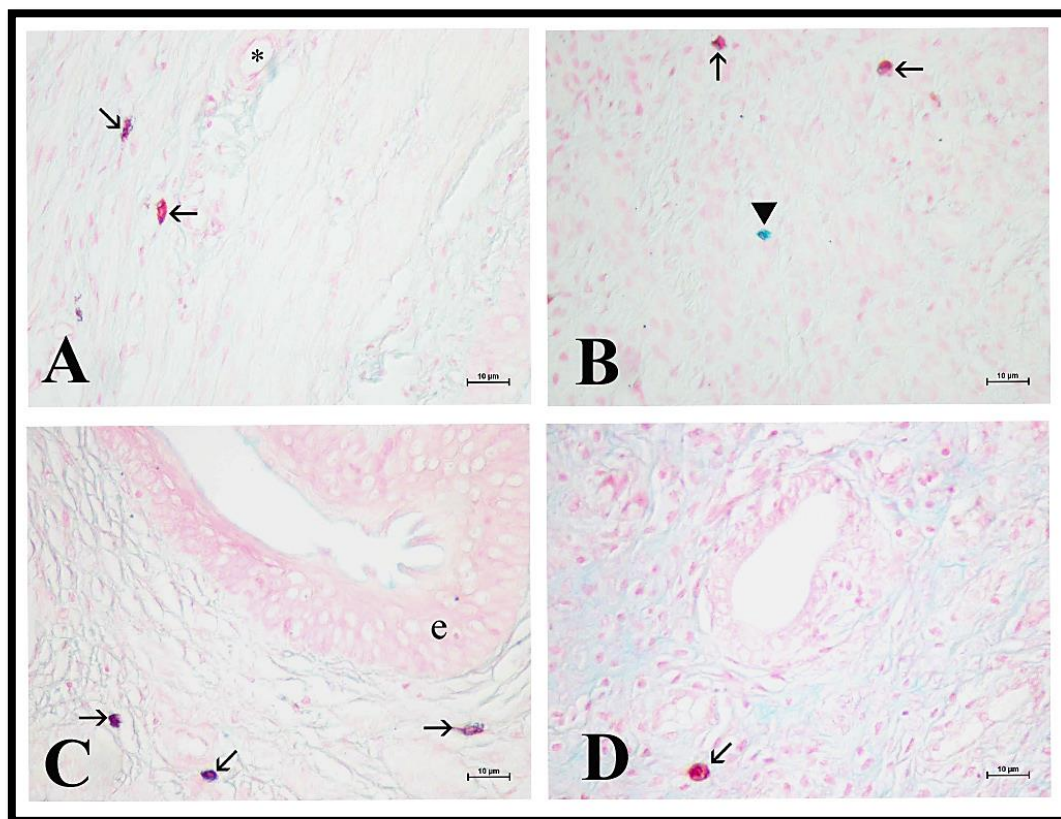


Fig 2. Control group (A), Capsaicin treated group (B) (42d old); Control group (C), Capsaicin treated group (D) (70d old). Alcian blue/safranin O combined staining method; arrow: safranin O (+) mast cell, arrowhead: alcian blue (+) mast cell, epithelium: e, blood vessel: asteriks, original magnification $\times 40$; range bar, 10 μm .

Immunohistochemical Results

Tryptase Immunohistochemistry: In the endometrium, tryptase-positive mast cells were found to be located in the lamina propria, especially around the blood vessels. Tryptase mast cells were seen to be located in the connective tissue between muscle bundles in the myometrium and around the blood vessels in the loose connective tissue in the perimetrium (Fig. 3A, 3C). More tryptase-positive mast cells were detected in the endometrium compared to the myometrium. No significant change in the number of tryptase positive cells was observed between pubertal and adult rats (Fig. 3A,3B,3C,3D). The number of tryptase-positive mast cells in the capsaicin treated group slight increase compared to the control group (Fig. 3A, 3B, 3C, 3D), (Table 2).

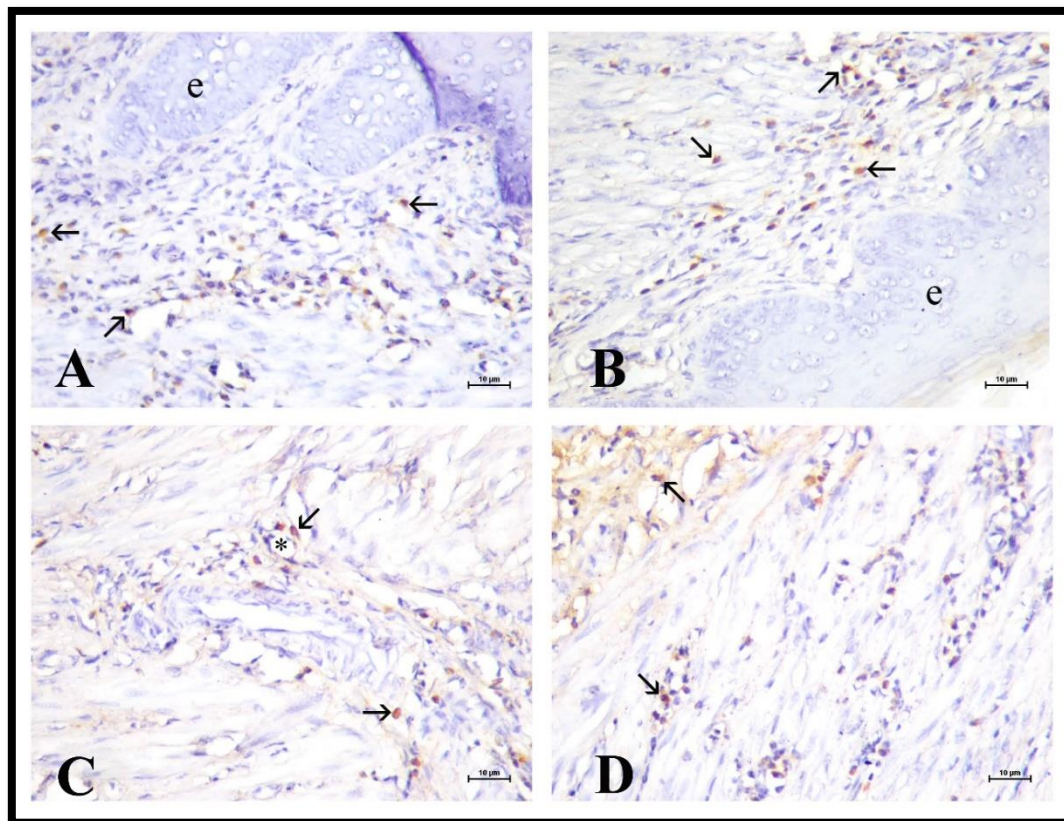


Fig 3. Control group (A), Capsaicin treated group (B) (42d old); Control group (C), Capsaicin treated group (D) (70d old). Tryptase immunostaining, arrow: tryptase - positive mast cells, epithelium: e, blood vessel: asteriks, original magnification $\times 40$; range bar, 10 μm .

Table 2. Tryptase positive cell reaction in uterus.

Tryptase	Puberty (42d old)	Adult (70d old)
Control group	++	++
Capsaicin treated group	++	+++

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

TNF- α Immunohistochemistry: In the control group, TNF- α was present in both the uterine stroma and the endometrial epithelial cells. It was determined that there were partially weak stained areas in the endometrium layer. Epithelial cells had weak to moderate intracytoplasmic staining. TNF- α immunoreaction was found to be more intensely in cytoplasm of epithelial cells close to lumen. When the myometrium and perimetrium layers of the uterus were evaluated, no immune positive reaction was observed. In the capsaicin treated group, TNF- α immunoreactivity increased compared to the control group, especially in epithelial cells (Fig. 4B, 4D).

When we evaluated the control and capsaicin treated groups among themselves; it was determined that the severity of immune reaction in the experimental group was more severe than in the control group (Fig. 4B, 4D).

In order to see possible differences in the developmental period, we examined the pubertal and adult preparations among themselves. As a result of detailed evaluations; it was observed that the severity of immune reaction was slightly increased in both control and capsaicin treated groups, in adult rats (Fig. 4C, 4D). Although differences were observed between the groups as a result of histological evaluations, no significant difference was determined as a result of statistical evaluations (Fig. 4A, 4B, 4C, 4D), (Table 3).

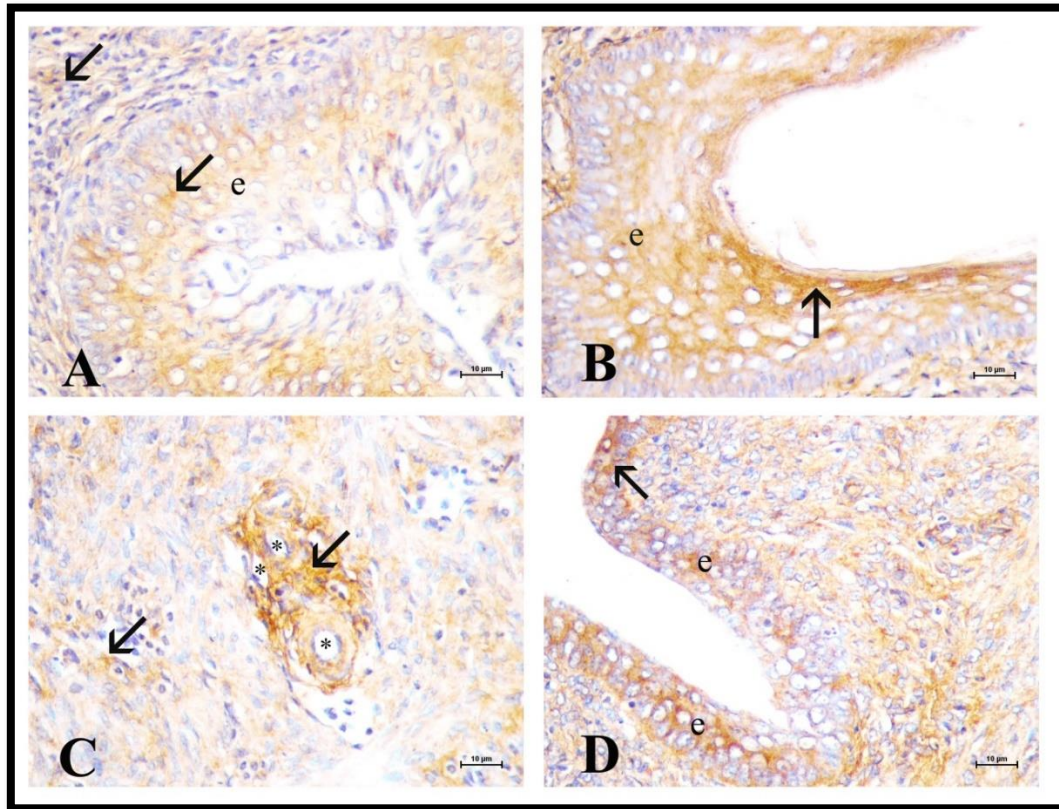


Fig 4. Control group (A), Capsaicin treated group (B) (42d old); Control group (C), Capsaicin treated group (D) (70d old). Tumor necrosis factor alpha immunostaining, arrow: TNF- α positive mast cells, epithelium: e, blood vessel: asteriks, original magnification $\times 40$; range bar, 10 μm .

Table 3. Intensity of TNF- α immunostaining in the different classes of uterus according to the developmental period (puberty and adult).

	Puberty (42d old) Control group	Puberty (42d old) Capsaicin treated group	Adult (70d old) Control group	Adult (70d old) Capsaicin treated group
Endometrium	\pm	\pm	+	+
Myometrium	-	\pm	-	\pm
Perimetrium	-	\pm	-	\pm

(-) no staining, (+) weak, (++) moderate and (+++) strong.

IFN- γ Immunohistochemistry: In all groups, IFN- γ immune positive reactions were detected in the endometrial epithelium. It was observed that the immunohistochemical reactions in the cells were intracytoplasmic. Weak immun reaction was observed in some areas of the myometrium and perimetrium layers of the uterus. Immunoreactivity was observed in the endometrium, especially around the vessels (Fig. 5A, 5B, 5C, 5D). When the preparations in the control and capsaicin treated groups were examined; it was determined that there were immune reactions with similar, but was observed that the reaction severity was increased in the capsaicin treated group in the 42-day-old group (Fig. 5A, 5B). In order to see the differences in the developmental period, we examined the pubertal and adult preparations among themselves. As a result of detailed evaluations; no significant difference was detected between the groups (Table 4).

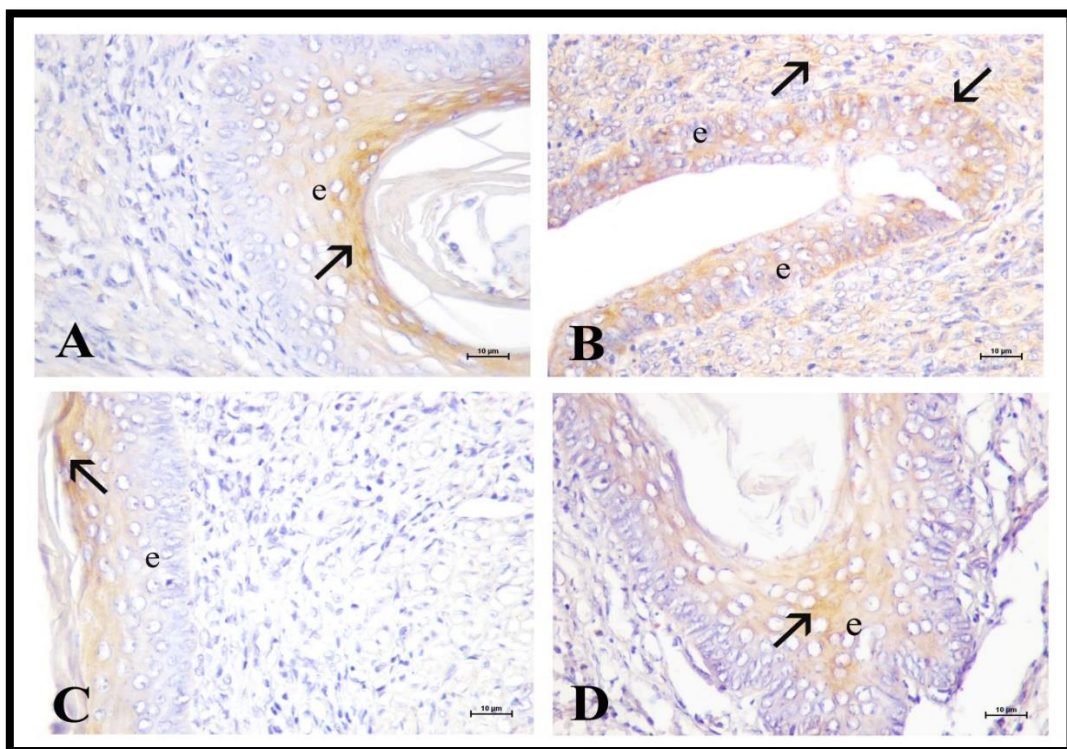


Fig. 5. Control group (A), Capsaicin treated group (B) (42d old); Control group (C), Capsaicin treated group (D) (70d old). Interferon gamma immunostaining, arrow: IFN- γ positive mast cells, epithelium: e, blood vessel: asteriks, original magnification $\times 40$; range bar, 10 μm .

Table 4. Intensity of IFN- γ immunostaining in the different classes of uterus according to the developmental period (puberty and adult).

	Puberty (42d old) Control group	Puberty (42d old) Capsaicin treated group	Adult (70d old) Control group	Adult (70d old) Capsaicin treated group
Endometrium	+	++	+	++
Myometrium	±	+	±	+
Perimetrium	±	±	±	+

(-) no staining, (+) weak, (++) moderate and (+++) strong.

In the present study, the effects of capsaicin and sexual development stage on mast cell density and tryptase, IFN- γ , TNF- α expressions were investigated in rats uterus. The results of this study show that capsaicin application may cause changes, in mast cell number in the uterus. It was determined that tryptase positive mast cell density was almost the same in all groups. Other important findings of this study are the increases IFN- γ and TNF- α expressions in experimental groups. In addition, it was observed that the severity of the reaction was slightly increased in the groups treated with capsaicin in adult rats in TNF- α expressions.

Mast cells are divided into subgroups considering histochemical differences, mediators they include, responses to the secreting agents and content of the proteoglycans they contain [22]. Aydın et al. [23] analysed the heterogeneity of mast cells in the oestrus cycle in female reproductive organs. SO (+) and AB (+) cells were found in uterus in their researches. Karaca et al. [24] detected only SO (+) and AB (+) cells after AB/SO staining in the uterus of rats. However, Eren et al. [25] observed that mixed granulose mast cells are present in the rat uterus as well as AB (+) and SO (+) mast cell. In the presented study, AB (+) and SO (+) and AB/SO (+) mixed mast cells were seen in the rat uterus. The findings of our study are compatible with the results of the studies mentioned [23-25]. Based on the findings we obtained in this study, we postulated that capsaicin and developmental period do not have much of an effect on mast cell heterogeneity.

It has been reported that the number of mast cell in rats increased in the early stages of pregnancy [26]. Researchers were reported that the number of mast cells increased in depending on the hormonal effect of different stages in the oestrus cycle on rat uterus [25]. A significant increase in the number of mast cells in the uterine tissue has been reported due to diabetes in rats [27] and cadmium exposure in mice [28]. The increase in mast cell counts in the capsaicin treated groups in our study is consistent with the findings of previous studies. Additionally, mast cell count among the pubertal and the adult groups was not significantly different.

It has been shown that tryptase-positive mast cells are localized in the endometrium and myometrium layers in the human uterus [29]. Tryptase containing mast cells have been reported to be found in the region where the between endometrium and myometrium [9], in the connective tissue around capillaries [30] and in uterine endometrial layer [31]. An increase in tryptase-positive mast cells has been reported in endometriotic lesions [32]. In another study conducted, tryptase-positive mast cell increase was found to occur in uterine tissue in rats which were experimental diabetes [27]. It was also noted that there is a significantly lower number of tryptase-positive mast cells in the pregnant woman uterus compared with the non-pregnant woman uterus [33]. In our study, the localization

of tryptase positive mast cells in the uterine tissue and a slight increase in the capsaicin treated group were parallel to the studies mentioned above. Although the increase in our study is very few, it can be said that capsaicin may affect tryptase mast cells.

Studies in dogs [34] and in monkeys [35] reported the presence of TNF- α expression in the uterus during pregnancy. In a study conducted in women after menopause, it was reported that TNF- α expression was not detected in uterine epithelial cells, but a weak reaction in the endometrium [36]. Immunohistochemical TNF- α cytokine localization was observed in different stages of oestrus cycles in the mouse uterus stroma and myometrium [37]. In addition studies conducted have reported that immunohistochemical TNF- α staining was observed in the cells of the epithelium lining the human [38] and dog uterus [34]. It has been found that the TNF- α staining ratio was higher in mice with experimental diabetes compared to healthy ones [13] and in myomas compared to myometrium and myometrium tissue adjacent to myoma [39]. To elucidate how capsaicin might affect TNF- α in rat uterus, we attempted to analyze TNF- α expressions in the uterus of pubertal and adult rats. In the present study, immunohistochemical data revealed that TNF- α was constitutively expressed by uterine epithelial cells in rats, and it was also observed that the localization of these cytokines in the epithelial layer increased in cells close to the lumen. We also observed that the application of capsaicin could affect TNF immune reactivity.

TNF- α has been observed to support mast cell development in mouse spleen cell cultures [40]. In hypertensive heart disease, it is mentioned that the expression of chymases and TNF- α shows a similar increase [41]. The fact that we observed an increase in TNF- α expression in parallel with the number of mast cells in this study supports previous studies [42,43].

Capsaicin has been reported to cause an increase in IFN-level in experimental studies in mice [44]. In studies conducted on mice [45], and rats [46], it was found that the number of mast cells and IFN-expression increased in parallel. Cencic et al. reported that IFN- γ expression was not observed in the lamina epithelial of the non-pregnant porcine uterus, and positivity was detected with the onset of pregnancy [47]. Platt et al. [14] showed that IFN-immune reaction was observed in uterine glands and lamina epithelialis during oestrus stages and increased staining intensity with pregnancy. These findings suggest that the expression of the IFN proteins in the endometrium, myometrium and perimetrium may differ, depending on the capsaicin applied. Furthermore, the expression of IFN- γ by the endometrium, myometrium and perimetrium even under normal conditions, suggests their potential role in normal uterus function. Moreover, the higher expression of IFN- γ in the uterus suggests that IFN- γ probably has an important protective function during the decidualization process of the endometrial stroma.

CONCLUSION

In conclusion, capsaicin was shown to have an effect on the expression of TNF- α , IFN- γ and tryptase in pubertal and adult rat uterus. The results confirmed that capsaicin could stimulate expression of TNF- α , IFN- γ and tryptase in the different development periods of rat uterus. It is thought that mast cells, which can affect physiological, immunological, and hormonal mechanisms in the organism, are effective against foreign factors that may affect the uterus. In the presented study the high expression of TNF- α and IFN- γ and mast cell number were observed in capsaicin group. These findings suggest that chemical substances such as capsaicin may have indirect effects on uterine tissue. As

a result, this study revealed the interaction between capsaicin, TNF- α , IFN- γ and mast cells in the uterus. It is thought that the findings we have obtained will contribute to more detailed studies to be carried out with biochemical, immunohistochemical and more advanced techniques. And future studies should be directed towards understanding the function of capsaicin in uterus physiology with mast cells.

Conflict of Interest. “The authors declared that there is no conflict of interest.”

Authorship Contributions. Concept: Ş.T., Design: Ş.T., Data Collection or Processing: Ş.T., M.Y., Analysis or Interpretation: Ş.T., T.E., Literature Search: Ş.T., T.E., Writing: Ş.T., T.E.

Financial Disclosure. This research received no grant from any funding agency/sector.

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