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Effects of *Vitis vinifera* L. cv. "Merlot" Seed Extract Against Thallium (I) Sulfate-Induced Reproductive Damage in Male Rats

Erkek Sıçanlarda Talyum (I) Sülfatın Neden Olduğu Üreme Hasarına Karşı *Vitis vinifera* L. cv "Merlot" Tohum Ekstraktının Etkileri

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ABSTRACT

The aim of the present study was to determine the effects of *Vitis vinifera* L. seed extract against reproductive damage induced by thallium sulfate in rats. For this purpose, a total of 24 male rats, 6 rats in each group, were used for the study. In the control group, the rats were given 0.5% carboxymethyl cellulose followed by physiological saline. In the thallium sulfate group, the rats were given thallium sulfate (10 mg/kg) by intraperitoneal injection. In thallium sulfate + *Vitis vinifera* L. extract groups,The rats were administered thallium sulfate (10 mg/kg/intraperitoneal) + *Vitis vinifera* L. extract (100 mg/kg) peros. In *Vitis vinifera* L. extract group, the rats were given *Vitis vinifera* L. extract (100 mg/kg) peros. After the experimental process was finished, the rats were sacrificed and the blood and testicular tissues were taken. The results of our study found that the malondialdehyde level in the blood and testicular tissues of the thallium sulfate group was statistically higher than the other experimental groups ($P < .05$). Catalase, glutathione peroxidase activity, and glutathione level were the lowest in the thallium sulfate group ($P < .05$). In the histopathological examination, necrotic and degenerative findings are seen in the thallium sulfate group. Cleaved caspase-3 was strongly expressed in seminiferous tubules in the thallium sulfate group, but cleaved caspase-3 level was inhibited in thallium sulfate + *Vitis vinifera* L. extract group. Total motility value was found to be lower in the thallium sulfate group compared to the other experimental groups ($P < .05$). As a result, the biochemical, histopathological, and spermatological effects of *Vitis vinifera* L. extract administered as a peros against the deteriorated physiological functions in rats given thallium sulfate were determined.

Keywords: Cleaved caspase 3, oxidative stress, testis, thallium, *Vitis vinifera* L.

ÖZ

Bu çalışmanın amacı sıçanlarda talyum sülfatın neden olduğu üreme hasarına karşı *Vitis vinifera* L. tohum ekstraktının etkilerini belirlemektir. Bu amaçla çalışmada her grupta 6 adet olmak üzere toplam 24 adet erkek sıçan kullanıldı. Kontrol grubundaki sıçanlara %0,5 karboksimetil selüloz ve ardından serumm fizyolojik verildi. Talyum sülfat grubundaki sıçanlara intraperitoneal enjeksiyon yoluyla talyum sülfat (10 mg/kg) verildi. Talyum sülfat + *Vitis vinifera* L. ekstresi gruplarında sıçanlara talyum sülfat (10 mg/kg/intraperitoneal)+Vitis vinifera L. ekstraktı (100 mg/kg) peros uygulama yapıldı. *Vitis vinifera* L. ekstresi grubundaki sıçanlara *Vitis vinifera* L. ekstresi (100 mg/kg) peros verildi. Sonra deney süreci tamamlanarak sıçanlar sakrifiye edildi ve kan ve testis dokuları alındı. Çalışmamızın sonuçlarına göre kan ve testislerdeki malondialdehit düzeyinin Talyum sülfat grubunda diğer deney gruplarına göre istatistiksel olarak daha yüksekti ($P < .05$). Talyum sülfat grubunda katalaz, glutatyon peroksidaz aktivitesi ve glutatyon düzeyi en düşük seviyedeydi ($P < .05$). Histopatolojik incelemede talyum sülfat grubunda nekrotik ve dejeneratif bulgular görülmektedir. Talyum sülfat grubunda kaspaz-3 güçlü bir şekilde ifade edilirken talyum sülfat+-*Vitis vinifera* L. ekstrakt grubunda kaspaz-3 seviyesi inhibe edildi. Talyum sülfat grubunda total motilite değerinin diğer deney grupları ile karşılaştırıldığında daha düşük olduğu görüldü ($P < .05$). Sonuç olarak sıçanlarda Talyum sülfat tarafından bozulan fizyolojik fonksiyonlara karşı peros olarak uygulanan *Vitis vinifera* L. ekstraktı'nın biyokimyasal, histopatolojik ve spermatolojik iyileştirici etkileri olduğu belirlendi.

Anahtar Kelimeler: Kaspaz 3, oksidatif stres, testis, talyum, *Vitis vinifera* L.

INTRODUCTION

Thallium is one of the heavy metals and is highly toxic on living things.[1] Thallium exposure is more toxic than other metals such as arsenic, cadmium, lead, and mercury on different organisms.[2] Indirect and direct exposure to Thallium adversely affects tissues and organs in the body.[3] Environmental exposure to thallium sulfate (TS) in zebrafish increased the level of superoxide dismutase (SOD) and Na^+/K^- ATPase activities in the liver and caused histopathological changes in the gills.[4] Thallium sulfate decreased the cell viability and induces cell apoptosis in cultured C6 glioma cells.[5] Thallium increases the oxidative stress, and phospholipid peroxidation causes mitochondrial depolarization by changing mitochondrial membrane potential (MMP).[6]

Vitis vinifera, a species belonging to the Vitaceae family, has several health benefits, including immune-enhancing, anti-inflammatory, neuroprotective, antioxidant, anti-apoptotic, and anti-carcinogenic properties.[7] *Vitis vinifera* leaves contain many antioxidant compounds such as flavonoids, procyanidins, organic acids, and vitamins.[8] In a study of *Vitis vinifera* species, catecin, epicatecin, resveratrol, rutin, and quercetin were detected in the seed and the skin.[9] *Vitis vinifera* seed oil was given to rats with lead (Pb) toxicity and it was observed that the histological changes in testicular tissue were reduced.[10] Studies with *Vitis vinifera* seed show that it has a protective effect against the male reproductive system disorders.[11]

However, no study has been found examining oxidative findings, sperm quality, and histopathological changes in the testicular tissue in rats treated with TS. Therefore, the aim of this study was to determine the protective effect of *Vitis vinifera* L. extract (VVE) on the testicular tissue in rats treated with TS.

MATERIALS AND METHODS

Plant Materials, Extraction, and Chemicals

All chemicals and TS used in the study were obtained from Sigma-Aldrich (St Louis, Mo, USA).

Vitis vinifera L. cv. was obtained from Merlot grapes harvested at optimum maturity from "Doluca" winery in Tekirdağ and identified by an expert. The extract was prepared by modifying the method described by Kordalı et al.[12] The seeds were dried in the shade and ground into flour with the help of a blender. The sample taken (1 g/5 mL) was diluted at 24°C with ethanol (99.8%) and left for incubation. It was filtered to remove coarse particles. After 48 hours, it was evaporated to dryness with a rotary evaporator at 40°C in a vacuum and stored at 4°C until and for the duration of the experiment. Before oral administration, the extract was prepared by diluting it with distilled water containing 0.5% aqueous carboxymethyl cellulose (CMC) and administered to rats.

Animals and Ethical Approval

Approval for this study was obtained from the Animal Experiments Local Ethics Committee of the Atatürk University (Date: 30.10.2018, Number: 2018/10-194).

Thallium sulfate was prepared by dissolving it in 0.9% physiological saline 5 mL. In this study, 24 Sprague–Dawley rats weighing approximately 250-300 g, aged 8 weeks, were used. Rats are grouped as follows:

Group 1 (control): The rats received only vehicle 0.5% CMC intraperitoneally (IP) once orally saline for 11 days.

Group 2 (TS group): The rats were given TS (10 mg/kg) single dose via IP once.

Group 3 (TS+VVE group): The rats were given TS (10 mg/kg IP) single dose+after 30 minutes VVE (100 mg/kg via oral gavage) for 11 days.

Group 4 (VVE group): The rats were given VVE (100 mg/kg) via oral gavage for 11 days.

All rats were anesthetized by administering intramuscular xylazine (10 mg/kg IM) and ketamine (50 mg/kg) at the end of the twelfth day. While the rats were under general anesthesia, all rats were euthanized by decapitation.

Collection of Samples

Following sacrification, both testes tissue of the rats were removed from the body and cleaned from other connective parts. The right cauda epididymis was used for total motility analyses. Oxidative stress parameter levels were measured from the right testis tissues. Other testicular tissue was used for histopathological examinations. The blood taken from the hearts of the rats was centrifuged and the plasma portion was stored in a deep freezer at −20°C until the experiments.

Total Motility Evaluations

The ratio of forward total sperm motility was evaluated using a light microscope with a heated stage (35°C), as described by Aksu et al.[13] The cauda epididymis from rats was incubated for 5 minutes in 5 mL of physiological saline heated to 35°C in a petri dish. Then, the cauda epididymis was trimmed with the help of a scalpel and the semen fluid was obtained. The sperm motility was performed with a light microscope (Primo Star; Carl Zeiss) placed on a warming plate. Approximately 20 µL of sperm fluid was dropped on the slide and the sample was covered with a coverslip and examined at 400× magnification. The final score was written in %.

Measurement of Blood Oxidative Stress Parameters

Measurements were determined according to the following methods. Malondialdehyde (MDA) levels were measured by the method applied by Yoshioka et al.[14] Glutathione (GSH) levels were measured by the method applied by Tietze.[15] Catalase (CAT) levels were measured by the method specified by Goth.[16] Superoxide dismutase levels were measured by the method specified by Sun et al.[17] Glutathione peroxidase (GPx) levels were measured by the method specified by Matkovics.[18] Measurements were made with the help of Biotek ELISA Reader.

Measurements of Testicular Tissue Parameters

Tissue CAT activity was determined by the method described by Goth.[16] Tissue MDA activity was determined by Placer et al.[19] Superoxide dismutase activity was determined by the method applied by Sun et al.[17] Tissue GPx activity was determined by Matkovics.[18] Tissue GSH analyses were measured with the method applied by Fernandez and Videla.[20]

Histopathological Examination

One of the testes was taken and fixed in a 10% neutral formalin for 1-2 days. Then the paraffin blocking process was started. First, 5-µm thick sections were stained with hematoxylin–eosin and 10 randomly selected tubules were examined under 20× magnification. It was assessed histopathologically using mean testicular biopsy score (JTBS) criteria.

Immunohistochemical Examination

Immunohistochemical analysis was performed by modifying the method described by Ömür et al.[21] To determine apoptosis, polyclonal rabbit cleaved caspase 3 antibody (Cat no; NB600-1235, Novus Biological, USA) was used.

In Situ Hybridization

In situ hybridization was performed by the method described by Sevim et al[22] with the Detection System Biotinylated Probes kit (Dako, Cat no. K0601) as recommended by the manufacturing company. Briefly, after deparaffinization, the sections were incubated in Pepsin–HCL solution for 5 minutes and washed with phosphate buffered saline (PBS). After the hybridization step, the sections were covered with a coverslip and passed through the alcohol and xylol series. Entellan was dropped and examined under a light microscope.

Statistical Analysis

The number of animals in the group was determined according to the results of the power analysis using 80% power and 5% margin of error. The degree of significance was determined at ($P < .05$). One-way analysis of variance (ANOVA) and post hoc Duncan test were used to determine the differences among the groups for sperm and biochemical findings. Immunohistochemistry and in situ hybridization parameters were measured by non-parametric Kruskal-Wallis test (Kruskal–Wallis). Dual comparisons between groups with significant values were assessed using a Mann–Whitney *U* test ($P < .05$). All statistical analyses were made with the help of Statistical Package for the Social Sciences (SPSS) version 20.0 software (IBM Corp.; Armonk, NY, USA).

RESULTS

Total Motility Findings

When the total motility findings were examined, it was seen that group 2 had the lowest value and group 4 had the highest motility value ($P < .05$). No difference was observed between group 3 and group 4. The best progressive motility value was seen in group 4 (Table 1).

Blood Oxidative Stress Findings

Malondialdehyde level was significantly higher in group 2 compared to other experimental groups ($P < .05$). Glutathione peroxidase, CAT, SOD activities, and GSH levels were lower than the other groups ($P < .05$). In group 3, on the other hand, it is seen that the oxidant balance improved compared to group 2 (Table 1).

Testis Tissue Oxidative Stress Findings

Malondialdehyde level of group 2 was significantly higher compared to other groups ($P < .05$). There was no statistically significant difference in SOD level between all groups. Glutathione, CAT, and GPx levels were the highest in the group 4 (Table 1).

Histopathological Examination

The seminiferous tubules in group 1 and group 4 were observed to have a normal structure. It was observed that the regular structure of the spermatogenic cells in group 2 was disrupted, and in some parts, the seminiferous tubules became necrotic and degenerative. It was determined that the necrotic and degenerative changes observed in group 3 decreased compared to group 2 ($P < .05$) (Figure 1).

Immunohistochemical and In Situ Hybridization Examination

Cleaved caspase 3 expression was found to be very low in group 1 and group 4. In group 2, cleaved caspase 3 was expressed as severe in the seminiferous tubules, but in group 3, the expression of cleaved caspase 3 was decreased ($P < .05$) (Table 1, Figures 2 and 3).

DISCUSSION

Experimental studies have shown that the heavy metals have a toxic effect on the male reproductive system and impair endocrine and reproductive functions.[23,24] As a result of heavy metal exposure, it has been reported that sperm count, motility, viability, and testosterone levels decrease, while lipid peroxidation and apoptosis increase.[25] Thallium is one of the rare heavy metals. Thallium salts have been used as pesticides in Germany since 1920 and later used as a rodenticide.[26] A study by Formigli et al[27] showed that there was a significant decrease in sperm motility in rats given 10 ppm of TS. In our study, the low motility observed in group 2 is similar to this situation. Low motility shows that the administration of TS is at a toxic level. It is thought that TS affects spermatogenesis by crossing the blood–testicular barrier and has an effect on the epididymal maturation process.

Testicular damage caused by TS is mostly due to increased oxidative stress. Oxidative stress causes testicular damage in many

Table 1. Spermatological, Biochemical, Histopathological, Immunohistochemical, and In Situ Hybridization Parameters of All Groups (Mean ± SEM)

	Group 1 (Control)	Group 2 (TS)	Group 3 (TS+VVE)	Group 4 (VVE)
Total motility	49.28 ± 3.84^{b}	35.00 ± 1.54^{a}	65.00 ± 2.18^{c}	67.85 ± 1.01^{c}
Testis tissue GSH level (mmol/g)	0.49 ± 0.06^{b}	0.27 ± 0.07^{a}	0.53 ± 0.08^{b}	0.79 ± 0.03^{c}
Testis tissue MDA level (nmol/g)	28.48 ± 1.38^{a}	43.65 ± 3.22^{b}	25.92 ± 0.85^{a}	25.39 ± 0.77^{a}
Testis tissue CAT level (catalase/g)	268.43 ± 11.54^{b}	190.72 ± 17.53^{a}	278.94 ± 4.90^{b}	291.71 ± 2.13^{b}
Testis tissue GPx level (U/mg)	0.06 ± 0.00^{b}	0.02 ± 0.00^{a}	0.08 ± 0.00^{d}	0.09 ± 0.00^{c}
Testis tissue SOD level (U/mg)	12.80 ± 0.33	11.91 ± 0.24	12.70 ± 0.29	13.00 ± 0.24
Blood GSH level mmol/g	2.74 ± 0.08^{b}	1.42 ± 0.09^{a}	2.65 ± 0.11^{b}	3.44 ± 0.06^{c}
Blood MDA level (nmol/g)	10.20 ± 0.52^{b}	24.33 ± 1.27^{d}	15.32 ± 1.36^{c}	6.94 ± 0.57^{a}
Blood CAT level (kU/L)	170.90 ± 16.78^{b}	77.06 ± 4.12^{a}	189.09 ± 24.08^{b}	267.08 ± 8.61^{c}
Blood GPx level (U/mg)	$0.13 \pm 0.01^{b,c}$	0.03 ± 0.01^{a}	0.11 ± 0.03^{b}	0.14 ± 0.00^{c}
Blood SOD level (U/mg)	9.95 ± 0.20	7.25 ± 0.60	11.49 ± 0.21	24.72 ± 13.60
Johnsen's score	9.13 ± 0.51^{a}	7.23 ± 0.40^{b}	8.16 ± 0.51^{c}	9.00 ± 0.51^{a}
Immunohistochemistry	0.50 ± 0.54^{a}	2.62 ± 0.18^{b}	1.16 ± 0.18^{c}	0.33 ± 0.18^{a}
In situ hybridization	0.50 ± 0.54^{a}	2.50 ± 0.18^{b}	1.16 ± 0.18^{c}	$0.33 \pm 0.18^{a-}$

[a-d]The values represented by different letters within the same line are significantly different from each other.
*$P < .05$.

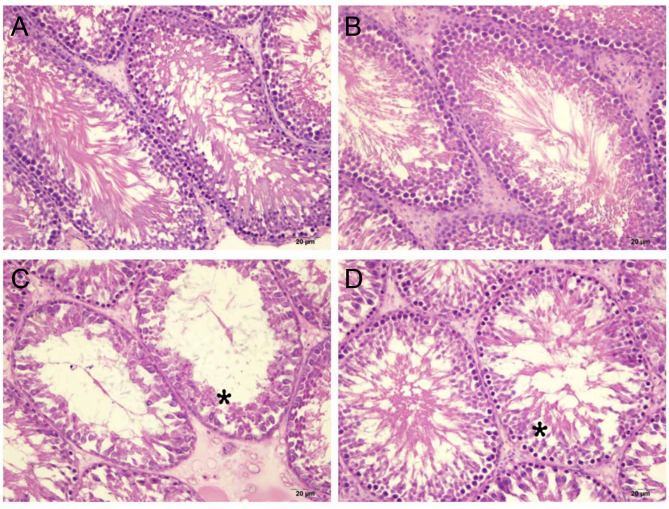

Figure 1. (A) Control group and (B) *Vitis Vinifera* L. extract groups. Normal structure. (C) Thallium sulfate group [severe necrotic and degenerative changes in seminiferous tubules (*)]. (D) HP +*Vitis Vinifera* L. extract group [mild necrotic and degenerative changes in the seminiferous tubules (*)]. (Staining: hematoxylin and eosin).

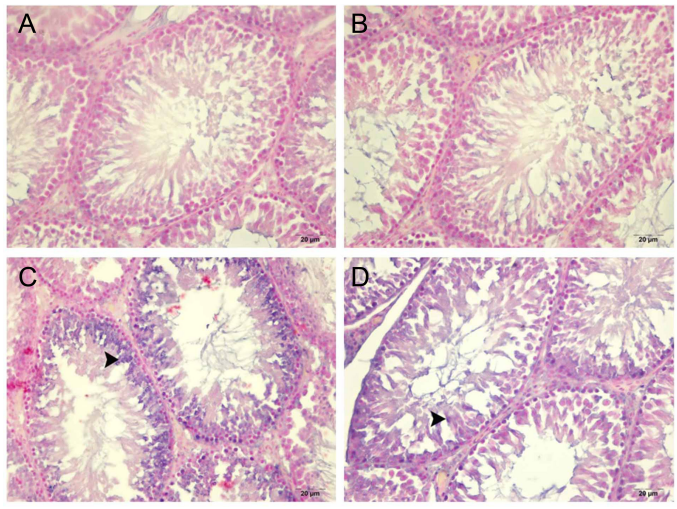

Figure 3. (A) Control group and (B) *Vitis vinifera* L. extract group. Cleaved caspase 3 negativity. (C) Thallium sulfate group [severe cleaved caspase 3 expression in seminiferous tubules (arrowhead)]. (D) *Vitis vinifera* L. extract + thallium sulfate group [moderate expression of cleaved caspase 3 in seminiferous tubules (arrowhead)]. (In situ hybridization.)

ways. Free radical types produced in the body during normal metabolism or through various external factors play an important role in the formation of diseases. It damages intracellular and extracellular components and thus causes loss of function.[28] Especially increased reactive oxygen species (ROS) production induces DNA integrity, lipid peroxidation, mitochondrial, and morphological lesions in germ cells, therefore testicular spermatogenesis is adversely affected.[29] In the present study, TS caused significant changes in GSH, CAT, GPx, SOD, and MDA levels by damaging the rat testicles. Antioxidant enzymes such as SOD, CAT, and GPx were observed to decrease, while MDA increased with the effect of TS in rat tissues. However, the administration of VVE turned the picture upside down, because of antioxidant property acting to protect testis tissues against the adverse effects of TS.

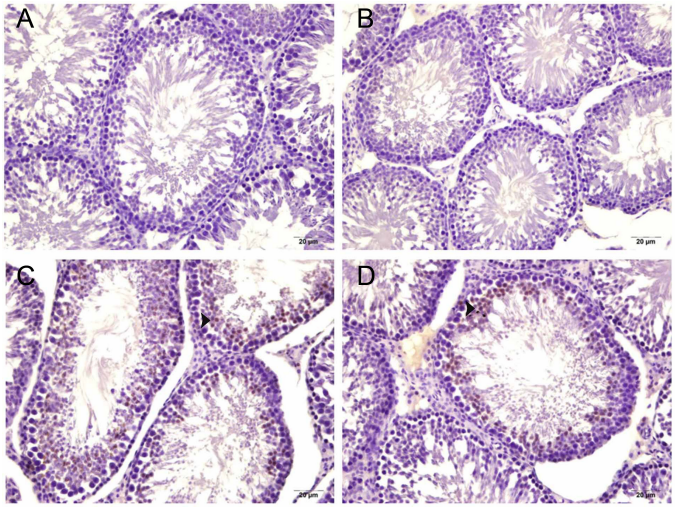

Figure 2. (A) Control group and (B) *Vitis vinifera* L. extract group. Cleaved caspase 3 negativity. (C) Thallium sulfate group [severe cleaved Caspase 3 expression in seminiferous tubules (arrowhead)]. (D) Thallium sulfate + *Vitis vinifera* L. extract group (moderate expression of cleaved caspase 3 in seminiferous tubules (arrowhead)]. (Staining: immunohistochemistry).

The effects of thallium on male reproduction have been investigated in rats in several studies. In the study of Formigli et al.,[27] peeling off the tubular epithelium, vacuolation, and changes in Sertoli cells were observed after the treatment. In our study result, it was determined that TS causes necrotic and degenerative changes in the testicles and decreases motility values in spermatozoa. However, it was observed that the VVE given in the TS group provided the regulation of this situation (Table 1)

In general, apoptosis is an event in which cells self-destruct, regulated by genes, programed, requiring RNA, protein synthesis, and energy, and maintaining homeostasis in the organism.[30] It has been reported in previous studies that toxic substances cause apoptosis in Sertoli cells in rats.[31] Caspases are biomarkers for apoptotic cell death. Apoptotic signals coming from inside or outside of the cell activate proteases in the cell. These proteases are called caspases.[32] Among these proteases, caspase 3 is an important biomarker to show apoptotic cell death.[33] In the presence of external stimuli that disrupt testicular physiology, non-physiological apoptosis occurs and a deterioration in spermatogenesis and infertility may occur.[34] The increase in the level of cleaved caspase 3 observed in group 2 in our study shows that TS administration causes apoptosis. It is seen that VVE given to rats slightly reduces the level of cleaved caspase 3 induced by TS.

Orally administered VVE in rats treated with TS resulted in decreased caspase 3 expression, normalization of sperm motility, and improvement of histopathological changes.

Ethics Committee Approval: Ethics committee approval was obtained from Atatürk University Animal Experiments Local Ethics Committee (Date: 30.10.2018, Number: 2018-10/194).

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B.M., A.S.M., A.G.; Literature Search – A.D.O., B.A.Y., S.A.A.; Writing Manuscript – S.A.A.; Critical Review – B.A.Y., A.D.O.

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