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Impact of Carvacrol on Ovarian and Uterine Changes Induced by Hyperthyroidism in Rats *

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This study evaluates the effects of carvacrol, used in hyperthyroidism, on ovarian reserve as well as inflammatory and oxidative stress. In the study, thirty-five female Wistar albino rats were randomly divided into groups with an equal number of animals in each group: control, solvent, hyperthyroidism, carvacrol, hyperthyroidism+carvacrol. At the end of the study, oxidative stress (MDA, GSH) and inflammatory cytokine (TNF-α and IL-6) analyses were performed in ovarian and uterine samples, while hormone (T_4 and AMH) measurements were performed in the blood. In the hyperthyroidism group, ovarian and uterine MDA levels increased, GSH levels decreased, ovarian TNF-α and IL-6 levels increased, while AMH levels remained unchanged. In the oxidative stress and inflammatory state caused by hyperthyroidism, carvacrol treatment showed antioxidant and anti-inflammatory effects but did not cause any statistical difference in ovarian reserves. In conclusion, it is thought that carvacrol has beneficial effects on ovarian activities in hyperthyroidism. However, it would be helpful to investigate different doses and carvacrol treatment regimens and carry out clinical studies.

Key Words: *Hyperthyroidism, carvacrol, ovarian reserve, oxidative stress, inflammation*

Sıçanlarda Hipertiroidizm Kaynaklı Ovaryum ve Uterus Değişiklikleri Üzerine Karvakrolün Etkisi

Hipertiroidizmde kullanılan karvakrolün ovaryum rezervi ile yangısal ve oksidatif stres üzerine etkilerinin değerlendirildiği bu çalışmada *Wistar albino* cinsi dişi otuz beş sıçan her grupta eşit sayıda hayvan olacak şekilde randomize olarak gruplara ayrıldı: kontrol, çözücü, hipertiroidi, karvakrol, hipertiroidi+karvakrol. Çalışmanın sonunda alınan ovaryum ve uterus örneklerinde oksidatif stres (MDA ve GSH) ve inflamatuvar sitokin (TNF-α ve IL-6) analizleri yapılırken kanda hormon (T_4 ve AMH) ölçümleri gerçekleştirildi. Hipertiroidi grubunda ovaryum ve uterus MDA düzeyinde artış GSH düzeyinde azalmalar; ovaryum TNF-α ve IL-6 düzeylerinde artışlar görülürken AMH düzeyinde değişiklik olmadığı görüldü. Hipertiroidinin neden olduğu oksidatif stres ve yangısal durumda karvakrol tedavisi antioksidan antiinflamatuvar etki gösterirken ovaryum rezervleri üzerinde istatiksel anlamda farklılığa neden olmadığı görüldü. Sonuç olarak hipertiroidi durumlarında karvakrol ovaryum faaliyetleri üzerinde faydalı etkiler oluşturduğu ancak karvakrolün farklı doz ve tedavi rejimlerinin incelenmesinin ayrıca klinik çalışmaların yürütülmesinin faydalı olacağı düşünülmektedir.

Anahtar Kelimeler: *Hipertiroidizm, karvakrol, ovaryum rezervi, oksidatif stres, yangı*

Introduction

Thyroid hormones play essential roles in processes such as folliculogenesis and placentation (1). For example, thyroid hormones contribute to glucose transport in the ovaries (2). Triiodothyronine (T3) hormone increases the effect of estrogen by modulating follicle-stimulating hormone (FSH) and luteinising hormone (LH) (3). In addition, thyroid hormones contribute to follicle development by increasing FSH stimulation (4, 5). Moreover, thyroid hormones affect ovarian activities by paracrine effects. Thyroid diseases are known to be the second most common endocrine disorder in the reproductive age (6). A close relationship exists between thyroid and female reproductive diseases (7). Retrospective studies show that women with hyperthyroidism experience primary or secondary infertility (3). In hyperthyroid patients, plasma estrogen levels are high in all menstrual cycle stages (8). In addition, hyperthyroidism may lead to biochemical and hormonal abnormalities and menstrual irregularities (7). In hyperthyroid patients, plasma estrogen levels are high in all menstrual cycle stages (8). In addition, it has been reported that oocytes, cumulus and granulosa cells are adversely affected directly or indirectly in cases of hyperthyroidism in females (9).

A coordinated relationship exists between thyroid hormone levels, oxidative stress, and inflammation. Thyroid hormones may act as oxidants on their own and may cause DNA damage (10). In hyperthyroidism, an increase in free radical levels and oxidative stress are observed in various tissues (11, 12). Moreover, it has been reported that oxidative stress and inflammation are closely related, and oxidative stress stimulates

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inflammation (10). In addition, many inflammatory chemokines and cytokine levels are increased in hyperthyroidism (13). Carvacrol (2-methyl-5-(1-methylethyl)- phenol) is a monoterpenic phenol found in many aromatic plants (14). Carvacrol, with its rich hydroxyl groups, instills confidence in its therapeutic potential, offering anti-inflammatory, antioxidant, antitumour, antibacterial, antifungal, analgesic, antiparasitic and antigenotoxic effects (14-16). It is also reported that carvacrol decreases proinflammatory cytokines and lipid peroxidation levels and increases total glutathione levels (17).

This study aimed to investigate the effects of carvacrol on ovarian damage caused by hyperthyroidism. To this end, the effects of carvacrol on ovaries in terms of inflammation, oxidative stress, and ovarian reserves in hyperthyroidism were investigated.

Materials and Methods

Research and Publication Ethics: Upon approval by the local ethics committee of Hatay Mustafa Kemal University (Decision No: 2024/06-06), the animals were obtained from Hatay Mustafa Kemal University Experimental Research and Application Center and the study was conducted at the corresponding center.

Design and Sample Collection: The sample size calculation was performed using G*Power software version 3.1, which determined a minimum requirement of 35 rats. This calculation was based on a medium effect size of 0.42, with an alpha value of 0.05 and a power of 0.80. Thirty-five female Wistar albino rats (250±25 g, 6-8 weeks old) were randomly divided into five groups of seven animals each: control (C), vehicle (V), hyperthyroidism (HT), carvacrol (CAR), hyperthyroidism+carvacrol (HT+CAR). For the hyperthyroidism model, L-thyroxine (levothyroxine) (0.3 mg/kg/day p.o) was dissolved in saline and administered by intragastric gavage for 25 days (9, 18, 19). Carvacrol (100 mg/kg/day p.o) was dissolved in 25% DMSO and administered by gavage for 14 days starting on day 11 of the study (20).

At the end of the study, animals were sacrificed under xylazine-ketamine anaesthesia and blood samples were collected from the intracardiac ventricles. Ovaries and uteri samples were collected and stored until analyses were performed.

Thyroxine (T_4) and Anti-Mullerian Hormone (AMH) Analyses: Blood samples were centrifuged (3000 rpm, 5 min), and sera were collected (21). Hormonal analyses were performed on blood sera. Serum free thyroxine (T_4) levels were measured by electrochemiluminescence immunoassay (Atellica IM, Siemens Healthineers) and expressed as ng/dL.

Ovary and Uterus Oxidative Stress Analysis (MDA and GSH): For oxidative stress analyses, ovaries (right and left) and uteruses were homogenised using Tris-buffered saline (pH 7.4) and then centrifuged (4000 rpm 60 min), and supernatants were collected (22). Malondialdehyde (MDA) and glutathione (GSH) analyses were performed to determine the oxidant-antioxidant status of the ovary and uterus. Oxidant status was evaluated by measuring MDA levels at 532 nm as a biomarker of lipid peroxidation level (23). Glutathione levels were measured spectrometrically at 412 nm to evaluate the antioxidant status (24). In addition, total ovarian and uterine protein concentrations were measured using a BCA protein assay kit (Takara Bio. Inc., Japan). MDA and GSH levels were expressed as nmol/gr protein.

Ovarian Inflammatory Cytokine Analyses (TNF-α and IL-6): For each animal, the right and left ovaries were pooled and homogenized after reconstitution with phosphate-buffered saline (PBS) (pH 7.4) at a ratio of (1/10, weight/volume). The homogenate was centrifuged at 5000 x g for 5 minutes, and the supernatant was collected and measured. Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels were measured using enzyme-linked immunosorbent assay (ELISA) kits. In addition, total protein concentrations in the samples were measured using a BCA protein assay kit (Takara Bio. Inc., Japan). Results were expressed as pg/mg protein.

Statistical Analysis: Data were statistically analyzed in the study. Parametric test assumptions were applied before significance tests for all variables were obtained. The variables were analyzed using the Shapiro-Wilk test for normality and the Levene test for homogeneity. Then, a one-way analysis of variance (ANOVA) was used to control the statistical difference between the variables. Duncan's test was used as a post hoc test for the variables when the difference between the groups was significant. All statistical analyses were analyzed with a minimum margin of error of 5%. The IBM SPSS 23 was used for all statistical analyses. All results are expressed as mean ± standard error of the mean (SEM).

Results

Thyroxine (T_4) and Anti-Mullerian Hormone (AMH) Analyses: The T_4 levels in the HT group, which were the highest among all groups, were found to be 3.487±0.158 ng/dL. The T_4 levels in the other groups were 1.611±0.070, 1.600±0.123, 1.533±0.087, and 2.326±0.175 ng/dL for C, V, CAR, and HT+CAR groups, respectively. Furthermore, the HT and HT+CAR groups showed a statistically significant difference from the control group, while the V and CAR groups were similar to the control group (p<0.001). The T_4 levels of the groups are visually represented in Figure 1a.

The AMH levels of the groups (C, V, HT, CAR and HT+CAR) were 1.572±0.067, 1.560±0.061, 1.724±0.040, 1.575±0.035, 1.718±0.041 ng/mL, respectively. The highest AMH levels were observed in HT and HT+CAR groups. In addition, V, HT, CAR, and HT+CAR groups were similar to the control group (p=0.045). The AMH levels of the groups are shown in Figure 1b.

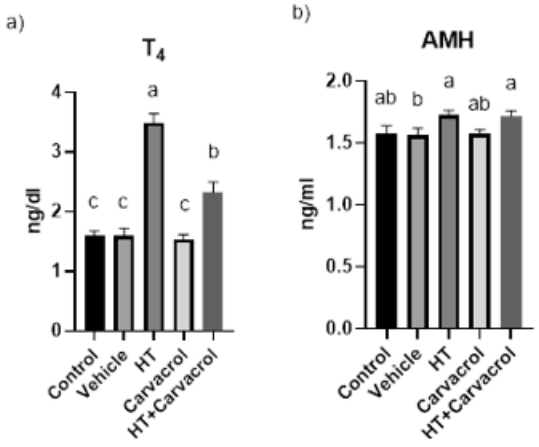

Figure 1. a. Serum-free T_4 levels **b.** Serum AMH levels. Values are expressed as Mean±Standard error of the mean. The letters shown in the columns indicate statistical differences. There is a statistical difference between different letters

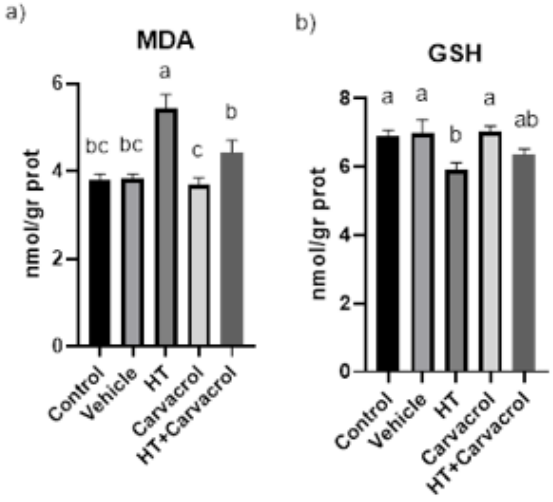

Figure 2. a. Ovarian MDA levels **b.** Ovarian GSH levels. Values are expressed as mean±standard error of the mean. The letters in the columns indicate statistical differences. There is a statistical difference between different letters

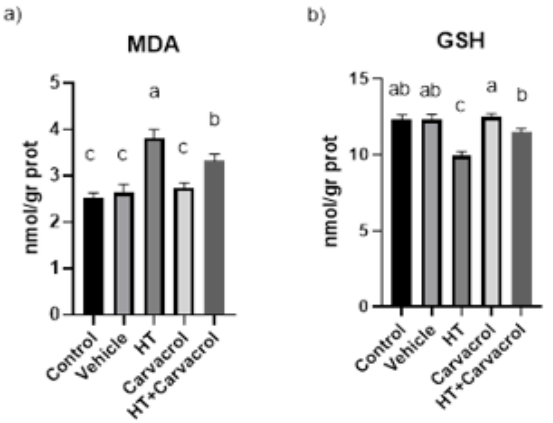

Figure 3. a. Uterine MDA levels **b.** Uterine GSH levels. Values were expressed as Mean±Standard error of the mean. The letters shown in the columns indicate statistical differences. There is a statistical difference between different letters

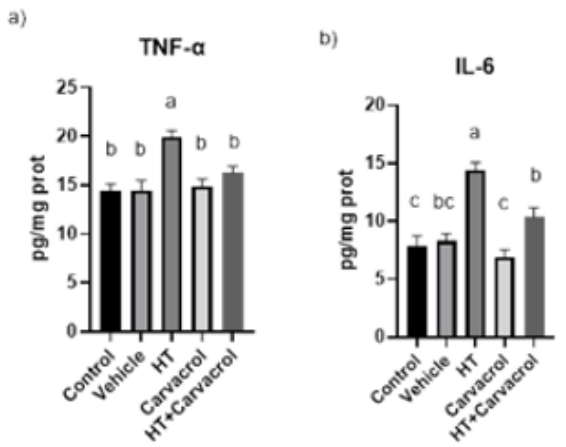

Figure 4. a. Ovary TNF-α levels **b.** Ovary IL-6 levels. Values are expressed as Mean±Standard error of the mean. The letters shown in the columns indicate statistical differences. There is a statistical difference between different letters

Ovarian MDA and GSH Analyses: The ovarian MDA levels of the groups (C, V, HT, CAR and HT+CAR) were measured as 3.792±0.141, 3.836±0.099, 5.431±0.319, 3.696±0.151, 4.434±0.271 nmol/g protein, respectively. The highest MDA levels were observed in the HT group. In addition, while V, CAR and HT+CAR were similar to the control group, the HT group differed from the control group (p<0.001). MDA levels of the groups are shown in Figure 2a.

The ovarian GSH levels of the groups (C, V, HT, CAR and HT+CAR) were measured as 6.900±0.175, 6.994±0.386, 5.900±0.232, 7.014±0.181, 6.367±0.154 nmol/g protein, respectively. The lowest GSH levels were observed in the HT group. In addition, while V, CAR and HT+CAR were similar to the control group, the HT group differed from the control group (p=0.009). GSH levels of the groups are shown in Figure 2b.

Uterus MDA and GSH Analyses: The uterine MDA levels of the groups (C, V, HT, CAR and HT+CAR) were measured as 2.517±0.117, 2.635±0.182, 3.818±0.186, 2.742±0.111, 3.318±0.152 nmol/gr protein, respectively. The highest MDA levels were found in the HT group. In addition, MDA levels were similar to the CAR control group but statistically different from the HT+CAR control group (p<0.001). The uterine MDA levels of the groups are shown in Figure 3a.

The uterine GSH levels of the groups (C, V, HT, CAR and HT+CAR) were measured to be 12.334±0.294, 12.309±0.360, 9.942±0.274, 12.487±0.206, 11.507±0.217 nmol/g protein, respectively. The lowest GSH levels were found in the HT group. In addition, while V, CAR and HT+CAR were similar to the control group, HT was statistically different from the control group (p<0.001). The uterine GSH levels of the groups are shown in Figure 3b.

Ovarian Inflammatory Cytokine Analyses (TNF-α and IL-6): TNF-α levels (C, V, HT, CAR and HT+CAR) were measured as 14.370±0.742, 14.428±1.050,

19.931±0.682, 14.890±0.742, 16.252±0.649 pg/mg protein, respectively. The highest ovarian TNF-α levels were observed in the HT group. In addition, V, CAR, and HT+CAR groups were similar to the control, whereas HT differed from the control (p<0.001). The TNF-α levels of the groups are shown in Figure 4a.

IL-6 levels (C, V, HT, CAR and HT+CAR) were measured as 7.879±0.847, 8.317±0.606, 14.411±0.664, 6.908±0.624, 10.352±0.824 pg/mg protein, respectively. The highest ovarian IL-6 levels were observed in the HT group. In addition, while V and CAR groups were similar to the control, HT and HT+CAR groups differed from the control (p<0.001). IL-6 levels of the groups are shown in Figure 4b.

Discussion

Hyperthyroidism, a condition known to cause hormonal disorders, menstrual irregularities, and abnormal ovarian activities in women (3), is closely linked to the hypothalami-hypophyseal thyroid (HPT) and hypothalami-hypophyseal gonadal (HPG) axes. These axes play a crucial role in regulating reproductive functions (25). Irregularities in the gonadotropin profile, a common occurrence in hypothyroidism, lead to changes in ovarian activities (26). In an experimental hyperthyroidism model in rats, disruptions in steroidogenesis, folliculogenesis, and ovulation were observed (27). A study on hyperthyroid rats revealed a decrease in estrogen levels (9). AMH, produced by granulosa cells of pre-antral and antral follicles, plays a vital role in follicular development (28) and is a key biomarker for evaluating ovarian reserves (29).

Increases in AMH levels were observed in thyroid autoimmune girls, and this was associated with the activation of inactive primordial follicles. It was stated that increased AMH may be a short-term reaction against autoimmunity (30). In other studies associating thyroid hormones with AMH, low TSH levels were associated with high AMH expression (31, 32). A study reported that the number of primordial, primary and secondary follicles increased in hyperthyroid rats while the size of primary and secondary follicles decreased (33). In addition, hyperthyroidism increases the number of secondary and tertiary follicles and decreases atretic follicles (34). In this study, it is thought that the increases in AMH levels in the hyperthyroidism group, even if not statistically significant, may have short-term effects and may be due to the effects of hyperthyroidism on ovarian metabolism.

Although the female reproductive system ages faster than the rest of the body, the quality and number of oocytes gradually decline, and ovarian reserves diminish with age (35). Oxidative stress and inflammatory processes accelerate ovarian ageing (36). A study of follicular fluid in women reported that oxidative stress and inflammation were associated with decreased ovarian reserve (37). In addition, hyperthyroidism is known to stimulate oxidative stress (19, 38). For example, free radical levels are known to be elevated in patients with Graves' disease (11). A study in rats reported that hyperthyroidism caused oxidative stress in the ovaries (9). Increases in MDA levels are observed in hyperthyroidism (39). This study observed statistical increases in MDA levels in the ovary and uterus in the hyperthyroidism group, consistent with previous studies. GSH is known to be one of the biological antioxidants against oxidative damage (40). In a study performed in rats, it was reported that GSH levels in various tissues decreased in hyperthyroidism (11). This study observed statistically significant decreases in ovarian and uterine GSH levels in the hyperthyroidism group. There is a close relationship between thyroid diseases and inflammatory mediators, and inflammatory cytokine levels increase in many thyroid diseases (13). For example, in a study conducted in mice, increases in TNF-α and IL-6 levels and decreases in anti-inflammatory levels, such as IL-10, were observed in hyperthyroidism (41). Similarly, in this study, it was observed that there were statistically significant increases in TNF-α and IL-6 levels in the hyperthyroidism group.

Carvacrol may have beneficial effects on female reproductive activity by affecting reproductive hormones in females (42, 43). Carvacrol administration was observed to increase estrogen levels in rats receiving radiation therapy (43). A study correlating carvacrol and ovarian reserve reported that carvacrol had a stimulating effect on ovarian reserve (20). Similarly, in another study conducted in rats, decreased AMH levels caused by radiation were restored to normal levels by carvacrol administration (43). However, in this study, it was observed that carvacrol administration did not cause a statistically significant difference in the ovarian reserves of both treated and healthy animals.

Carvacrol is a bioactive substance with antioxidant and anti-inflammatory effects (14, 44). A study has shown that carvacrol has antioxidant activity in radiation-induced ovarian damage (43). It has been reported that carvacrol treatment in rats causes antioxidant effects by reducing MDA and TOS and increasing TAS and total glutathione in ovarian ischemia and reperfusion damage (45). Similarly, carvacrol treatment in cisplatin-induced ovarian damage causes decreases in ovarian MDA levels and increases in total glutathione levels (20). In this study, similar to previous studies, although there was no statistically significant difference, carvacrol application was observed to have an antioxidant effect by reducing MDA and increasing GSH. It has also been shown that carvacrol causes anti-inflammatory effects by inhibiting inflammatory cytokine levels (45). In one study, carvacrol was observed to reduce the levels of many inflammatory cytokines in ovarian ischemia-reperfusion injury in a dose-dependent manner (46). Similarly, in another study, carvacrol treatment in ovarian injury reduced the levels of inflammatory cytokines such as NF-kB, TNF-α, IL-1b and IL-6 (20). Similarly, in this study, carvacrol administration was observed to statistically reduce the increases in inflammatory cytokine levels caused by hyperthyroidism.

In conclusion, carvacrol, used in hyperthyroidism, has been found to have promising beneficial effects through antioxidant and anti-inflammatory mechanisms. However, future studies investigating different treatment doses and clinical studies would help reveal the long-term effects of carvacrol.

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