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RESEARCH ARTICLE

Investigation of the Effects of Rutin on Valproic Acid Induced Testicular Damage in Rats

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ABSTRACT

Valproic acid (VALP) is a drug used for many psychiatric diseases such as epilepsy. However, the use of VALP has potential side effects on various tissues, including the testicles. Rutin (RUT) is a flavonoid with protective effects against oxidative stress-induced diseases and lipid peroxidation. In this study, the protective effects of RUT against testicular damage caused by VALP were investigated. For this purpose, 35 male Spraque-Dawley rats weighing 220-250 g were used in the study. The rats were randomly divided into 5 groups as Control (physiological saline), RUT (100 mg/kg/bw), VALP, (500mg/kg/bw), VALP+RUT 50 (500 mg/kg/bw VALP+50 mg/kg/bw RUT), and VALP +RUT 100 (500 mg/kg/bw VALP+100 mg/kg/bw RUT). At the end of the RUT and VALP administrations, the rats were sacrificed and testicular tissues were taken to be used for biochemical and spermatological analyzes. According to the results of this study, the MDA level in the testicular tissues of the VALP group was found to be statistically higher than the other experimental groups ($p<0.05$). Testicular tissue GSH and Nrf-2 level was the lowest in the VALP group. TNF-α, IL-1B and MAPK14 levels in testicular tissue were highest in the VALP group, while it was decreased in the RUT treatment groups. Similarly, the highest levels of Bax, Caspase-3 and MMP-9 were observed in the VALP group, while RUT treatment decreased this value. When the spermatological analyzes were examined, it was observed that the total motility value decreased significantly in the VALP group, but the total motility value increased in the RUT treatment group ($p<0.05$). A significant increase in the rate of dead and abnormal sperm was found in the VALP group, but these parameters improved in the RUT treatment group. As a result, RUT has protective effects against VALP-induced testicular damage in rats.

Keywords; Oxidative stress, rat, rutin, semen, valproic acid

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Sıçanlarda Valproik Asite Bağlı Testis Hasarına Rutin Etkisinin Araştırılması

ÖZ

Valproik asit (VALP), epilepsi gibi birçok psikiyatrik hastalık için kullanılan bir ilaçtır. Ancak VALP kullanımının testisler de dahil olmak üzere çeşitli dokularda potansiyel yan etkileri vardır. Rutin (RUT), oksidatif stres kaynaklı hastalıklara ve lipid peroksidasyonuna karşı koruyucu etkileri olan bir flavonoiddir. Bu çalışmada VALP'in neden olduğu testis hasarına karşı RUT'un koruyucu etkisi araştırıldı. Bu amaçla çalışmada ağırlıkları 220-250 g olan 35 adet erkek Sprague-Dawley cinsi rat kullanıldı. Ratlar kontrol (serum fizyolojik), RUT (100 mg/kg/va), VALP, (500mg/kg/va), VALP+RUT 50 (500 mg/kg/va VALP+50 mg/kg/va RUT) ve VALP +RUT 100 (500 mg/kg/v VALP+100 mg/kg/va RUT) olarak 5 gruba ayrıldı. Uygulamalarının sonunda ratlar sakrifiye edilerek testis dokuları biyokimyasal ve spermatolojik analizlerde kullanılmak üzere alındı. Sunulan çalışmanın sonuçlarına göre VALP grubunun testis dokularındaki MDA düzeyi diğer deney gruplarına göre istatistiksel olarak yüksek bulundu ($p<0,05$). Testis dokusu GSH ve Nrf-2 düzeyi VALP grubunda en düşüktü. Testis dokusunda TNF-α, IL-1β ve MAPK14 seviyeleri VALP grubunda en yüksek iken, RUT tedavi gruplarında azaldı. Benzer şekilde en yüksek Bax, Caspase-3 ve MMP-9 seviyeleri VALP grubunda gözlenirken, RUT tedavisi bu değeri azaltmıştır. Spermatolojik analizler incelendiğinde total motilite değerinin VALP grubunda anlamlı olarak düştüğü, ancak RUT tedavi grubunda total motilite değerinin arttığı görüldü ($p<0,05$). VALP grubunda ölü ve anormal sperm oranında önemli bir artış bulundu, ancak bu parametreler RUT tedavi grubunda düzeldi. Sonuç olarak RUT, sıçanlarda VALP'nin neden olduğu testis hasarına karşı koruyucu etkilere sahiptir.

Anahtar kelimeler; Oksidatif stres, rat, rutin, sperma, valproik asit.

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INTRODUCTION

Valproic acid (VALP, 2-propylpentanoic acid) or valproate is an eight-carbon branched chain fatty acid used as an antiepileptic drug (Tolou-Ghamari & Palizban, 2015). VALP and its salts are among non-specific histone deacetylase inhibitors (Zhou et al., 2020). VALP has been used for its anti-migraine, neuroprotective, and anti-manic properties (Safdar & Ismail, 2022). Despite its medical use, side effects are reported after VALP administration (Abdelkader et al., 2020; Pourahmad et al., 2012; Saleh et al., 2012). VALP can cause dyspepsia, obesity, hematological toxicity, teratogenicity, hepatotoxicity, nephrotoxicity and reproductive toxicity (Adewole et al., 2021; Galaly et al., 2014; Kandemir et al., 2022). VALP induces oxidative stress and inflammation, leading to toxication (Jin et al., 2014; Tong et al., 2005). Like other histone deacetylase (HDAC) inhibitors, VALP has the potential to alter the expression of protein levels (Bradbury et al., 2005). Increased levels of phospho-nuclear factor kappa beta (p-Nf-kB) and Caspase-3 were observed in testicular damage induced by VALP (Savran et al., 2020). Moreover, VALP causes a decrease in testicular weight and sperm quality in rats (Alsemeh et al., 2022).

Flavonoids are known for their anti-inflammatory and anti-apoptotic properties (Akaras, Gur, et al., 2023; Akaras, Ileriturk, et al., 2023; Ileriturk et al., 2023; Kandemir et al., 2017; Şimşek et al., 2023; Şimşek et al., 2023; Yardim et al., 2020). Rutin (RUT) is a flavone with antioxidant and anti-inflammatory effects, consisting of quercetin and the disaccharide rutinose (Aktaş et al., 2017; Kandemir, Caglayan, et al., 2020). Previous studies indicate that RUT has antidiabetic (Kamalakkannan & Prince, 2006), neuroprotective (Ola et al., 2015), and cardioprotective (Wang et al., 2015) effects. In addition, RUT also has protective effects against testicular toxicity (Abarikwu et al., 2013; Hozayen, 2012). It has also been determined that RUT has protective effects in rats with testicular ischemia perfusion (Akondi et al., 2011; Wei et al., 2011). In acrylamide administered rats, RUT reduced testicular toxicity (Salem et al., 2017). In addition, RUT has protective effects against testicular toxicity caused by doxorubicin (Hozayen, 2012), lead acetate (Ansar et al., 2015) and busulfan (Abarikwu et al., 2022) induced in rat.

In the literature review, we could not find a study examining the effects of RUT against VALP-induced testicular damage. In this study, the protective properties of RUT against VALP induced testicular toxicity were investigated.

MATERIAL AND METHODS

Animals and Ethical Approval

Presented study 35 male Sprague Dawley rats, 10-12 weeks old and weighing 220-250 g, were used. Animals were purchased from …… University Medical Experiment Application and Research Center and the study was conducted here. Animals were provided with feed and water ad libitum throughout the study. Ethical approval was obtained for the study from …… University Animal Experiments Local Ethics Committee (Protocol No: 2023-4-5). VALP (Depakin) was purchased from the pharmaceutical company Sanofi (Sanofi, France). Other chemicals were purchased from Sigma (Sigma Aldrich Company, USA) unless otherwise stated.

In the study, five different groups consisting of seven rats in each group were formed. The rats in the control group were given oral saline solution daily for 14 days. Rats in the RUT group were given oral RUT at a dose of 100 mg/kg/bw for 14 days. The rats in the VALP group were given sodium valproate orally at a dose of 500 mg/kg/bw for 14 days. Rats in the VALP+ RUT 50 group were given orally 500 mg/kg sodium valproate + RUT 50 mg/kg/bw. Rats in the VALP+ RUT 100 group were given orally 500 mg/kg/bw sodium valproate + RUT 100 mg/kg/bw. At the end of the study, the rats were sacrificed under mild sevoflurane (Abbvie, England) anesthesia and testicular tissues were taken. While testicular tissues were used for biochemical analysis, cauda epididymis parts were used for semen analysis.

Testicular tissue oxidative stress analysis

The measurement of malondialdehyde (MDA) was determined as a lipid peroxidation marker by its reaction with thiobarbituric acid. Testicular tissue MDA analyzes were determined by the method described by Placer et al.(1966) Glutathion (GSH) level was measured for antioxidant status in testicular tissue. Testicular tissue GSH analyzes were determined by the method described by Sedlak and Lindsay's(1968).

RT-PCR Analysis

After VALP and RUT treatments in testis tissues, mRNA transcript levels of tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), mitogen-activated protein kinase 14 (MAPK14), Bcl-2-associated X protein (Bax), Caspase-3, nuclear factor erythroid 2-related factor 2 (Nrf-2) and matrix metallopeptidase 9 (MMP-9) genes were analyzed by RT-PCR method. In the first step of the assays, total RNA was isolated from tissues using QIAzol Lysis Reagent (79306; Qiagen) according to the manufacturer's instructions. The concentration of total RNAs was measured in the NanoDrop (BioTek Epoch) device. In the second step, total RNAs were converted into cDNAs with the iScript cDNA Synthesis Kit (Bio-Rad). In the third and final step, a mixture of primers of the relevant genes, cDNAs, iTaq Universal SYBR Green Supermix (BIORAD) and DNase/RNase-free water was prepared

according to the manufacturer's instructions. The mixture was then reacted in the Rotor-Gene Q (Qiagen) device at the temperature cycles given by the manufacturer. After the cycles were completed, the CT values taken from the device were normalized according to β -actin with the 2-^{deltadeltaCT} method developed by Livak KJ and Schmittgen TD (2001). Primer sequences are given in Table 1.

Table 1. Sequences of primers

Gene	Sequences (5'-3')	Length (bp)	Reference No
Nrf2	F: TTTGTAGATGACCATGAGTCGC R: TCCTGCCAAACTTGCTCCAT	161	NM_031789.2
TNF-α	F: CTCGAGTGACAAGCCCGTAG R: ATCTGCTGGTACCACCAGTT	139	NM_012675.3
IL-1β	F: ATGGCAACTGTCCCTGAACT R: AGTGACACTGCCTTCCTGAA	197	NM_031512.2
MAPK14	F: GTGGCAGTGAAGAAGCTGTC R: GTCACCAGGTACACATCGTT	170	NM_031020.2
Bax	F: TTTCATCCAGGATCGAGCAG R: AATCATCCTCTGCAGCTCCA	154	NM_017059.2
Caspase-3	F: ACTGGAATGTCAGCTCGCAA R: GCAGTAGTCGCCTCTGAAGA	270	NM_012922.2
MMP9	F: AGCTGGCAGAGGATTACCTG R: ATGATGGTGCCACTTGAGGT	230	NM_031055.2
β-Actin	F: CAGCCTTCCTTCTTGGGTATG R: AGCTCAGTAACAGTCCGCCT	360	NM_031144.3

Semen Analysis

Testes taken from rats were separated from the cauda epididymis. The cauda epididymis was trimmed in 5 mL saline in a Petri dish. The resulting liquid was used in semen analysis. Sperm motility determination was determined by the method described by Aksu et al.(2021) Approximately 20 µL semen sample was dripped onto the heating plate, covered with a coverslip and examined with a light microscope (Zeiss Primo Star; Carl Zeiss, Oberkochen, Germany). The results were calculated as percentage.

For density analysis, 10 µL of semen fluid was taken and 990 µL physiological saline was added to it. It was vortexed for 15 sec at 2500 rpm and counted at 400X magnification on a Thoma slide to ensure homogeneity. The mean value of both compartments was multiplied by 5×10^6 and the result was considered as semen density (Aksu et al., 2018; Gur, Akarsu, et al., 2022). The rate of dead spermatozoa and sperm abnormalities were performed by the method described by Aksu et al. (2017). 10 µL of semen sample was taken and 10 µl of eosin-nigrosin mixture was dripped onto it, mixed with a coverslip and smear was taken. Dried smears were evaluated under a light microscope. To calculate the rate of dead spermatozoa and abnormal spermatozoa, 200 sperm cells were examined on each slide. Results were expressed as percentage.

Statistical Analysis

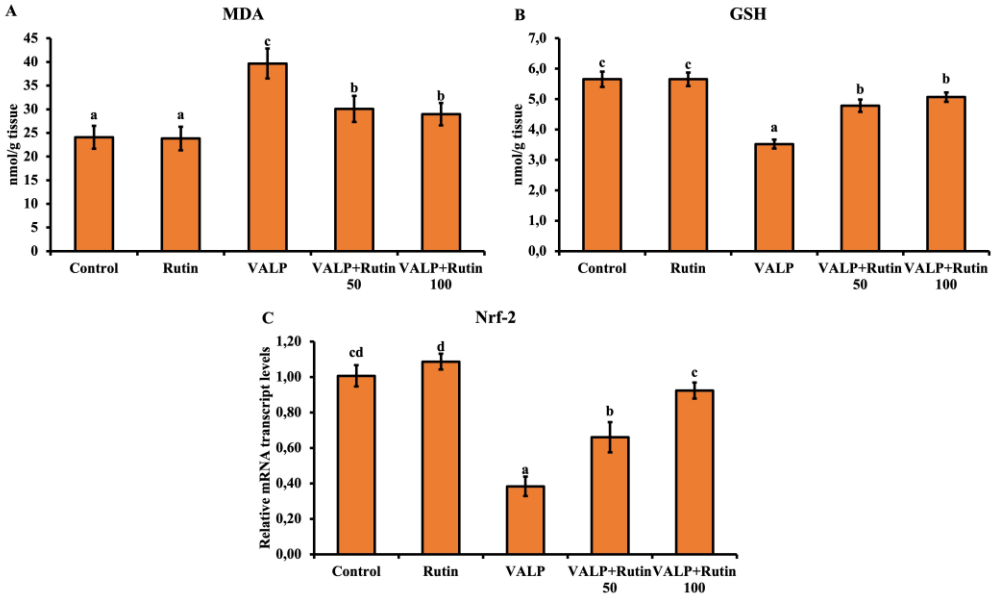
Statistical evaluation of the data obtained from the study was made in IBM SPSS (Version 26.0) program. One-way ANOVA and Tukey post hoc tests were used to determine whether there was a statistical difference between the groups. The results were given as mean ± standart deviation. $p<0,05$ was considered statistically significant.

RESULTS

Oxidative stress analysis results

MDA and GSH levels and Nrf-2 mRNA transcript levels in testicular tissue are shown in Figure 1. Accordingly, it is noteworthy that in the VALP group, the level of MDA, which is a biomarker of lipid peroxidation, increased, and the level of GSH and Nrf-2 mRNA transcripts decreased ($p<0,05$). However, it was observed that the MDA level decreased and the GSH level increased in the RUT treatment groups. Nrf-2 level increased dose-dependently with RUT treatment ($p<0.05$).

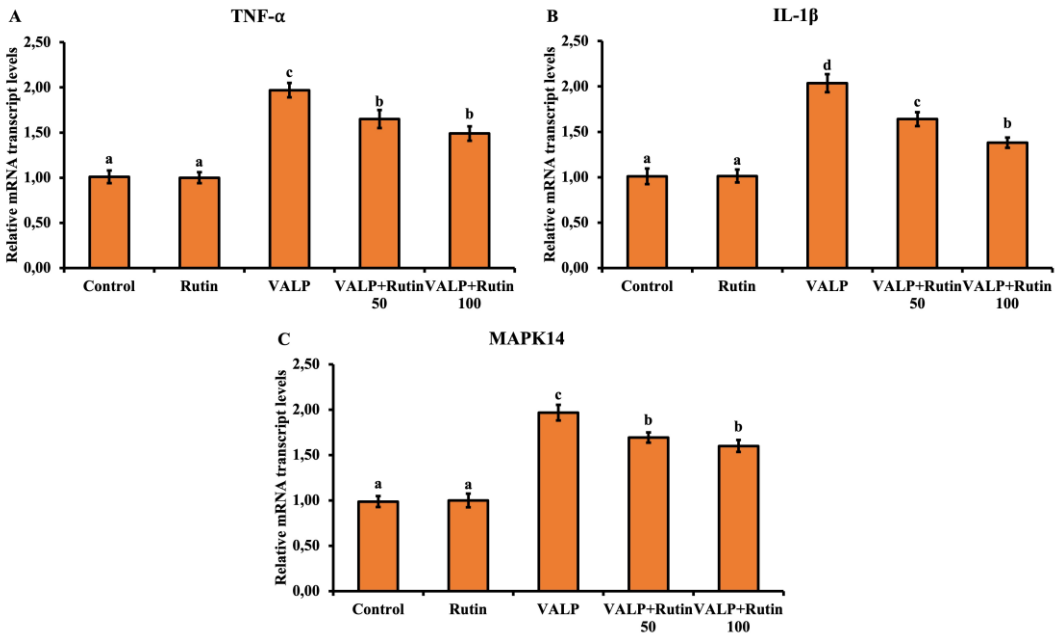
Figure 1. Testicular tissue MDA, GSH and Nrf-2 levels.



Inflammation-related gene expression level results

TNF-α, IL-1β and MAPK14 mRNA transcript levels in testicular tissue are shown in Figure 2. TNF-α and MAPK14 gene expression levels were significantly decreased in the RUT treatment groups compared to the VALP group. IL-1β mRNA transcript level decreased in a dose-dependent manner with RUT treatment ($p<0,05$).

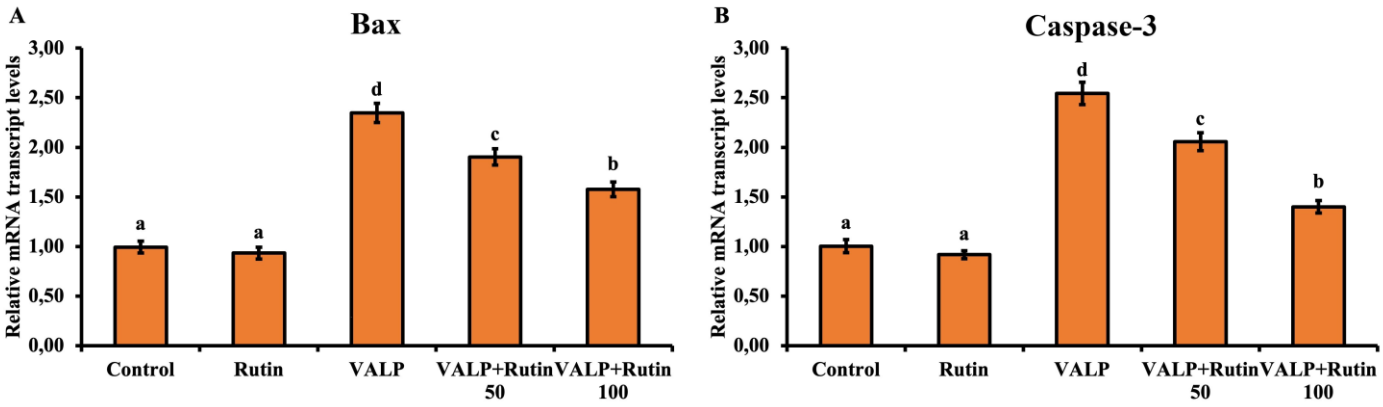
Figure 2. Testicular tissue TNF-α, IL-1β and MAPK14 mRNA transcript levels.



Bax, Caspase-3 level results in testis tissue

The mRNA transcript level results of Bax and Caspase-3, which are apoptosis-related genes in testis tissue, are shown in Figure 3. The expression levels of Bax and Caspase-3 genes were increased in the VALP group. Bax and Caspase-3 levels were significantly decreased in the RUT treatment groups compared to the VALP group in a dose-dependent manner ($p<0,05$).

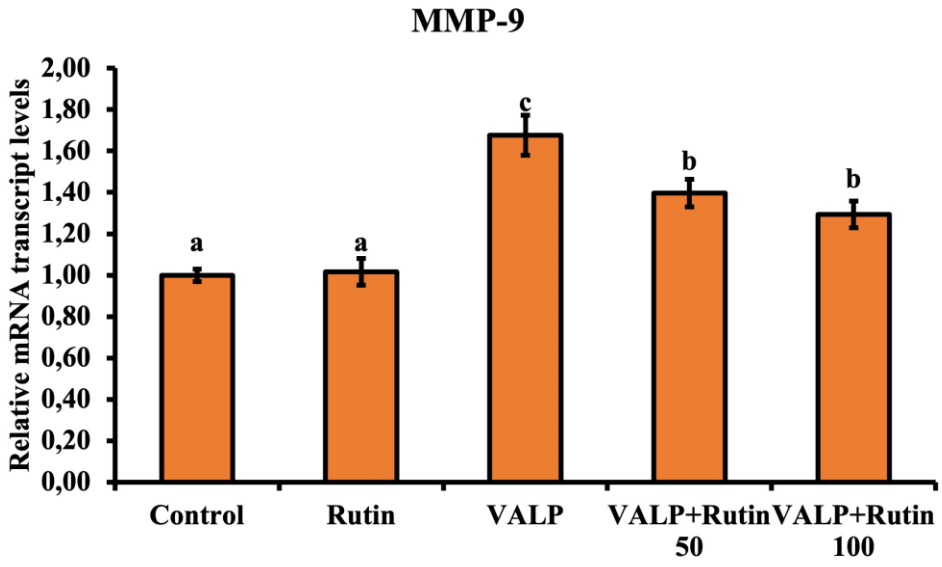
Figure 3. Testicular tissue Bax and Caspase-3 mRNA trancripts levels.



mRNA Transcript Levels of Metalloproteinases in Testis Tissue

The testicular tissue MMP9 gene expression level of rats in the whole experimental group is shown in Figure 4. Accordingly, it was determined that MMP9 expression level increased in testicular tissue after VALP application, while RUT treatment decreased these expression levels ($p<0.05$).

Figure 4. Testis tissue MMP9 levels.



Semen Analysis Results

The epididymal semen analysis results of the rats in the study groups are shown in Table 2. In the VALP group, it was observed that the total motility value decreased significantly compared to the other experimental groups. In addition, the rate of dead and abnormal sperm was significantly increased in the VALP group ($p<0,05$). Sperm quality improved in the RUT treatment group. However, there was no difference between the groups in terms of sperm density and testicular weight.

Table 2. Spermatological parameter results

	Total Motility (%)	Density x10⁶	Live-Dead Spermatozoa Rate(%)	Abnormal Spermatozoon Rate(%)	Total Testis Weight (mg)
Control	72,75±2,48[a]	73±2,36	11,06±2,04[ab]	11,01±1,67[ab]	2935,66±168,71
RUT	82,21±4,03[b]	76,5±3,55	8,83±0,98[a]	10,16±0,98[a]	2881,33±243,16
VALP	62,18±4,02[c]	74,863±2,4	16,33±2,16[c]	15,01±4,43[b]	2956,33±218,42
VALP+RUT 50	78,80±5,54[b]	72,33±3,14	12,83±1,83[b]	13,51±1,04[ab]	3089,16±385,02
VALP+RUT 100	79,95±4,22[b]	73±2,01	12,23±2,92[b]	12,33±0,81[ab]	2961,33±160,12

DISCUSSION

VALP has long been widely used as a broad-spectrum anticonvulsant drug (Nalivaeva et al., 2009). However, VALP has a toxic effect on many tissues, including the reproductive system (Bairy et al., 2010; Kandemir et al., 2022; Mandana et al., 2013). In this study, the effects of RUT against testicular toxicity induced by VALP were tried to be determined by biochemical pathways and spermatological analyzes.

Toxic compounds are sources of oxidative stress in testicles (Akaras et al., 2020; Belhan et al., 2017; Gur, Kandemir, et al., 2022). Oxidative stress is one of the main mechanisms in VALP toxicity. Measurement of MDA levels is necessary to determine lipid peroxidation, which is an indicator of oxidative stress (Kandemir et al., 2022). Previous studies show that VALP induces an increase in MDA level in various tissues (Hamza & Amin, 2007; Raza et al., 1997). In the presented study, it was determined that VALP caused an increase in lipid peroxidation and thus in MDA levels. However, it was determined that RUT administration in the treatment groups decreased the MDA level. This may be due to increased free radicals released by VALP.

GSH is a non-enzymatic antioxidant compound (Kucukler et al., 2020). Decreased levels of antioxidant substances cause oxidative damage in testis tissue (Akaras et al., 2017; Aksu et al., 2019). It has been stated in previous studies that RUT is a good antioxidant scavenger (Moshahid Khan et al., 2012). In our study, it was observed that there was a significant decrease in GSH levels in the VALP group and RUT treatment provided a significant improvement in GSH levels. Nrf2 is one of the cellular responses to oxidative damage (Ma, 2013). Nrf2 is suppressed when ROS is excessive (Gur & Kandemir, 2023). VALP leads to a decrease in Nrf2 levels in various tissues(Adewole et al., 2021).

In our study, a significant decrease in Nrf2 level occurred in the VALP group. There was a dose-dependent increase in Nrf2 levels in the RUT treatment groups. This indicates that cellular response to oxidative damage occurs in RUT groups and that RUT is a good reactive oxygen species scavenger.

Inflammation occurs in response to a chemical, physical or biological substance in the body (Ambriz-Pérez et al., 2016). TNF-α, IL-1β and MAPK14 are proinflammatory cytokines (Gur, Kandemir, et al., 2022; Temel et al., 2020) Previous studies have shown that RUT inhibits TNF-α and IL-1β levels in various tissues (Cihan et al., 2022; Kandemir, Caglayan, et al., 2020; Youssef et al., 2022). In the presented study, it was determined that there were significant increases in TNF-α, IL-1β and MAPK14 mRNA transcript levels in testicular tissue triggered by VALP application to rats. It was observed that RUT administration alleviated oxidative stress and suppressed the expression of TNF-α, IL-1β and MAPK14. Previous studies have reported that RUT reduces oxidative stress and alleviates inflammation, thus protecting target tissues from damage by toxic agents (Caglayan, Kandemir, Darendelioğlu, et al., 2019; Caglayan, Kandemir, Yildirim, et al., 2019).

In testicular toxicity, apoptosis occurs, which is programmed cell death (Gur, Kandemir, et al., 2022; Tuncer et al. 2023). It is stated that ROS produced in mitochondria in the apoptotic process have a function (Kandemir, Yıldırım, et al., 2020). Bax and Caspase 3 are used as indicators to determine the level of apoptosis (Kucukler et al., 2020). It has been stated that VALP triggers apoptosis in cells (Phillips et al., 2003). In the present study, it was determined that VALP application increased the mRNA transcript levels of Bax and Caspase-3 in testicular tissue, thus causing apoptosis. On the other hand, it

was observed that RUT treatment suppressed Bax and Caspase-3 expressions, thus protecting the testicular tissue from the destructive effect of VALP. In previous studies, it has been reported that phytochemicals suppress the apoptotic pathway induced by toxic agents (Kandemir, Caglayan, et al., 2020). In this respect, our study is compatible with previous studies.

MMPs are enzymes that have an important function in cell membrane disruption (Yıldız et al., 2022). These enzymes are inhibited when the cell Zinc (Zn) and Copper (Cu) levels are too low (De Souza et al., 2000). The increase in oxidative stress causes an increase in MMP level in parallel. In our study, there was a significant increase in MMP9 levels in the VALP group. It is thought that this situation may be related to the decrease in the concentration of Zn or Cu, which is suppressed against increasing oxidative damage.

Sperm motility provides information about the health status of rats (Aksu et al., 2017). In our study, there was a significant decrease in sperm motility in the VALP group. This may have occurred due to increased oxidant activity. Germ cells are extremely sensitive to toxic substances due to their high mitotic activity and low antioxidant capacity (Martin et al., 1999). In our study, the increase in the ratio of dead and abnormal spermatozoa in the VALP group can be explained by this mechanism. The decrease in the ratio of dead and abnormal sperm in RUT-treated groups can be explained by the fact that RUT scavenges ROS with increased antioxidant activity.

CONCLUSION

As a result, VALP administration cause to an increase in oxidative stress, inflammation and apoptosis in testicular tissue of rats, thus decreasing sperm quality. RUT treatment improved sperm quality by partially ameliorating the testicular damage induced by VALP.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: All authors contributed to the study. SAA, NAK, and EE contributed to the project idea, design and execution of the study. SAA, NAK and EE contributed to obtaining the data. SAA analyzed the data. NAK and EE drafted and wrote. The SAA critically reviewed the manuscript. All authors have read and approved the finalized article.

Ethical approval: Ethical approval was obtained for the study from Atatürk University Animal Experiments Local Ethics Committee (Protocol No: 2023-4-5).

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