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Protective Effect of Subsieve Grape Extract (*Vitis vinifera* L.) on Testes, Reproductive Hormones, and Some Spermatological Parameters in Heat Stress-Induced Rats

Elekaltı Üzüm Ekstraktının (*Vitis Vinifera L.*) Sıcaklık Stresi Uygulanan Ratlarda Testis, Reprodüktif Hormonlar ve Bazı Spermatolojik Parametreler Üzerine Koruyucu Etkisi

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ABSTRACT

Heat stress (HS) has negative effects on reproductive parameters in male animals. This study was designed to investigate the protective effects of *Vitis vinifera* L., which has a natural antioxidant property, on reproductive parameters, testicular oxidative stress, reproductive hormones, and testicular histopathology in male rats treated with HS. Fifty male rats were divided into 5 different groups (control, HS, HS+100, HS+200, and HS+300). It was determined that subsieve *V. vinifera* extract given at different doses eliminated the negative effects of HS administration on reproductive parameters. It was determined that *V. vinifera* increased sperm motility and density and decreased the rate of abnormal sperm regardless of dose ($P < .001$). It was determined that increased oxidative stress and decreased luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone levels as a result of HS administration improved in the extract-treated groups ($P < .05$), and FSH and testosterone levels increased in the HS+200 and HS+300 groups compared to the control group ($P < .001$). Histopathologically, it was determined that the degeneration, edema, and congestion caused by HS disappeared due to the extract administration. As a result of this study, the protective effect of subsieve *V. vinifera* extract on HS on male reproductive parameters was revealed.

Keywords: Heat stress, sperm, testis, *Vitis vinifera* L.

ÖZ

Sıcaklık stresi (HS), erkek hayvanlarda üreme parametreleri üzerinde olumsuz etkilere sahiptir. Bu çalışma, doğal antioksidan özelliği olan *Vitis vinifera* L.'nin HS'ye maruz bırakılan erkek sıçanlarda üreme parametreleri, testiküler oksidatif stres, üreme hormonları ve testis histopatolojisi üzerindeki koruyucu etkilerini araştırmak amacıyla planlandı. 50 erkek rat 5 farklı gruba ayrıldı (Kontrol, HS, HS+100, HS+200, HS+300). Farklı dozlarda verilen elekaltı *V. vinifera* ekstraktının üreme parametreleri üzerindeki olumsuz etkilerini ortadan kaldırdığı tespit edildi. *V. vinifera*'nin dozdan bağımsı olarak sperm motilite, yoğunluğunu arttırdığı ve anormal sperm oranını azalttığı belirlendi ($P < ,001$). HS uygulaması sonucu artan oksidatif stres ve azalan lüteinleştirici hormon (LH), folikül uyarıcı hormon (FSH) ve Testosteron düzeylerinin ekstrakt uygulanan gruplarda iyileştiği ($P < ,05$), ayrıca HS+200 ve HS+300 gruplarında FSH ve Testosteron düzeylerinin kontrol grubuna oranala arttığı belirlendi ($P < ,001$). Histopatolojik olarak HS'nin neden olduğu dejenerasyon, ödem ve konjesyonların ekstrakt uygulamasına bağlı olarak ortadan kalktığı belirlendi. Bu çalışma sonucunda elekaltı *V. vinifera* HS uygulamasına bağlı olarak bozulan erkek üreme parametreleri üzerindeki koruyucu etkisi ortaya konmuştur.

Anahtar Kelimeler: Sıcaklık stresi, sperm, testis, *Vitis vinifera* L.

INTRODUCTION

Heat stress (HS) occurs when the animal is exposed to temperatures that exceed its physiological range and stabilizing ability. Although it may be limited to a specific organ or anatomical region, it usually affects the entire body.[1] HS is one of the parameters that negatively affect the animal's performance, health, and product value.[2] Depending on HS, mammalian reproductive functions and spermatogenes are impaired and reproductive parameters are decreased.[3]

In mammals, the testicles are located in the scrotum outside the abdominal cavity after fetal or neonatal development. Here, the temperature is 2-7°C lower than normal body temperature, and testicular temperature is maintained through special heat exchange mechanisms to ensure normal sperm production.[4,5] It regulates testicular temperature through the scrotum, tunica dartos, musculus cremaster, and plexus phampiniformis.[6]

Grapes are one of the fruits that have been grown frequently throughout the world in the past. Phenolic compounds in its structure prevent lipoprotein oxidation. Grape is a rich source of monomeric phenolic compounds such as (−)-epicatechin, (+)-catechins, (−)-epicatechin-3-O-gallate, and procyanidins. The polyphenols, vitamins, and minerals it contains have a high antioxidant capacity, protective effect on cells, immune system regulator, anti-mutogenic effect, and anti-aging properties.[7-9]

In this study, it was aimed to investigate the effect of different doses of subsieve grape extract (*Vitis vinifera L.*) on the percentage of sperm motility, abnormal sperm rate, semen density, and changes in testicular histopathology in HS-induced rats.

MATERIAL AND METHODS

Ethics Committee Approval

Ethical committee approval was received from the Ethics Committee of Van Yüzüncü University (Date: 23.02.2023, Number: 2023/05-18).

Extract

The feed additive material (subsieve grape) used in this study was obtained from a private feed company in Manisa province. The waste part of the Sultaniye seedless grape (*V. vinifera*) belonging to the Manisa region, which was not put up for sale and passed through the sift during the selection process and which was not used as human food, constituted the additive material. The subsieve grapes were dried, and the extraction process was performed.[10] The lethal dose was determined by the administration of the extract, according to Organisation for Economic Co-operation and Development (OECD) 423 guidelines.[11] Three different doses (100, 200, and 300 mg/kg) determined below the lethal dose level were used in heat-stressed groups.

Animals

Male rats with an average weight of 200-250 g used in the study were fed ad libitum at 21 ± 2°C in a 12-hour light/dark environment. For HS rats, they were kept at 37 ± 2°C for 4 hours between 08:00 and 12:00. Extract and saline administration were given by gavage every day at 08:00.

Groups

Fifty male rats were randomly divided into 5 different groups.

Group 1 (n = 10) (control): The group was given 1.5 mL daily saline via gavage for 28 days.

Group 2 (n = 10) (HS): Only heat stress was applied to the group for 28 days, and 1.5 mL of saline was given by gavage for 28 days.

Group 3 (n = 10) (HS + extract 100 mg/kg): Heat stress was applied to the group for 28 days, and the extract was given by gavage at a dose of 100 mg/kg in 1.5 mL saline every day.

Group 4 (n = 10) (HS + extract 200 mg/kg): Heat stress was applied to the group for 28 days, and the extract was given by gavage at a dose of 200 mg/kg in 1.5 mL saline every day.

Group 5 (n = 10) (HS + extract 300 mg/kg): Heat stress was applied to the group for 28 days, and the extract was given by gavage at a dose of 300 mg/kg in 1.5 mL saline every day.

Motility Examination

Immediately after sacrification, semen samples were taken by epididymis puncture. A minimum of 3 regions were examined with a 38 °C heating plate microscope.[12]

Density Analysis

Sperm sample (0.1 mL) was diluted with 0.5 mL of Hayem solution. It was counted on the Thoma slide.[12]

Abnormal Sperm Ratio

Sperm sample (0.1 mL) was diluted with 0.5 mL of Hancock solution. A minimum of 400 spermatozoa were counted for each preparation, and the ratio was determined.[12]

Histopathological Examination

At the end of the experiment, necropsies of the rats were performed. Testis tissue samples were taken. After the tissue pieces were fixed in Bouin solution, routine tissue follow-up was performed and embedded in paraffin blocks, and 4 μm sections were taken with a microtome. They were stained with hematoxylin–eosin (H&E) and examined under a light microscope, and morphological findings were photographed and evaluated.

Advanced Oxidation Protein Product, Malondialdehyde, Catalase

Advanced oxidation protein products (AOPP) were determined via the method described by Witko-Sarsat et al (1996).[13] Testis tissue malondialdehyde (MDA) level was measured by the method identified by Ohkava et al (1979), and MDA level was presented as mmol/g tissue.[14] Catalase (CAT) activity was spectrophotometrically analyzed at 240 nm according to the Lartillot and Kedziora (1988) method.[15]

Enzyme-Linked Immunosorbent Assay (Follicle-Stimulating Hormone, Luteinizing Hormone, Testosterone)

The blood samples were centrifuged at 2500 rpm for 15 minutes in EDTA tubes, and the serums were separated. Serum samples were analyzed according to the follicle-stimulating hormone (FSH) (AD3200 Ra), luteinizing hormone (LH) (AD1683 Ra), and testosterone (AD 1386 Ra) enzyme-linked immunosorbent assay (ELISA) kit protocols of Andy Gene.

Statistical Analysis

The Statistical Package for the Social Sciences Statistics software, version 20.0 software (IBM Corp.; Armonk, NY, USA) was used for statistical analyses. All data were expressed as mean ± standard deviation. Statistical analyses of the groups were carried out using the one-way analysis of variance followed by post hoc multiple comparisons (Bonferroni) for a comparative analysis between the groups. $P < .05$ was regarded as statistically significant.

Table 1. Sperm motility, Density, Anormal Sperm Rate, and Testis Weight

	Control	HS	HS+100	HS+200	HS+300	*P*
Motility (%)	75.55 ± 5.27	45.55 ± 8.81*	75.45 ± 7.26	73.33 ± 5.14	83.33 ± 7.07	<.001
Density (× 10^9/mL)	2.01 ± 0.12	1,44 ± 0.13*	2,02 ± 0,09	2.06 ± 0.14	2.07 ± 0.16	<.001
Abnormal sperm rate (%)	16.88 ± 1.69	35.66 ± 5.51*	18.11 ± 1.05	18.77 ± 1.09	17.22 ± 0.83	<.001
Testis weight (g)	1.43 ± 0.01	1.38 ± 0.11	1.42 ± 0.06	1,48 ± 0.11	1.49 ± 0.12	>.05

*Statistically significant difference between the groups in the same row ($P < .001$).
HS, heat stress.

RESULTS

Spermatological Parameters

Statistically, in the HS group, it was determined that motility and density decreased, and the rate of abnormal sperm increased ($P < .001$) (Table 1). It was determined that testicular weight did not differ between the groups ($P > .05$).

Histopathological Findings

Microscopically, the testis of the control (Figure 1A) group had normal histological appearances. In the HS group, loss of spermatozoa was also observed in the tubular lumens, and germ cells were dissociated from the basal membrane widespread. Additionally, degeneration of spermatogonia and interstitial spaces were expanded with edema, and congestion was present in the vessels (Figure 1B-C). In group HS+100, when compared to group HS, pathomorphological changes significantly decreased and spermatogenesis continued. It was found to have a close-to-normal histological appearance (Figure 1D). In the HS+200 and HS+300 groups, the histological structure of the testicular tissue was normal and was similar to that of the control group (Figures 1E and F).

Oxidative Stress Parameters Results

Heat stress group testicular tissue MDA, AOPP levels and CAT activity were found to be significantly higher than those of the

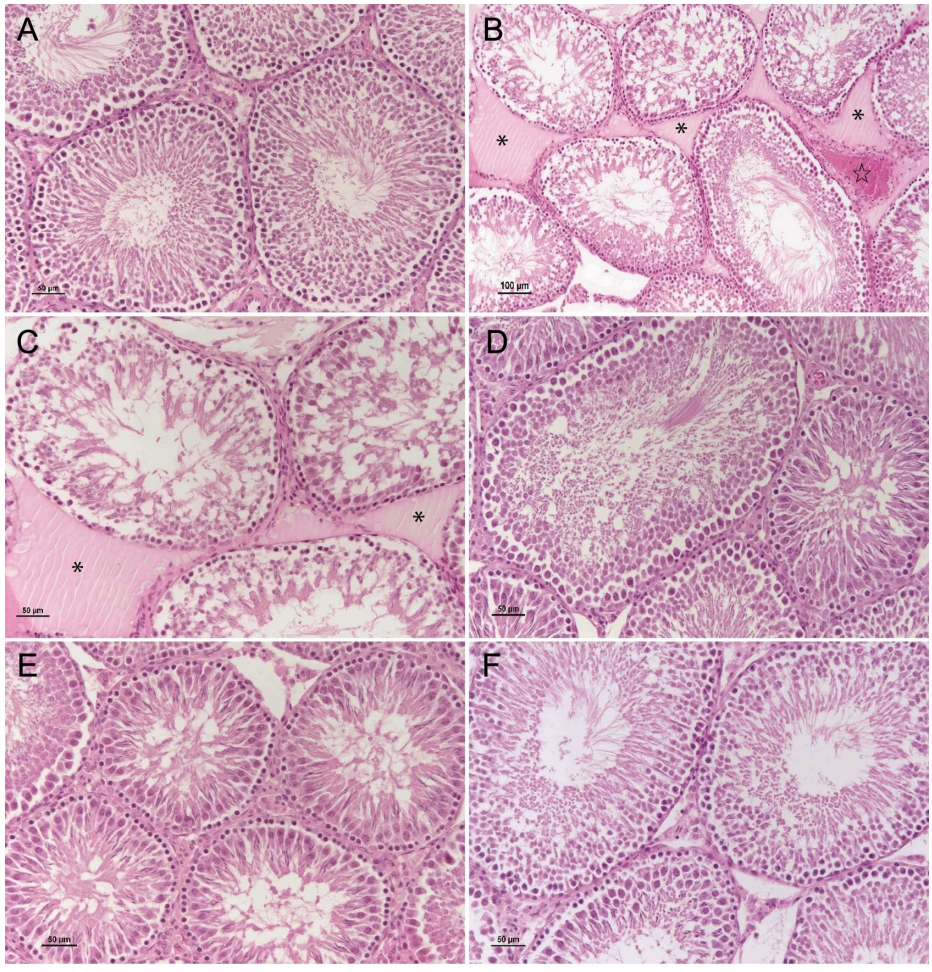

Figure 1. Testicular histopathology. (A) control group showed normal seminiferous tubule morphology and spermatogenic cells in advanced stages. (B, C) The testes of the HS group arrested spermatogenesis. Moreover, degeneration of spermatogonia, edema (*), and congestion (stars) in the interstitial spaces were observed. (D) In the HS+100 group, degeneration of spermatogonia was decreased, and edema and congestion in the interstitial spaces were significantly reduced. (E-F) In the testes of the HS+200 and HS+300 groups, the seminiferous tubule morphology was almost normal. All tissues were stained with hematoxylin and eosin. HS, heat stress.

Table 2. Oxidative Stress Parameters (MDA, AOPP, Catalase)

	MDA (mmol/g)	AOPP (mmol/g)	Catalase (U/L)
Control	0,486 ± 0,050[b]	10.59 ± 1.70[b]	357.76 ± 29.06[b]
HS	0.614 ± 0.103[a]	13.30 ± 1.69[a]	469.92 ± 50.10[a]
HS+100	0.522 ± 0.048[b]	11.56 ± 1.16[a, b]	384.88 ± 56.56[b]
HS+200	0.543 ± 0.054[a, b]	11.23 ± 1.02[a, b]	429.60 ± 60.69[a, b]
HS+300	0.491 ± 0.040[b]	11.21 ± 2.16[a, b]	371.27 ± 70.12[b]

Different letters in the same column represent statistical significance ($P < 0.05$).
AOPP, advanced oxidation protein products; HS, heat stress; MDA, malondialdehyde.

control group ($P < .05$) (Table 2). When the control group, HS+100, HS+200, and HS+300 groups were compared, no significant difference was found between testicular tissue MDA and AOPP levels and CAT activities.

Enzyme-Linked Immunosorbent Assay (Follicle-Stimulating Hormone, Luteinizing Hormone, Testosterone Results

It was determined that LH level increased statistically in the HS+300 group ($P< .05$) (Table 3). It was determined that the ratio of FSH levels to other groups decreased in the HS group and increased in the HS+300 group ($P < .05$). Testosterone levels decreased in the HS group compared to the other groups ($P < .05$); a statistically significant difference was found between the HS+300 group and the HS group in a pairwise comparison ($P < .001$).

DISCUSSION

It is known that HS has biologically negative effects on many metabolic systems. This study was designed to investigate the effects on the male reproductive system and the effects of *V. vinifera* extract on the damage.

The ideal ambient temperature for rats should be between 20 and 24°C. The testicles should be 2-7°C lower than the body temperature. Exposure of testicles to temperatures higher than body temperature disrupts the testicular thermoregulation mechanism; it causes spermatogenesis and sperm DNA damage. To create HS, many researchers used ambient temperatures in the range of 35-42°C.[6,16-18] In the presented study, the ambient temperature was increased to 37 ± 2°C and kept for 4 hours. The temperatures used in this study and other studies to induce HS are similar.

It has been reported by many researchers that HS has negative effects on testicular histopathology, oxidative stress, sperm motility rate, semen density, and abnormal sperm rate.[19-23] Similar to these findings, in the current study, histopathologically, the lumens of the tubulus seminiferus contortus were empty; MDA, AOPP, and CAT levels increased ($P < .05$); sperm motility rate and density decreased ($P < .001$); abnormal sperm rate was found to increase ($P < .001$).

Table 3. LH, FSH, Testosterone levels

	Control	HS	HS+100	HS+200	HS+300
LH (ng/L)	237.30 ± 9.18	228.34 ± 16.16	232.01 ± 23.01	252.08 ± 19.77	250.63 ± 23.67[a]
FSH (pg/mL)	672.64 ± 167.44	644.51 ± 118.50[a]	737.16 ± 72.87	771.47 ± 74.38	842.35 ± 108.75[b]
Testosterone (ng/L)	333.81 ± 30.53[a]	296.24 ± 22.71[b, c, x]	322.42 ± 16.72	355.56 ± 13.74[d]	376.2952 ± 14.31[b, y]

Statistically significant difference between the groups in the same row is represented by different letters (a, b, c, d) ($P < .05$). Statistically significant difference between the groups in the same row is represented by different letters (x, y) ($P = .001$).

V. vinifera, which has natural antioxidant properties, has been studied using different chemicals: lead,[24] holoperidol,[25] lindane,[26] manganese,[27] and aluminium chloride.[28] In this study, the oxidative stress parameters MDA, AOPP, and CAT were examined in order to investigate the protective effect of *V. vinifera* extract against HS. It was determined that the oxidative stress did not increase in the groups whose antioxidant property extract was used.

When serum testosterone levels were examined (Table 3), it was found in this study and many other studies that heat stress lowered testosterone levels. In addition, it was determined that testosterone levels increased in the 2 groups (200 mg/kg and 300 mg/kg) given *V. vinifera* extract compared to the control, HS, and HS+100 mg/kg groups ($P < .001$). At the same time, it was determined that LH and FSH levels increased in the group given 300 mg/kg of *V. vinifera* extract ($P < .05$). Heat stress causes reduced testosterone levels by causing suppression of star protein, disruption of mitochondrial activities, decreased cholesterol transport, and increased ROS levels.[29,30]

In the present study, when the histopathological effects of *V. vinifera* on reproductive parameters in male rats exposed to HS were examined, germ cells were dissociated from the basal membrane, and widespread loss of spermatozoa was also observed in the tubular lumens. In spermatological parameters, in all groups given *V. vinifera*, sperm motility rate, density, and abnormal sperm rate were similar to the control group. This, and the data obtained as a result of Halder's (2018) study, reveal that *V. vinifera* has a protective effect on spermatological parameters against HS.[31]

Considering all the findings in this study, its use should be recommended due to its protective effects on spermatological parameters, oxidative stress, and reproductive hormones against HS, including subsieve *V. vinifera* in rations as a feed additive.

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