TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2013) 43: 326-332
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doi:10.3906/sag-1205-120

Immunohistochemical distribution and gene expression of transforming growth factor alpha in ovarian tissue of rats treated with capsaicin in puberty*

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Received: 31.05.2012 • **Accepted:** 16.07.2012 • **Published Online:** 15.03.2013 • **Printed:** 15.04.2013

Aim: In this study, the effects of capsaicin on the immunohistochemical distribution and gene expression of transforming growth factor alpha (TGF-α) in ovarian tissue of rats at the pubertal age were examined.

Materials and methods: Rats (50 days old) were divided into experimental, alimentation, and sham groups. The experimental group was injected with 1 mg/kg doses of capsaicin (10% ethanol and 1% Tween in an 80% distilled water mixture) subcutaneously every day for a period of 1 week. The alimentation group received 1 mg/kg doses of capsaicin (10% ethanol and 1% Tween in an 80% distilled water mixture). The sham group was injected with a solution of 10% ethanol and 1% Tween in a mixture of 80% distilled water. Body weights and ovary weights were compared within the groups. Triple staining methods were used for the histological examinations. The tissue localization of TGF-α was analyzed using an immunohistochemical method and gene expression level was determined using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique.

Results: No difference in the structure of the 3 groups was observed histologically. Immunohistochemically, TGF-α immunoreactivity was detected in the primordial follicles, primary follicles, theca cells, and interstitial cells at a medium density in all groups. The effect of capsaicin on TGF-α with mRNA was not found to be significantly different among the 3 groups.

Conclusion: The results showed that capsaicin had no effects on ovaries at different developmental stages.

Key words: Capsaicin, TGF-α, ovary, RT-PCR, immunohistochemistry, rat

1. Introduction

Transforming growth factor alpha (TGF-α) is a small polypeptide whose biological effects and functional structure are similar to those of epidermal growth factor (EGF). It is roughly the size of 50 amino acids (1), and its molecular weight varies between 5 and 20 kDa depending on the secretion region (2). TGF-α synthesizes with induced macrophages, thrombocytes, keratinocytes, and certain other cells in the body. TGF-α is produced and oscillated by the theca interna cells in the ovary. It has been emphasized that TGF-α creates phenotypic change (which has been identified as reversible), exists in both the granulose and theca interna cells, and directs the follicular activity that arises (3,4).

Plants found in the wild are extremely beneficial for the field of medicine, which is constantly developing and advancing, and for the chemical industry. *Capsicum annuum* L., also known as paprika, is an important plant frequently used in chemistry, medicine, veterinary medicine, and the pharmaceutical industry. The active substance in *Capsicum annuum* L. is capsaicin, a substance that dissolves easily in acetone, methyl alcohol, ethyl alcohol, and warm water and is spicy, white, and odorless (5,6). Numerous experimental studies have been conducted on the effect of capsaicin (6–16). Some studies state that capsaicin decreases the level of liver and serum triglycerides and the amount of fatty tissue by increasing lipid peroxidation as well as by having an inhibitor effect on the glycogen metabolism of skeletal muscles in an in vitro environment (17,18). Studies also emphasize that capsaicin affects the cholesterol level in serum and decreases the risk of developing arteriosclerosis by decreasing blood, serum, cholesterol, and triglyceride levels (17).

The purpose of this study is to determine the effect of TGF-α on gene expression and the localization of immunohistochemicals in ovarian tissue by using the reverse transcriptase-polymerase chain reaction (RT-PCR) method to determine the gene expression of TGF-α in the

* This study was presented at the 11th National Histology and Embryology Congress, 16–19 May 2012, in Denizli, Turkey.

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ovarian tissue of rats that were administered capsaicin during puberty.

2. Materials and methods

This study, approved by the Animal Testing Local Ethics Committee of Kafkas University, was conducted on 45 female Sprague Dawley rats. Rats that were going through puberty (day 50) were included in the study. The rats were divided into 3 groups: experimental, alimentation, and sham. The rats were kept in standard cages at 22 ± 2 °C and were exposed to 12 h of sunlight and 12 h of darkness. The rate of capsaicin used in our study was determined based on the studies conducted by Moran et al. (19) and Tütüncü (20). For the rats in the experimental group, 1 mg/kg of capsaicin (Sigma, M2028) was dissolved with 10% ethanol and 1% Tween 20 (Merck, M8170772100) in a mixture of 80% distilled water on a daily basis. The rats were subcutaneously injected with this capsaicin mixture equal to their daily body weight at the same time every day for 1 week using an insulin injector. For the rats in the alimentation group, 1 mg/kg of capsaicin was dissolved with 10% ethanol and 1% Tween 20 in a mixture of 80% distilled water, and this mixture was added to their drinking water according to their daily body weight in order to make them drink. The rats in the sham group (n = 15) were subcutaneously injected with 10% ethanol and 1% Tween in a mixture of 80% distilled water. The rats were subcutaneously injected with this mixture based on their weight using an insulin injector. The weights of the rats were measured on day 50, during the puberty period. The rats from all 3 groups were weighed every day for a week before they were injected. After 1 week, the rats from all 3 groups were weighed, and then all were killed using ether anesthesia before removing their ovaries.

2.1. Tissue preparation for histological analysis

The ovarian tissue taken from the rats was identified in a 10% formaldehyde solution and 4-μm-thick serial sections were taken from the paraffin blocks. The sections were incubated in 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity after undergoing deparaffinization and rehydration. The maximum possible heat was applied in a microwave for 10 min in citrate buffer solution to reveal antigens after washing with phosphate buffer saline (PBS). Blocking solution A (Invitrogen Histostain plus Broad Spectrum (AEC) Ref. 85.9943) was applied to the sections to reduce the nonspecific binding of antibodies. Primary antibodies diluted with 1/50 PBS and an anti-TGF-α antibody (Merck (Ab-2) Mouse mAb (213-4.4) Calbiochem, Cat. No. GF10) were applied to the sections in a humid environment at room temperature for 1 h. Only PBS was applied to the tissues of the negative control group. The streptavidin–biotin peroxidase technique, one of the indirect methods, was used after primary antibody incubation (21). For this purpose, a broad-spectrum antibody (Invitrogen Histostain plus Broad Spectrum (AEC) Ref. 85.9943), counter to the type produced by the primary antibody, was applied to the sections and left at room temperature for 15 min. HRP streptavidin (Invitrogen Histostain plus Broad Spectrum (AEC) Ref. 85.9943) was applied to the sections, which were then incubated at room temperature for 15 min. After adding the AEC solution, the sections were placed under a light microscope to check for immunoreactivity and the reaction was deactivated with distilled water in accordance with the immunoreactivity status. The sections were then dipped in hematoxylin for negative staining and covered with a lamella using a water-based glue (Lab Vision, Large Volume Vision Mount, TA-060-UG). The samples were examined using a light microscope, and their photographs were taken. Scoring was conducted using a semiquantitative method on an area of the sections, based on the criteria of percentage of stained cells and degree of staining. The degree of staining was evaluated as follows: 0 (no staining), +1 (weak staining), +2 (moderate staining), and +3 (strong staining). Crossman's triple staining technique was also applied to the sections in order to illustrate which tissue was being studied, and to observe the tissue as a whole (22).

2.2. Gene expression

TRI Reagent, as defined by Chomczynski and Sacchi (23) and established as a result of modifications to the guanidine isothiocyanate/phenol-chloroform method, was used to obtain the total RNA. The RNA amount per microliter was measured using a spectrophotometer with a 260-nm wavelength. RNA integrity was observed using 1% agarose gel. Total mRNA was isolated using Oligo dT primers (Promega, C1101). The ideal temperature of these primers is 70 °C. The cDNA synthesis of the obtained mRNA samples was conducted using the following procedure. First, the master mixture that contains the Maloney murine leukemia virus reverse transcriptase (MMLV RT) enzyme was prepared with 8 μL of MMLV enzyme buffer (Promega, M1701), 8 μL of dNTP (Sigma, DNTP100-1KT), 1 μL of RNasin ribonuclease inhibitor (Promega, N2511), 1.6 μL of MMLV RT enzyme, and 6.4 μL of nanofiltrated water in each 25 μL aliquot of this mixture. A 25-μL aliquot of the MMLV RT master mixture was then added to every Eppendorf tube containing mRNA under a laminar flow hood. The mixture was kept at 37 °C for 1 h, 95 °C for 5 min, and 4 °C for 5 min. The forward primer sequence of the β-actin gene, used for control, was 5' to 3' TCA TGA AGT GTG ACG TTG ACA TCC GT, and the reverse primer sequence was CCT AGA AGC ATT TGC GGT GCA CGA TG. The forward primer sequence for TGF-α was 5' to 3' GCG CTG GGC TTC TCG TG, and the reverse primer sequence was TGG

AGA ACA GCA CGT CC (24). A 50-μL Taq mixture was added to tubes containing 2 μL of cDNA after the primer and Taq mixtures (5 μL Taq buffer, 1 μL dNTP, 1 μL forward primer, 1 μL reverse primer, 1 μL Taq enzyme (Sigma, D1806-250UN), and 41 μL nanofiltrated water) were prepared. The final mixture was kept at 94 °C for 5 min, 62 °C for 1 min, and 72 °C for 1.5 min in order for the DNA production process to take place. Mixtures were kept at these temperatures for the said times for 35 cycles and then were left at 4 °C after being kept at 72 °C for 10 min. The RT-PCR final products were examined using gel electrophoresis (75 min, 100 V) on a 1.5% agarose gel. The photographs taken of the obtained gel product under UV lighting were recorded on a computer to be used for assessment and statistical analysis.

3. Results

3.1. Live weight results

The weights of the rats going through puberty were measured in the experimental, alimentation, and sham groups throughout the application and the means of the live weights were calculated. The data obtained were used to determine whether or not there was a difference within the groups between day 0 and day 14 (Table 1). As illustrated in Table 1, there was an increase in the body weight of the rats in all 3 groups; however, the greatest increase was in the sham group. The increase in weight was greater in the alimentation group than in the experimental group.

3.2. Ovarian weight results

A Kruskal–Wallis analysis of variance was used to determine whether or not there was a difference in the ovarian weight of the rats in all 3 groups on day 14 (25) (Table 2).

As illustrated in Table 2, the mean ovarian weight of the rats in the experimental group was statistically significantly different from those in the alimentation and sham groups at a significance level of $P < 0.05$.

3.3. Histological results for the ovarian tissue

Normal histological results were obtained in the ovaries of rats in the experimental, alimentation, and sham groups at the conclusion of the microscopic analysis. No difference was observed in the histological structure of the rat ovaries in all 3 groups (Figure 1).

3.4. Immunohistochemical results

TGF-α staining was done in every group at the conclusion of the microscopic analysis. In the ovarian tissue of rats receiving capsaicin and going through puberty in the experimental group, there was moderate TGF-α immunoreactivity in the primordial follicle and the cytoplasm of the primary follicles (Figure 2). Similarly, moderate TGF-α immunoreactivity was observed in the primordial follicle and the cytoplasm of the primary follicles of rats in the alimentation and sham groups (Figures 3 and 4). TGF-α immunoreactivity, with a moderate staining degree of +2, was also identified in the theca cells and interstitial cells of all 3 groups (Figures 3, 5, and 6). There was no immunoreactivity in the sections set aside for the negative control.

3.5. Gene expression results

The expression levels of the β-actin genes, a control gene, and TGF-α in the ovarian tissue taken from the experimental, alimentation, and sham groups were measured using the RT-PCR method in order to identify the

Table 1. The mean weight (g) of rats in all 3 groups on day 0 and day 14.

Group	Number (n)	Day 0	Day 14	Difference (Day 14 vs. day 0)
Experimental	15	155.20 ± 14.46	159.40 ± 16.51	4.20
Alimentation	15	131.47 ± 19.10	136.93 ± 18.40	5.46
Sham	15	143.20 ± 22.65	159.47 ± 15.86	16.27

Table 2. The statistical difference tests of ovarian weight (g).

Group	Number (n)	Mean rank	Chi-square	P
Experimental	15	35.47	21.390	0.000
Alimentation	15	18.10		
Sham	15	15.43		

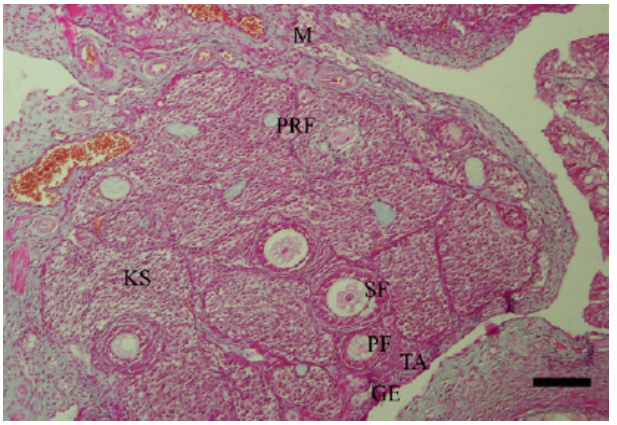

Figure 1. An ovarian section from an experimental animal on day 14. PRF = primordial follicle, SF = secondary follicle, TH = theca cells, TA = tunica albuginea, GE = germinative epithelium, M = medulla, and IH = interstitial cells. The triple staining method was used. Bar = 100 μm.

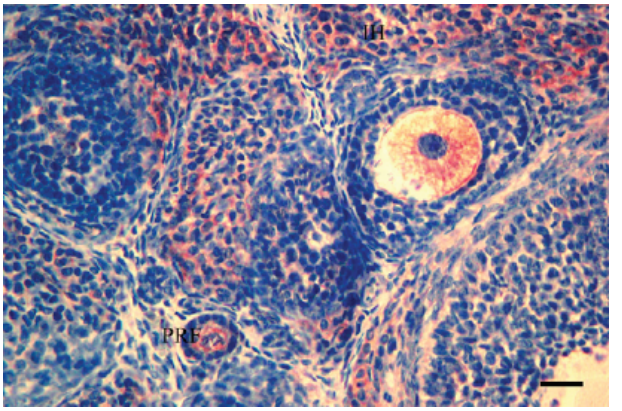

Figure 2. The TGF-α immunoreactivity in the primordial follicles and interstitial cells of ovarian tissue belonging to rats in the experimental group and going through puberty. PRF = primordial follicle, TH = theca cells, and IH = interstitial cells. Bar = 100 μm.

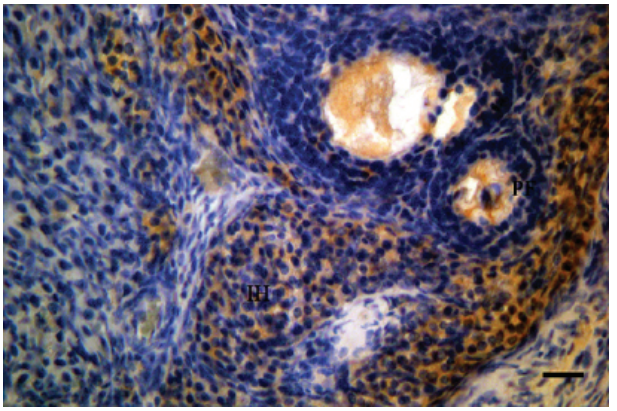

Figure 3. The TGF-α immunoreactivity in the primary follicles and interstitial cells of ovarian tissue belonging to rats in the alimentation group, receiving capsaicin, and going through puberty. PF = primary follicle and IH = interstitial cells. Bar = 50 μm.

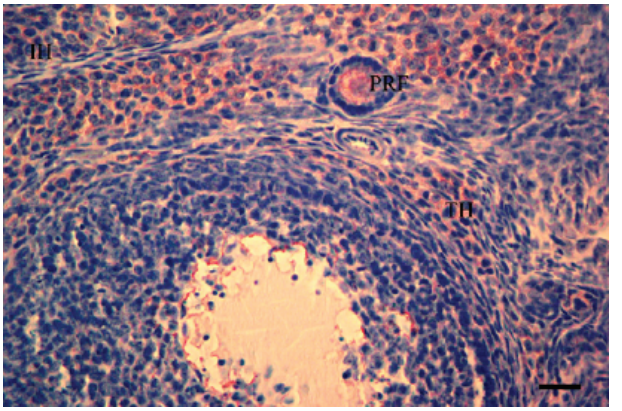

Figure 4. The TGF-α immunoreactivity in the primary follicles, theca cells, and interstitial cells of ovarian tissue belonging to rats in the sham group, receiving capsaicin, and going through puberty. PRF = primordial follicle, TH = theca cells, and IH = interstitial cells. Bar = 100 μm.

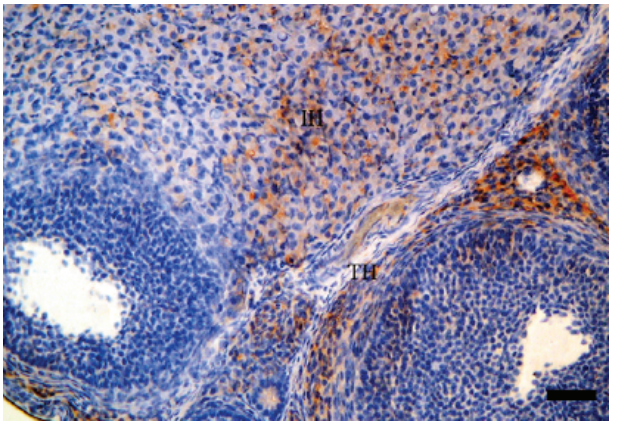

Figure 5. The TGF-α immunoreactivity in the theca cells and interstitial cells of ovarian tissue belonging to rats in the experimental group, receiving capsaicin, and going through puberty. TH = theca cells and IH = interstitial cells. Bar = 50 μm.

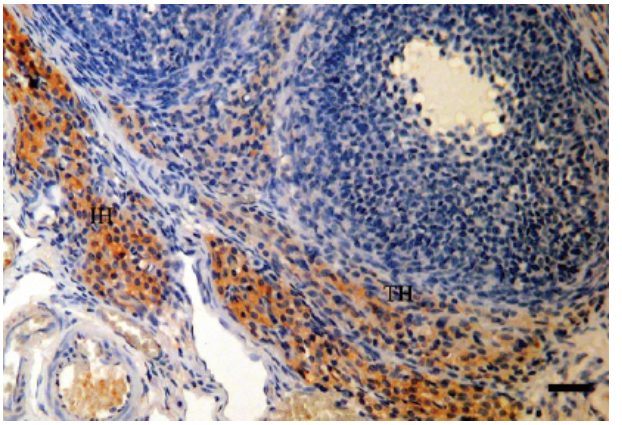

Figure 6. The TGF-α immunoreactivity in the primordial follicles and interstitial cells of ovarian tissue belonging to rats in the alimentation group, receiving capsaicin, and going through puberty. TH = theca cells and IC = interstitial cells. Bar = 100 μm.

effect of capsaicin. The values for TGF-α were normalized using β-actin. A statistical analysis was conducted on the measurements obtained to compare all 3 groups. TGF-α gene expression was identified in the ovarian tissue of all 3 groups; however, there was no statistically significant difference between the TGF-α gene expressions of all 3 groups (Table 3; Figures 7–9).

4. Discussion

The changes in body weight and ovarian weight, the changes in the histological structure of the ovarian tissue, the immunohistochemical localization of TGF-α, and the gene expression (measured using RT-PCR) of rats receiving capsaicin during puberty were analyzed in this study.

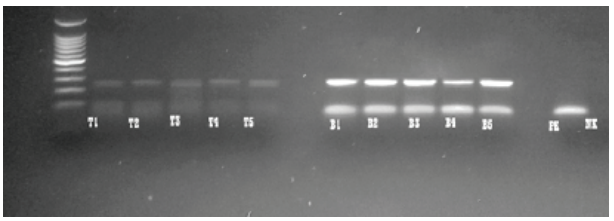

Figure 7. The TGF-α gene expression in the ovarian tissue of rats receiving capsaicin during puberty in the experimental group. L = 100-bp DNA ladder, T = TGF-α, B = β-actin, PK = positive control, and NK = negative control.

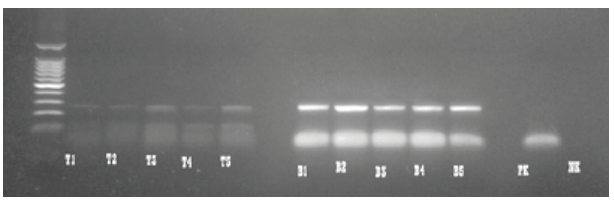

Figure 8. The TGF-α gene expression in the ovarian tissue of rats receiving capsaicin during puberty in the alimentation group. L = 100-bp DNA ladder, T = TGF-α, B = β-actin, PK = positive control, and NK = negative control.

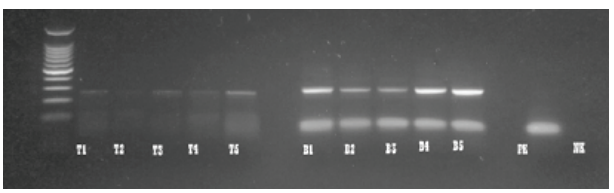

Figure 9. The TGF-α gene expression in the ovarian tissue of rats receiving capsaicin during puberty in the sham group. L = 100-bp DNA ladder, T = TGF-α, B = β-actin, PK = positive control, and NK = negative control.

Table 3. A comparison of TGF-α gene expression in the ovarian tissue of rats receiving capsaicin during puberty.

Group	Number (n)	Mean rank	Chi-square	P
Experimental	5	10.60		
Alimentation	5	6.00	2.780	0.249
Sham	5	7.40		

Tütüncü (20) reported that the body weight of rats receiving capsaicin in the experimental groups was lower in comparison to the rats receiving capsaicin in the other groups. Numerous studies support the view that capsaicin reduces the amount of body weight gain (7,10–13,18,19), increases capsaicin carbohydrate metabolism and the activity of liver enzymes, stimulates lipid metabolism, and makes lipid mobilization from fatty tissue easier (26). Our study concluded that there was an increase in the body weight of rats in the experimental, alimentation, and sham groups, which is similar to study results obtained by Srinivasan and Satyanarayana (7), Moran et al. (19), Kempaiah and Srinivasan (18), Özer et al. (10), Özgüden Akkoç (12), Erdost et al. (11), and İlhan (13). The greatest increase was recorded for the sham group, but the increase in the alimentation group was higher than the increase in the experimental group. In terms of the difference among groups, the difference was greater in the sham group in comparison to the 2 other groups.

In terms of ovarian weight, Özer et al. (10) concluded that while the body weight gain in roosters fed with paprika decreased, their testicle weight increased. The ovarian weight results of our study illustrated differences among the groups in line with the results obtained by Özer et al. (10) and Özgüden Akkoç (12). In terms of which treatment caused the difference, the results of the experimental group were higher in comparison to those of the alimentation and sham groups. Contrary to our results, Erdost et al. (11) and Tütüncü (20) emphasized that both the mean live weight gain and the mean ovarian weight of the experimental group during puberty decreased in parallel with one another. The difference between results can be due to the way in which the capsaicin was applied, the dose of the capsaicin, and the age of the animals.

Normal histological results were obtained in the ovaries of rats in the experimental, alimentation, and sham groups at the conclusion of the microscopic analysis. This proves that capsaicin did not create structural differences in all 3 groups.

In their studies, Akkoyunlu et al. (4) and Ozcakir et al. (27) proved that TGF-α exists in both granulosa and theca interna cells and in primordial follicles, and directed follicular activity. EGF and TGF-α bond with a common receptor, and both factors are seen in the ovarian follicles of humans. The role of these factors is to stimulate the reproduction of granulose cells (3). Our study shows similarities to numerous studies conducted in the past (4,26–29). In our study, there was positive TGF-α immunoreactivity, at different densities, in all

of the primordial follicles, primary follicles, theca cells, and interstitial cells of the ovarian tissue belonging to the experimental, alimentation, and sham groups. In our study, TGF-α immunoreactivity in the ovarian follicles of all 3 groups was identified by a moderate degree of staining. The fact that all 3 groups experienced the same density of TGF-α immunoreactivity is because the capsaicin, injected subcutaneously and given orally, did not show its full effect in a short period. In future studies, capsaicin may be injected subcutaneously or given orally for a longer period in order to identify the effect that TGF-α has on ovaries.

Similarly, Kudlow et al. (30), Skinner and Coffey (31), Yeh et al. (32), Harada et al. (33), and Pehlivan et al. (34) identified TGF-α in granulose cells of ovarian tissue belonging to rats using the RT-PCR method, and highlighted that TGF-α interacts with other growth factors. The RT-PCR method was also used in another study in which TGF-α was identified in granulose cells and theca cells (26). These results prove that TGF-α plays a role in follicle growth. Similar to the results of studies conducted by Kudlow et al. (30), Skinner and Coffey (31), Yeh et al. (32), Harada et al. (33), Pehlivan et al. (34), and Lobb (26), our study also identified TGF-α in ovarian tissue using the RT-PCR method. There was no statistically significant difference between the TGF-α density values identified in each group, leading to the conclusion that TGF-α in the ovarian tissue of rats receiving capsaicin via RT-PCR and immunohistochemically did not make a significant difference in the experimental, alimentation, and sham groups. In future studies, capsaicin should be applied over a longer period and at different doses in order to identify the changes that could occur at both the gene level and immunohistochemically.

In conclusion, this study investigated the effect of TGF-α in the ovarian tissue of rats receiving a low dosage of capsaicin (in the form of a subcutaneous injection or orally) on the live weight and ovarian weight, the histological structure of ovaries, immunohistochemical localization, and gene expression (together with RT-PCR). This study will form a basis for other future studies that may use advanced techniques, such as real-time and microarray techniques. In this study, the effect that capsaicin, applied at a low dosage, had on TGF-α in the ovarian tissue was analyzed both immunohistochemically and using the RT-PCR method.

Acknowledgment

This research was supported by a grant from the Scientific and Technological Research Council of Turkey (TÜBİTAK-TOVAG, project no. 109O829) and was summarized from PhD thesis of the first author.

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