Short Paper

Immunohistochemical expression of ghrelin in capsaicin-treated rat ovaries during the different developmental periods

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Summary

Red hot pepper is a plant that belongs to the Solanaceae family and is known as *Capsicum annuum*. Capsaicin is the active ingredient of cayenne pepper. Ghrelin is a hormone, which consists of polypeptide structure. Ghrelin also contributes to growth hormone secretion, energy balance, food intake and body weight regulator. The aim of this study was the localization and expression of ghrelin in the ovaries of rats treated with capsaicin during the postnatal development. Ninety female Sprague-Dawley rats (21 d) were used. The rats were randomly divided into 3 groups (n=30 each) as pubertal, post pubertal and adult. Each group was subdivided into three groups. The first subgroup (control) was given no injections. The second subgroup (vehicle) received only 0.3 cc solvent and the third subgroup (experiment) received subcutaneous injection of equal volume of capsaicin (1 mg/kg/d) for 42, 56, and 70 days. Ghrelin immunoreactivity was determined in ovarian follicular granulosa cells, interstitial cells and corpus luteal cells. A ghrelin immunopositive reaction located in the cytoplasm of cells in all groups. These results indicate that prolonged administration of low dose capsaicin does not affect ghrelin expression. However, follicular atresia was seen in lower rate in capsaicin treated group in comparison to other groups.

Key words: Capsaicin, Ghrelin, Immunohistochemistry, Ovary, Rat

Introduction

Chili pepper is a plant which is a member of the Solanaceae family and is known as *Capsicum annuum*. Capsaicin is an alkaloid ($C_{18}H_{17}NO_3$) form substance that is bitter, caustic, white and odorless (Lopez-Hernandez *et al*., 1996). Capsaicin is affective on several systems in the organism, primarily on gastrointestinal, cardio-vascular and respiratory systems (Pyan *et al*., 1984; Kress *et al*., 1999). Rat ovaries receive neural stimuli along the symphatic, cholinergic, peptidergic and sensoric nerve fibers. Studies demonstrated that tachykinins like substance-P (SP), neurokinin A (NKA) and neurokinin B (NKB) have roles in forming reproductive functions (Traurig *et al*., 1988; Patak *et al*., 2000). Kojima *et al*. (1999) first identified ghrelin in mice stomach in 1999. It is a hormone in polypeptide form secreted by endocrine cells in the stomach (Kojima *et al*., 1999; Miller *et al*., 2005). However, in studies using gene expression in humans and rats, ghrelin and its receptor were observed in several organs such as bowels, heart, kidneys, lungs, pancreas, placenta, pituitary gland, gonads and brain (Papotti *et al*., 2000; Kojima *et al*., 2001; Gnapavan *et al*., 2002). Fernandez *et al*. (2004) stated that ghrelin in prepubertal rats reduced LH secretion significantly and did not affect follicle stimulating hormone (FSH) secretion. As is known, LH is secreted from frontal hypophysis gonadotrop cells, and stimulates estrogen formation and secretion from granulose, interstitial and corpus luteum cells (Yılmaz, 1999). Therefore, it has been postulated that ghrelin has an indirect effect on oogenesis while it has a direct effect on hypophysis in LH secretion regulation.

This study aims to investigate the possible changes on ghrelin expression in the ovaries of rats with different phases of development that were administered capsaicin.

Materials and Methods

Ninety 21 days-old female Sprague-Dawley rats were studied. The animals were divided into three main groups, as puberty (42 d), post puberty (56 d) and adult (70 d) and they were also further divided into three sub-groups, as experimental, vehicle control (which was administered 10% tween, 10% ethanol, 80% distilled water solution) and control groups. Rats were fed with standard rat chowand drinking water *ad libitum*, kept under 12 h light and 12 h darkness and in an environment with a temperature of 21-23°C and 50-60% humidity. Experimental procedures were approved by the Uludag University Ethical Committee for Animal Experimentation (Protocol Number: 2015-06/07). Rats were live-weighed each time prior to capsaicin injection to determine the capsaicin amount to be administered. Experimental groups were administered capsaicin subcutaneously (1 mg/kg/d) during their stated periods

until 42, 56, and 70 days; vehicle groups were administered only vehicle solution and control groups were not administrated capsaicin.

All animals were sacrificed one day after the last injection. Specimens were fixed in 10% formalin and routinely processed using standard procedures and then stained with crossmon's trichrome staining (Crossmon, 1937).

Immunohistochemical staining

Rabbit polyclonal ghrelin primary antibody was used for immunohistochemical staining. As a secondary antibody, Histostain Plus IHC Kit was used. The sections were submitted to deparaffin process and permeabilized in citrate buffer solution at 700 watt power for proteolysis. The sections were then washed in phosphate buffer solution (PBS) and endogenous peroxidase activity was blocked by 3% H_2O_2 at room temperature. After washing with PBS, the sections were blocked with rabbit serum for 30 min, followed by incubation with ghrelin antibody 1/1000 at 4°C overnight. After washing, the sections were incubated in biotinylated secondary antibody for 30 min. Then, the sections were washed, and incubated in streptavidin-HRP complex for 30 min. Antibody binding was detected with a 3,3'-diaminobenzidine kit, and the sections were counter-stained with haematoxylin.

Results

Histological findings

Crossmon's trichrome staining revealed that ovaries from all groups were encircled with germinal epithelium and tunica albuginea underneath. In cortex, follicular (primordial, primary, secondary, graff and atretic) interstitial cells and corpus luteums were observed in different phases of growth. In medulla, within connective tissue, blood veins and connective tissue cells in different sizes were observed (Fig. 1). Although follicle count was not performed, general examination of slides showed no difference between the control, vehicle and experimental groups 42 days-old rats for the follicle density. While atretic follicle numbers were observed more in control and vehicle groups, developing follicle density was observed to be higher in the experimental group. It has been established that atretic follicle density in the control group was higher than the vehicle group (Figs. 1A, B, C). Upon examination at day 56 there was no difference for the developing follicle density levels between groups. Atretic follicle density was decreased, respectively (Figs. 1D, E, F). In 70 days-old rats, there was no significant difference in three groups for the developing and atretic follicle densities. In all groups, corpus luteum numbers were increased (Figs. 1G, H, I).

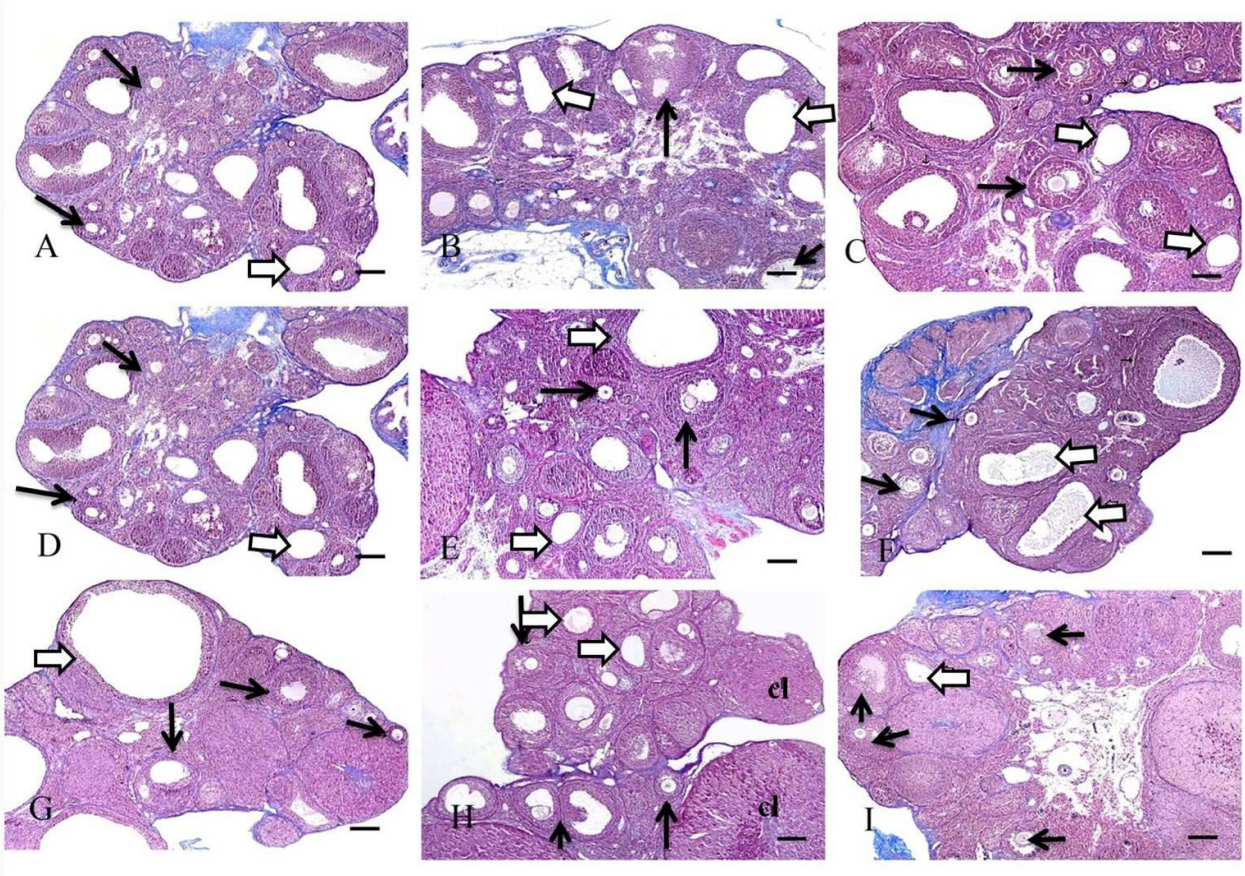

Fig. 1: Histological structure of ovary in experimental groups. ⇨ : atretic follicle, ↑ : developing follicles, cl: corpus luteum. Crossman's triple stain, A, B, C: 42 d; D, E, F: 56 d; G, H, I: 70 d, (bar: 50 µm)

Immunohistochemical findings

Immunohistochemical expression of ghrelin was implemented by the evaluation of ovary slides, and by observing reaction densities of the granulosa cells in follicles, interstitial cells, theca follicle cells and corpus luteal cells from all groups and sub-groups. Evaluation is executed by independent observers by assigning points between 0 and 3, depending on non-staining (-), weak staining (+), moderate staining (++) and intense staining (+++) properties (Tables 1, 2, 3) (Adams *et al*., 1999). Different strengths of staining were observed in all three main groups. No staining was observed in negative control slides (Fig. 2). Rats in the main group at day 42 were evaluated within the group. Staining densities in interstitial and granulosa cells were found to be similar and at moderate level in control group. In the experimental group, reaction density in granulosa cells was moderate, while in interstitial cells and theca follicles staining was weak. No corpus luteum was detected in this group (Fig. 3A, Table 1). In corpus luteal cells and theca follicles cells, staining density was determined to be weak (Fig. 3B). Vehicle group evaluation showed that, while reaction density was moderate in granulosa cells, theca follicles and corpus luteal cells, the reaction in interstitial cells was lower (Fig. 3C). In the group of 56 days rats staining intensity in experimental group the most intense reaction was observed in corpus luteal cells; the reaction was dense in granulosa and interstitial cells and theca follicles (Fig. 3D, Table 2). In the control group was moderate in corpus luteal cells, where it was denser in granulosa cells. In the same group, in interstitial cells and in theca follicle cells reaction density was similar and weak (Fig. 3E). In the vehicle group reaction density was moderate in granulosa cells, weak in interstitial cells and corpus luteal cells, and very weak in theca follicles (Fig. 3F). At

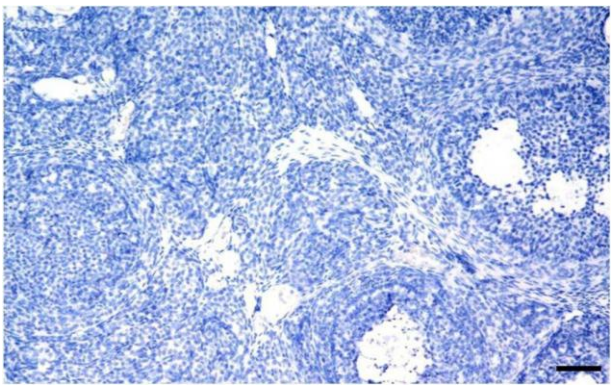

Fig. 2: Ghrelin immunostaining negative control slides, (bar: 50 µm)

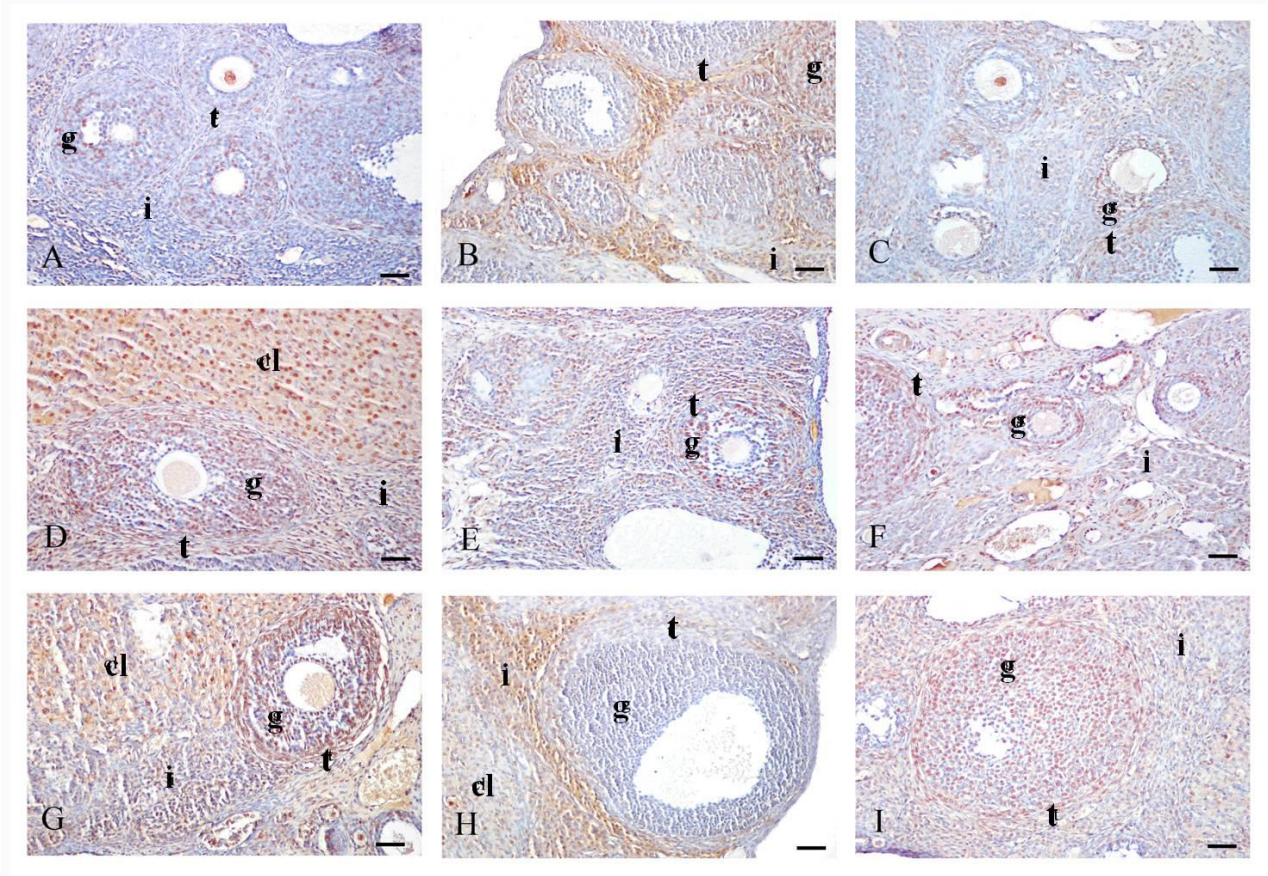

Fig. 3: Ghrelin immunostaining in the ovarian cells from experimental group during the puberty period (42 d) (A), the postpuberty period (56 d) (D), and in adult rat (70 d) (G). Vehicle group rats during the puberty period (42 d) (C), the postpuberty period (56 d) (F), and in adult rat (70 d) (I). Control rats during the puberty period (42 d) (B), the postpuberty period (56 d) (E), and in adult rat (70 d) (H). g: granulosa cells, i: interstitial cells, t: theca follicle cells, and cl: corpus luteal cells, (bar: 25 µm)

Table 1: Intensity of ghrelin immunostaining in ovary cells 42 d group rats

42 d	Granulosa cells	Interstitial cells	Theca cells	Corpus luteal cells
Control	++	++	+	+
Vehicle	++	+	++	++
Experimental	++	+	++	–

(–) no staining, (+) weak, and (++) moderate

Table 2: Intensity of ghrelin immunostaining in ovary cells 56 d group rats

56 d	Granulosa cells	Interstitial cells	Theca cells	Corpus luteal cells
Control	++	+	+	++
Vehicle	+++	+	±	+
Experimental	++	+	++	+++

(–) no staining, (+) weak, (++) moderate, and (+++) strong

Table 3: Intensity of ghrelin inovary cells 70 d group rats

70 d	Granulosa cells	Interstitial cells	Theca cells	Corpus luteal cells
Control	+	++	+	+
Vehicle	++	+	+	++
Experimental	+++	+	+	+

(+) weak, (++) moderate, and (+++) strong

day 70, in the experimental group, intense reaction density was observed in granulosa cells, it was measured weak in corpus luteal cells, interstitial cells and theca follicle cells (Fig. 3G, Table 3). Although the reaction density was low in the control group, granulosa cells, corpus luteal cells and theca follicles, it was moderate in interstitial cells (Fig. 3H). Finally, in the vehicle group, moderate reaction strength was observed in granulosa cells and corpus luteal cells, while poor reaction was obtained in interstitial and theca follicle cells (Fig. 3I).

Discussion

Capsaicin is known to affect many systems in the organism, including nervous, cardiovascular, respiratory, immune, gastrointestinal systems and reproductive system (Tutuncu and Ozfiliz, 2011). Little is known about the effects of capsaicin on the female reproductive system. Many researchers used high doses of capsaicin as a specific tool to examine the function of sensory neurons in the reproductive system (Moran *et al*., 2003; Nance *et al*., 1987). Literature review shows ghrelin and its receptor exist in several organs such as intestine, heart, kidney, lung, pancreas, placenta, hypophysis, gonads and brain (Pyan *et al*., 1984; Lopez-Hernandez *et al*., 1996; Kress *et al*., 1999). Ghrelin is found in human, rat, pig, sheep, and chicken ovary (Caminos *et al*., 2003; Sirotkin *et al*., 2006). Expression of the functional ghrelin receptor has been reported in oocytes as well as follicular, luteal, and surface epithelium and interstitial hilus cells in rat ovary (Caminos *et al*., 2003; Dupont *et al*., 2010).

Developing follicle density was higher in 42 and 56 days-old rats in experimental groups than the other two groups. In the group of 70 days, there was no difference between three groups for the follicle density. Contrary to our findings, Moran *et al*. (2003) showed that high doses of capsaicin administration to newborn rats caused a decrease in follicle count in ovaries and an increase in atretic follicle count. The difference between our finding and the findings of Moran *et al*. (2003) could be due to duration of administration and the dose of capsaicin. In our study immunohistochemical staining for the presence of ghrelin antibody revealed certain differences, in terms of staining density between groups and sub-groups for the interstitial and granulosa cells, corpus luteal cells and cells in theca follicles. Similar to our finding Miller *et al*. (2005) demonstrated that, follicles in different growth stages were positive for ghrelin in granulosa and theca cells and in corpus luteum. Furthermore, Cominos *et al*. (2003) have demonstrated that ghrelin is expressed in the rat ovary and corpus luteum. In their study, Cominos *et al*. (2003) showed that ghrelin induced estradiol secretion in prepubertal rat ovaries (Rak *et al*., 2008) because of elevated number of granulosa cells. Similar to these findings, in the present study the highest ghrelin expression, during the prepubertal period, was detected in the granulosa cells indicating increased estrogen secretion due to elevated number of granulosa cells.

In conclusion, this study demonstrates *in vivo*, the localization and expression of ghrelin in ovary tissues and analyzes capsaicin-ghrelin interaction. Ghrelin expression was displayed in the ovaries of all groups in granulosa cells, interstitial cells, theca follicle cells and corpus luteal cells. Ghrelin expression in all groups shows that low-dose administration of capsaicin for long periods does not inactivate ghrelin, in addition, the fact that follicle atresia reduced in experimental groups that were administered capsaicin and the immunoreactions were denser, indicating that capsaicin may affect pituitary-gonad axis and having a positive effect on the growth of gonads.

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