The Effect of Capsaicin on IGF-I and IGF-IR Expression in Ovarian Granulosa Cells

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ABSTRACT. Capsaicin (trans-8-methyl-N-vanillyl-6-noneadamide) is a pungent ingredient in red peppers from the Capsicum family. Insulin-like growth factor-I (IGF-I) is expressed in granulosa cells and has an important role in ovarian development. However, there are no data about the IGF-I expression in ovarian granulosa cells after capsaicin treatment. The aim of this study was to investigate the expression of IGF-I and its receptor (insulin-like growth factor-I receptor [IGF-IR]) in primary rat ovarian granulosa cells after low and high doses of capsaicin treatment. For this, granulosa cells were isolated and cultured from ovaries of 30-day-old female Sprague-Dawley rats. Granulosa cell plates were divided into four groups as cell control (C), vehicle control (V), and 50 µM and 150 µM capsaicin groups. In experimental groups, granulosa cells were exposed to capsaicin for 24 hours and immunocytochemistry was performed afterwards using anti-IGF-I and anti-IGF-IR antibodies. Both IGF-I and IGF-IR expressions were found to be significantly increased in parallel to the capsaicin doses. Elevated levels of IGF-I may be a risk factor for ovarian development. Because of the crucial role of IGF-I in ovary development, capsaicin treatment can be effective on follicular development and/or disorders characterized by high IGF-I levels.

Keywords: ovary, capsaicin, IGF-I, IGF-IR

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INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a basic peptide comprised of 70 amino acids and promotes differentiation of various cell types with its anti-apoptotic activity and anabolic effect (Daughaday and Rotwein 1989). In addition to ubiquitous distribution in various tissues, the ovary is a major site of IGF-I production in mammalians (Daughaday and Rotwein 1989). IGF-I is highly expressed in the ovary and its mRNA is concentrated in all stages of developing follicles’ granulosa cells (Kadakia et al., 2001). IGF-I and its receptor (IGF-IR) are important factors that regulate ovarian cells’ proliferation and differentiation as well as follicular development and ovulation (Armstrong and Webb 1997; Zhou et al., 2013; Baumgarten et al., 2017). IGF-I enhances FSH-stimulated estrogen and progesterone production by increasing steroid biosynthetic enzyme activities and induce LH receptors (Davoren and Hsueh 1884; el-Roeiy et al., 1993). In vitro studies demonstrated that IGF-I stimulates granulosa cell proliferation in the ovary of rat (Adashi et al., 1985), human (Wood et al., 1993), pig (Xia et al., 1994), sheep (Campbell et al., 1995) and cow (Armstrong et al., 1996; Stubbs et al., 2013). IGF-I null mice are infertile with an arrest at the preantral follicle stage similar to FSHβ- and FSHR-deficient ovaries (Baker et al., 2000). In addition, IGF-IR stimulates the development, transformation and differentiation of cells (Baserga 1995 and 2000; Chen and Sharon 2013). Previous studies showed that removal of the cell membrane IGF-IR by the abolition of the IGF-IR gene, suppression of cell expression or inhibition of function could lead to cell transformation (Baserga 1995; Baumgarten et al., 2017).

Capsaicin (CAP) is the pungent ingredient in hot chili peppers of the family Capsicum. It is widely consumed as food additive and topical analgesic (Surh and Lee 1996; Arora et al., 2011). Besides, CAP is currently being utilized for therapeutic treatment of various clinical conditions such as pain relief, rheumatoid arthritis, diabetic neuropathy, obesity, cardiovascular and gastrointestinal conditions (Josse et al., 2010; Sharma et al., 2013). CAP excites sensory neurons by binding to its receptor (TRPV1- capsaicin-sensitive receptor transient receptor potential, vanilloid type 1), localize on primary afferent neurons (Wardle et al., 1997; Nagy et al., 2004; Nakagawa and Hiura 2006). CAP-sensitive sensory neurons are nociceptive neurons that are known to activate ligand-gated, nonselective cation channels such as CGRP, substance P (SP) and neurokinin A (Jessell et al., 1978; Saria et al., 1987). Some researchers have suggested that CAP-sensitive sensory nerves could play a role in regulating the fertility and follicle development in females (Traurig et al., 1984; Pintado et al., 2003). Little is known about the effects of CAP on the female reproductive system. Pintado et al. (2003) treated female rats neonatally with a high dose of CAP (50 mg/kg) and found that rats exhibited an apparently normal courtship behavior but a lower reproductive success and litter size compared with control animals. On the contrary, low dose CAP protected the follicles from apoptosis and atresia, and stimulated follicular development (Zik et al., 2010). Ozer et al. (2005) fed laying hens with a diet containing red hot pepper and demonstrated that follicular development was stimulated and laying performance was improved.

Previous studies have identified a link between the insulin family of trophic factors and TRPV1, showing that IGF-I sensitize TRPV1 receptors (van Buren et al, 2005). But to our knowledge, the mechanism of the effect of capsaicin in connection with IGF-I and IGF-IR in rat ovarian granulosa cells has not been reported. The objectives of this in vitro study, thus, were to examine (1) the expression of IGF-I and IGF-IR in rat ovarian granulosa cells, (2) effect of low and high dose CAP treatment on the expression of IGF-I and IGF-IR in rat ovarian granulosa cells.

MATERIALS-METHODS

Animals

Female Sprague-Dawley rats (30 days old) obtained from the Bursa Uludag University Experimental Animals Breeding and Research Center were used throughout the experiments. All procedures were performed after the approval of the Bursa Uludag University Animal Research Local Ethics Committee (Approval No. 2011-09/03). Handling and euthanasia of rats were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. The animals were housed five per cage at 20-24°C with a 60-70% humidity and 12/12 h light/dark cycle, and fed ad libitum. Euthanasia was performed by cervical dislocation after ether inhalation and the ovaries were harvested for culturing.

In Vitro Culture and Treatment

Granulosa Cell Culture

Granulosa cells were prepared as described previously with some modification (Uzumcu and Lin 1994; Zachow and Uzumcu 2006). Briefly, ovaries from the animals were rinsed in cold Hanks’ Balanced...
Salt solution (HBSS) medium (PAN-Biotech GmbH; P04-34500; Germany) supplemented with 1% 1,000 
U/ml penicillin G, 10 mg/ml streptomycin sulfate 
(Bio-Ind; 03-031-1B; CT, USA). Afterwards, the ova-
ries were cleaned of all connective tissues and fat and 
were moved into Dulbecco’s Modified Eagle Medi-
um/Nutrient Mixture F-12 (DMEM/F12) medium 
(Gibco™; 11039-021) supplemented with 10% fetal bovine serum (FBS) (Bio-Ind; 04-007-1A) and 0.1% 
1,000 U/ml penicillin G, 10 mg/ml streptomycin sul-
fate. Ovaries were punctured using a non-enzymatic 
needle puncture method with 27-gauge needle to ex-
trude granulosa cells and the extract was then filtered 
through a 70 µm filter. The cell suspension was cen-
trifuged at 200 g for 5 minutes, resuspended in a culture 
containing DMEM/F-12 medium supplemented with 
10% FBS, and 0.1%, 1,000 U/ml penicillin G, 10 mg/ 
ml streptomycin sulfate and then plated (Uzumcu and 
Lin 1994; Zachow and Uzumcu 2006).

The following day (day 0), the media were replaced 
with fresh media. Four groups were assigned: (1) cell 
control group (C), (2) media containing vehicle solu-
tion (0.01% DMSO; Ambresco; N182) for vehicle 
control (V), (3) low dose (50 µM) and (4) high dose 
(150 µM) of CAP (Sigma-Aldrich; M2028) diluted in 
vehicle solution. The cells were cultured at 37°C in 
a humidified atmosphere of 5% CO₂. Twenty-four hours 
after treatment, the experiments were terminated.

Immunocytochemistry
Granulosa cells were grown on coverslips in 24-
well plates. The cells on the coverslips were washed 
three times in PBS. The cells were then fixed in 4% 
paraformaldehyde at room temperature for 15 min and 
permeabilized with 0.1% Triton X-100 for 10 min. The 
cells were blocked (Vector Lab., MP7401) for 20 
min and incubated with anti-IGF-I (1:200), and anti-
IGF-IR (1:250) antibodies at 4°C overnight and with 
the secondary antibody (Vector Lab.; MP7401) for 30 
min. Cells were then treated with 3,3’-diaminoben-
zidine (DAB) for 5 min, counterstained with Harris 
Hematoxylin for 2 min, and the slides were then ex-
amined under Nikon Eclipse 80i microscope at a mag-
nification of x400. Five microscopic areas were ran-
domly counted and the percent value of stained cells 
was calculated for each experiment groups by both 
investigators. The average of two readings was taken.

Immunofluorescence
The cells were blocked with 5% BSA (in PBS) for 1 
h followed by incubation with IGF-I (1:200) and IGF-
IR (1:250) antibodies at 4°C overnight. Cells were then 
incubated with bovine anti-rabbit IgG-FITC (Santa 
Cruz; sc2365) secondary antibody (1:200) for 1 h in 
a dark room. Then coverslips were mounted and cells 
were visualized under Nikon Eclipse 80i microscope.

Statistical Analysis
The data were analyzed using the IBM SPSS Sta-
tistics 22. The normality of the data were determined 
by the Shapiro-Wilk test. Statistical significance be-
tween the groups was analyzed by the Kruskal-Wal-
lis Test, followed by Mann-Whitney U posthoc test. 
Bonferroni correction was applied in order to control 
of alpha (α/k=0.008). All experimental data are ex-
pressed as mean±SD of three separate experiments, 
each carried out in replicate. A value of p≤0.05 was 
taken as statistically significant.

RESULTS
Granulosa cells in well plates were examined 24 h 
after CAP addition. Regular epithelioid structure and 
compact cell-cell interaction were observed in low 
dose CAP (50 μM) and control groups (Figure 1A). 
However, in the high dose group (150 μM), the struc-
ture of granulosa cells appeared deformed and cell-
cell interactions were disrupted (Figure 1B).

The expression of IGF-I and IGF-IR was observed 
in the cytoplasm of granulosa cells ; the intensity 
was more prominent in the perinuclear area (Figures 
2, 3). Rat ovarian granulosa cells with/without CAP 
addition were able to release the IGF-I and IGF-IR 
(Figures 2-4). Dose of CAP (50 and 150 µM) had a 
significant (p≤ 0.05) influence on IGF-I and IGF-IR 
expression. Highest number of cells expressing both 
IGF-I and IGF-IR was found after CAP treatment at 
the highest dose when compared to control and vehi-
cle groups (p≤ 0.05) (Figure 4).

There was no statistical significance between 
the control and the vehicle group (p>0.05) regarding 
IGF-I immunoreactive cells, while CAP treated 
groups had significantly higher immunoreactive cells 
compared to the control groups (p≤ 0.05) (Figures 2, 
4). Also, there is statistical significance between the 
groups administered dose 50 µM and 150 µM of CAP 
(p≤ 0.05) (Figures 2, 4). When IGF-IR results were 
evaluated, there was no significant difference between 
the control groups, but the difference was significant 
between the control and CAP groups difference of 
CAP groups. (p≤ 0.05) (Figures 3, 4). The number 
of granulosa cells expressing IGF-IR was more than 
IGF-I positive cells in all groups (Figure 4).
Figure 1. Morphological structure of ovarian granulosa cells A. Healthy ovarian granulosa cells (arrow) after 24 h, 50 µM CAP treatment, (Bar: 100µM ), B. Apoptotic ovarian granulosa cells (arrowhead) after 24 h, 150 µM CAP treatment, (Bar: 100µM).

Figure 2. A. Positive (arrow) IGF-I expression in ovarian granulosa cells; 24 h, 50 µM CAP treatment (IF method) (Bar: 25µM ). B. Positive (arrow) and negative (arrowhead) IGF-I expression in ovarian granulosa cells; control group (ICC method), (Bar: 50µM ), C. Positive (arrow) and negative (arrowhead) IGF-I expression in ovarian granulosa cells; 24h, 50 µM CAP treatment (ICC method), (Bar: 100µM ), D. Positive (arrow) and negative (arrowhead) IGF-I expression in ovarian granulosa cells; 24h, 150 µM CAP treatment (ICC method), (Bar: 50µM ).
Figure 3. A. Positive (arrow) IGF-IR expression in ovarian granulosa cells; 24 h, 50 µM CAP treatment (IF method) (Bar: 25µM ), B. Positive (arrow) and negative (arrowhead) IGF-IR expression in ovarian granulosa cells; control group (ICC method), (Bar: 25µM ), C. Positive (arrow) and negative (arrowhead) IGF-IR expression in ovarian granulosa cells; 24h, 50 µM CAP treatment (ICC method), (Bar: 25µM ), D. Positive (arrow) and negative (arrowhead) IGF-IR expression in ovarian granulosa cells; 24h, 150 µM CAP treatment (ICC method), (Bar: 25 µM ).

Figure 4. A. IGF-I expression, B. IGF-IR expression in ovarian granulosa cells. *, **, ***; difference between groups, p≤0.05. C, cell control ; V, Vehicle control.
DISCUSSION

Many growth factors have been reported to participate in ovary physiology (Sirotnik 2011). IGF-I is one of them that amplifies gonadotropin action in granulosa and theca interstitial cells by acting on the IGF-IR (Maestro et al., 1997). Stimulating effect of IGF-I on granulosa cell proliferation has been shown in many in vitro studies (Adashi et al., 1985; Campbell et al., 1995; Armstrong et al., 1996; Surh and Lee 1996). IGF-I stimulates initiation of preantral follicles development by revealing mRNA and protein levels. The presence of IGF-IR was demonstrated in the same study (Stubbs et al., 2013). Baumgarten et al. (2017) reported that IGF-I and IGF-IR expression were found in granulosa cells of follicles from the primary to the antral stage and expression of IGF-IR in granulosa cells is essential for reproduction and lack of IGF-IR leads to apoptosis in granulosa cells. IGF-I gene expression in cell type-specific in ovary, 10-fold greater abundance of IGF-I transcripts in granulosa cells as compared with theca-interstitial cells (Hernandez et al., 1989). IGF-I staining localization and intensity in granulosa cells in our study also support previous data.

Effect of capsaicin on various type of cells is influenced by the administered dose (Pintado et al., 2003; Ozer et al., 2005; Zhang et al., 2008; Zik et al., 2010; Alatriste et al., 2013). Many researchers have suggested that high dose of CAP affects steroidogenesis by creating degeneration in the sensory nerves of the hypothalamus-pituitary-ovarian pathway (Moran et al., 2003; Pintado et al., 2003; Alatriste et al., 2013). Moran et al. (2003) injected CAP (50 mg/kg, sc) at birth and in 3 day-old rats which resulted in a significant delay of puberty and first vaginal estrus, as well as lower preantral and antral follicles. The studies about the effect of the low dose of CAP are limited (Ozer et al., 2005; Zik et al., 2010). Zik et al. (2010) observed that low dose of CAP inhibits apoptosis and stimulates follicular development and proliferation of the granulosa cells. In addition, Ozer et. al. (2005) determined that low dose of red hot pepper (10 g/kg) added in diet of laying hens caused a significant increase in ovarian weight, follicle number and earlier onset of puberty compared to the control group.

In our previous studies, we found that low doses of CAP (10, 50 and 100 µM) increased cell proliferation in granulosa cells, but high doses (150 and 200 µM) induced apoptosis (Guler and Zik 2018). However, there are no studies on the expression of IGF-I and IGF-IR after CAP treatment on ovarian granulosa cells.

Many researchers have observed that IGF levels increase by applying toxic substances to ovarian cells (Halloway et al., 2007; Cansu et al., 2008; Ozden-Akkaya et al., 2017). Halloway et al., (2007) administered dichlorodiphenylchloroethylene (DDT), a pesticide that can negatively affect ovarian function, and investigated IGF expression in vivo in rat ovaries and in vitro primary culture of human granulosa cells. They observed that IGF-I expression was increased in parallel to the increasing concentration of DDT. Ozden-Akkaya and et al., (2017) injected methoxychlor to fetal and neonatal rats and they found that IGF-I expression increased in granulosa cells. On the other hand, the increase in IGF-I expression in interstitial cells was directly related to the polycystic ovary syndrome (Schildkraut et al., 1996; King et al., 2013). To our knowledge, the studies have been conducted on the expression of IGF-I and IGF-IR after CAP treatment on ovarian granulosa cells have not been reported. In some studies, the relationship between IGF-I and TRPV1 and IGF-IR and TRPV1 have also been demonstrated in different tissues and cells (Caprodossi et al., 2011; Li et al., 2013; Lilja et al., 2007). Li and et al. (2013) showed that IGF-I expression increased in tibia bone marrow and it is responsible for the up-regulation of TRPV1 expression and function in the peripheral nerves. In another study IGF-I and insulin enhance TRPV1 protein expression in neublastoma cell line (Lilja et al, 2007).

In our experiments, the cell culture lasted 24 h with two different doses of CAP. Our results showed that the higher dose of CAP increased number of cells expressing IGF-I and IGF-IR. The expression results of IGF-I in other studies is consistent with our results (Halloway et al., 2007; Cansu et al., 2007; Ozden-Akkaya et al., 2017). Several researchers observed co-existence of suppressed IGF-I expression and increased IGF-IR expression (Ozden-Akkaya et al., 2017). But in our study, number of IGF-I and IGF-IR immunopositive granulosa cells increased with increasing doses of CAP.

Our results show that IGF-I and IGF-IR expressions were CAP dose-dependent. As the application dose of CAP increased, IGF-I and IGF-IR expressions increased in parallel, but morphological deformation was observed in cells.
CONCLUSION

In conclusion, this study demonstrates for the first time that CAP, a widespread food additive, can increase the expression of the essential ovarian growth factor, IGF and its receptor IGF-1R. High dose of CAP may be a risk factor and result in adverse reproductive outcomes. Our results are expected to lead to a focus of future in vivo studies about ovarian infertility in connection with IGF and CAP treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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