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MALE REPRODUCTION: ORIGINAL ARTICLE

Histochemical, Immunohistochemical, and Biochemical Investigation of the Effect of Resveratrol on Testicular Damage Caused by Methotrexate (MTX)

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Abstract

Cancer is one of the world's major causes of death. The aim of this study is to examine the acute effects of resveratrol on testicular toxicity, oxidative stress, and apoptosis caused by MTX, which is widely used in the treatment of many diseases, especially cancer, histochemically, immunohistochemically, and biochemical methods using different parameters. A total of 32 Wistar albino male rats were randomly divided into 4 groups: control, resveratrol (RES), MTX, and MTX + RES, with 8 animals in each group. At the end of the experiment, tissue and blood samples were taken, and histochemical, immunohistochemical, and biochemical parameters were examined. In this study, where parameters were compared for the first time, total thiol (TT) and native thiol (NT) are the highest in the RES group, disulfide (DS), and ischemia-modified albumin (IMA) are the highest in the MTX group. Total oxidant status (TOS) and oxidative stress index (OSI) are the highest in the MTX group, and total antioxidant status (TAS) is the highest in the RES group. Separation and deterioration in the tunica albuginea, congestion and edema in the interstitial region, vacuolization in the seminiferous epithelium, and spermatogenic serial cells spilling into the lumen without completing their maturation were observed. When examined in terms of histochemical, immunohistochemical, and biochemical examinations, our study revealed that resveratrol has positive effects on methotrexate-induced acute testicular damage, oxidative stress, and apoptosis.

Keywords Carnitine palmitoyl transferase 1C · Caspase · Heat shock protein 70 · Kisspeptin1 · Methotrexate

Introduction

The number of cancer deaths is increasing every day. Chemotherapy is one of the most utilized treatments. This type of treatment uses chemotherapeutic agents to eliminate cancer cells [1]. This is accomplished through the use of cytotoxic anti-neoplastic agents [2]. Chemotherapy treatments cause oxidative damage and may alter organ structure, sexual hormones, and function [3, 4].

MTX (N-10 methyl aminopterin) is an antiproliferative, anti-inflammatory, and immunomodulatory folic acid antagonist [5]. It is one of the most extensively used antimetabolites in the treatment of numerous diseases, including cancer, rheumatoid arthritis, and psoriasis [6-8]. MTX has been documented to damage healthy tissues and cells, producing functional abnormalities in both somatic and reproductive cells due to its inhibitory effect on DNA synthesis, repair, and cellular replication [9]. By boosting the generation of reactive oxygen species, MTX induces toxic reactions and severe alterations in testicular tissue [10]. Recently, research

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has focused on the utilization and efficacy of antioxidant materials in reducing MTX adverse effects [11, 12].

RES (trans-3,5,4′-trihydroxy-stilbene) is an antioxidant phytoalexin present in a range of foods, particularly grape seeds. Recent in vivo and in vitro research has revealed that RES protects spermatocytes from lipid peroxidation and enhances testicular sperm motility [13]. It has also been proven to stimulate sperm production [14], decrease apoptosis in germinal cells [15], and protect against environmental toxins [16]. According to several studies, RES is a potent antioxidant that functions by enhancing the production of antioxidant enzymes and decreasing lipid peroxidation [18]. As a result, resveratrol is employed as a key antioxidant in applications such as chemotherapy to minimize oxidative stress [16].

There is only one article on the effects of resveratrol on oxidative stress and apoptosis against testicular damage caused by methotrexate [Yulug et al. 2013]. However, only hematoxylin–eosin (HE) and tunel (terminal deoxynucleotidyl transferase dUTP nick end labeling), which is an indicator of programmed death, were studied in histopathological examination, while malondialdehyde (MDA) levels, superoxide dismutase (SOD), and catalase (CAT) were studied in biochemical examination. The aim of the present study is to examine the acute effects of resveratrol on testicular toxicity, oxidative stress, and apoptosis caused by MTX, which is widely used in the treatment of many diseases, especially cancer, with histochemical, immunohistochemical, and biochemical methods by using different parameters (Fig. 1).

Methods

Ethical Approval

This research was conducted in the Experimental Animal Research Laboratory, Histology and Embryology Research Laboratory, and Biochemistry Research Laboratory of Suleyman Demirel University's (SDU) Faculty of Medicine. It was funded by the SDU Scientific Research Projects Management Unit under project TDK-2020–8034. The SDU Animal Experiments Local Ethics Committee approved this study, and ethical guidelines were observed throughout. The SDU Faculty of Medicine Animal Experiments Local Ethics Committee (Ethics Committee No. 13–2/2019) granted the required authorization for animal studies.

Experiment Animals and Their Features

Thirty-two male Wistar albino rats weighing 300–400 g were used in the study. Throughout the experiment, the

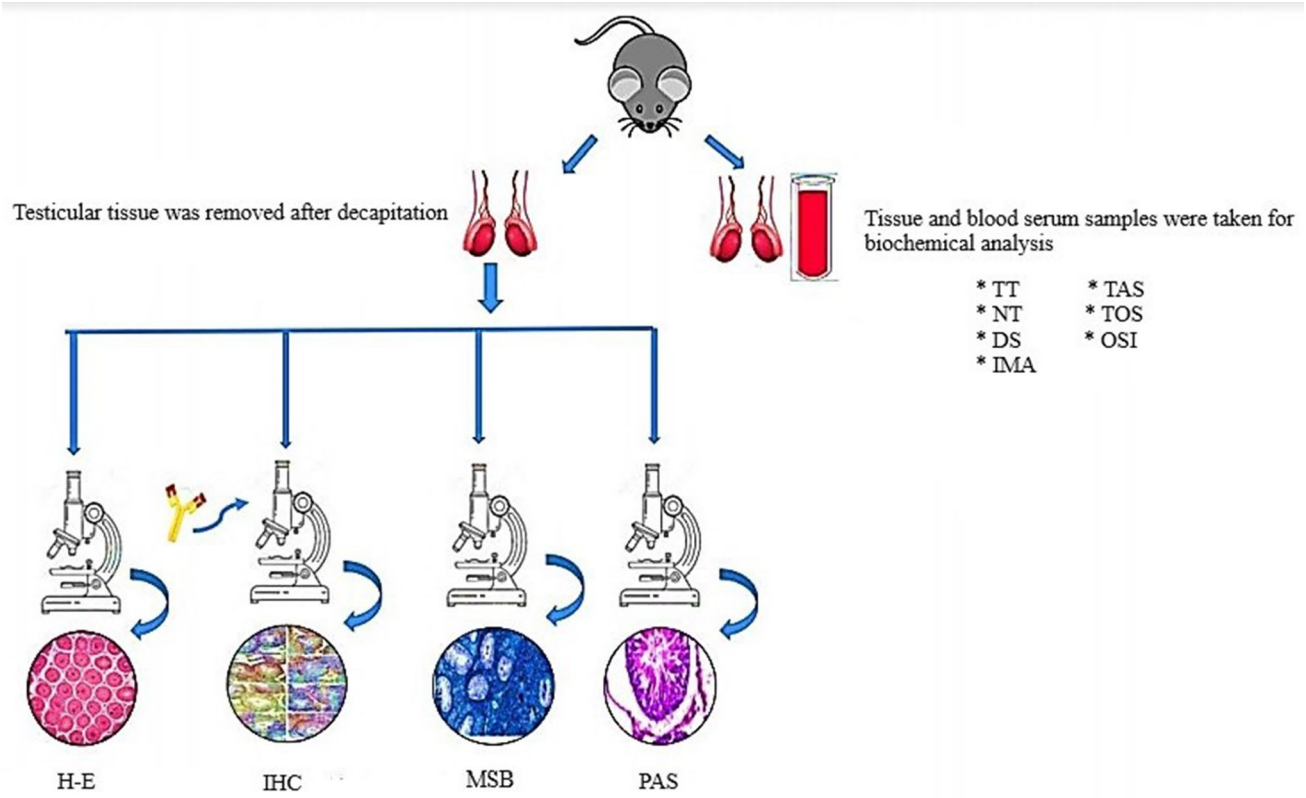


Fig. 1 Summary of application method

rats were maintained in standard humidity, light (12 h of daylight/12 h of darkness), and temperature (25 °C) conditions. The rats were raised at the Production Unit of the Experimental Animal Research Laboratory of the Suleyman Demirel University (SDU) Faculty of Medicine. Throughout the trial, rats had unrestricted access to food and water (ad libitum).

Thirty-two male Wistar albino rats were chosen at random and divided into four groups of eight animals each (Fig. 1). Table 1 contains information on this categorization as well as application details.

Anesthesia, Tissue, and Blood Samples

After the last dose of medication (end of day 5) given to rats who had been fasting since the previous night, anesthesia was induced by i.p. administration of a mixture of ketamine hydrochloride (75 mg/kg) (ketamin 10%, Bremer Pharma) and xylazine (alfazyne 2%, Ege Vet) hydrochloride (10 mg/kg). In order to collect tissue samples and intracardiac blood samples, all rats were sacrificed. For histochemical and immunohistochemical analyses, certain tissue samples were included in Boin's solution. Blood samples were examined the same day they were collected, while the remaining tissue samples were stored in a – 20 °C deep freezer for biochemical analysis.

Biochemical Analysis of Blood Samples

Rat blood samples were placed in biochemistry tubes without additives and centrifuged for 10 min at 4000 rpm. Serum samples were tested for total thiol (TT), native thiol (NT), disulfide (DS), and ischemia-modified albumin (IMA) levels. On a Beckman-Coulter AU5800 autoanalyzer, spectrophotometric calculations were made (Brea, CA, USA). Disulfide quantities (SS), disulfide/total thiol percentages (SS/SH + SS), disulfide/natural thiol percentages (SS/SH), and natural thiol/total thiol percentages (SH/SH + SS) were estimated after determining natural thiols (SH) and total thiols.

Calculating Total Oxidant Status (TOS), Total Antioxidant Status (TAS), and Oxidative Stress Index (OSI)

Cold phosphate buffer was used to homogenize testicular tissues to a tissue concentration of 10%. For measurement from homogenized tissues, TAS (Rel Assay®, Diagnostics kits, Mega Tp, Gaziantep, Turkey) and TOS (Rel Assay®, Diagnostics kits, Mega Tp, Gaziantep, Turkey) commercial kits were utilized. The TOS test reagent was calibrated using a reference solution containing 10 µmol H_20_2 Equiv/L, and the results were represented as M hydrogen peroxide equivalent per liter (µmol H_2O_2 Equ/L). The antioxidant standard solution containing 1 mmol Trolox equivalent/L vitamin E analog was used to calibrate the TAS reagent. The results were expressed in mmol Trolox equivalent per liter. TOS and TAS assays were conducted spectrophotometrically on a Beckman Coulter AU5800 autoanalyzer (Brea, CA, USA) and assessed in the SDU Department of Medical Biochemistry's Research Laboratory. OSI is the TOS/TAS ratio and was determined as follows: OSI = TOS (µmol H_2O_2 Eq/L)/TAS (mmol Trolox Eq/L) × 100 [19].

Tissue Follow-up, Sectioning, and Routine Staining

Testicular tissues were fixed in Bouin's solution, washed in 70% alcohol, and submitted to a series of routine histologic examinations. They were xylol polished and implanted in paraffin blocks. Sections of 3 µm thick were cut from the prepared paraffin blocks. HE, PAS, and MSB were used to stain the sections. They were studied and assessed using a Nikon ECLIPSE Ni-U-type binocular microscope, and pictures were taken using imaging equipment. The light microscopic staining differences are shown in the figure (Fig. 2).

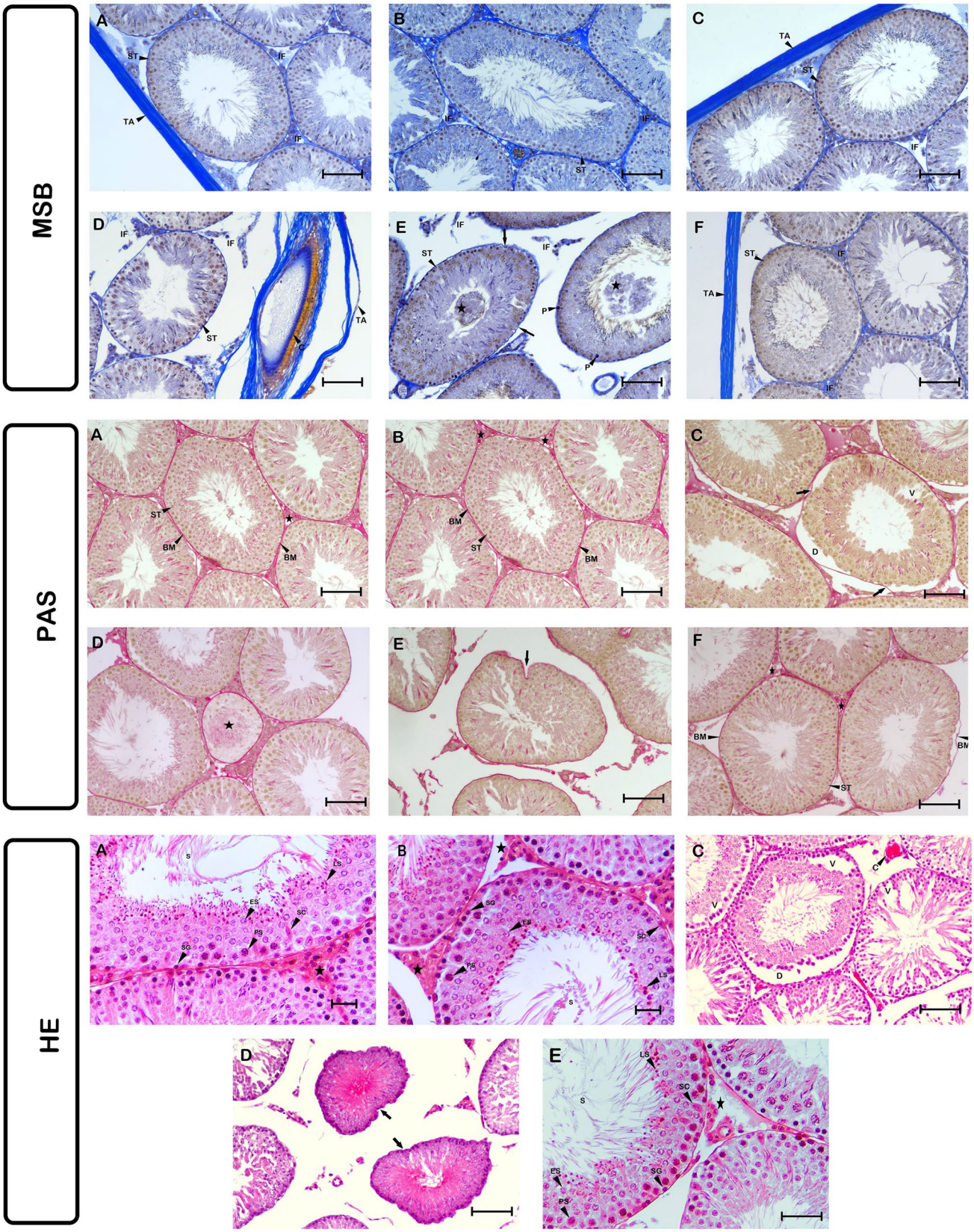
Table 1 Animal groups and features

Group name	Group information
Group I (control) ($n = 8$)	Group administered saline i.p.* for 5 days
Group II resveratrol (RES** group) ($n = 8$)	Group administered 20 mg/kg RES (R5010-500 mg, Sigma-Aldrich) i.p.* for 5 days
Group III methotrexate (MTX*** group) ($n = 8$)	Group administered a single i.p.* dose of 30 mg/kg MTX (methotrexate flacon-50 mg, Kocak Farma) on the first day
Group IV MTX + RES ($n = 8$)	Group administered a single i.p.* dose of 30 mg/kg MTX on the first day and 20 mg/kg i.p.* RES 30 min before the treatment and for the following 5 days

*i.p. (intraperitoneal) (references: Gulgun et al. [11], Yulug et al. [17])

**RES (R5010-500 mg, Sigma-Aldrich, St. Louis, MO, USA). It was dissolved in dimethyl sulfoxide (DMSO) and applied (reference: Yulug et al., [17])

***MTX (methotrexate flacon-50 mg, Kocak Farma, Tekirdag, Turkey) (reference: Yulug et al. [17])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◂**Fig. 2** Control (**A**, **B**), RES (**C**), and MTX + RES (**F**). Tunica albuginea (TA), seminiferous tubule (ST), interstitial space, and Leydig cells (IF). MTX (**D**, **E**). Disorganization, separation, disruption, and vascular congestion in the TA (C), disruption in the ST and IF, ST invagination (arrow), spermatogenic serial cells (asterisks), and pyknotic bodies (P) shed into the lumen prior to maturation (MSB × 20, scale bar = 100 µm). Control (**A**), RES (**B**). ST, basement membrane (BM), interstitial space and Leydig cells (asterisk). MTX (**C**, **D**, **E**). Invagination (arrow), deletion (D), and vacuolization (V) in the basement membrane of the seminiferous tubule, edema in the interstitial space (asterisk), and invagination in the basement membrane of the seminiferous tubule (arrow). MTX+RES (**F**). The BM, ST, interstitial space, and Leydig cells (denoted by an asterisk) are mostly intact (PASx20, scale bar=100 µm). Control (**A**), RES (**B**), MTX+RES (**E**). Spermatogonium (SG), primary spermatocyte (PS), early spermatid (ES), late spermatid (LS), spermium (S), Sertoli cell (SC), and Leydig cells (asterisk) (HEx40, scale bar=50 µm). MTX (**C**, **D**). Epithelial deletion and vacuolization of seminiferous tubules (D and V), congestion (C), irregular contours, and atrophic seminiferous tubules (arrow) (HEx20, scale bar=100 µm)

Immunohistochemical Studies in Tissue Samples

In testis tissue, caspase-3 (catalog: FNab01289, 1:200), caspase-8 (catalog: FNab01293, 1:100), caspase-9 (catalog: FNab01295, 1:200), kisspeptin1-KISS1 (catalog: FNab10525, 1:100), heat shock protein 70-HSP70 (catalog: FNab04048, 1:200), carnitine palmitoyl transferase 1C-CPT1C (catalog: FNab10524, 1:100) immunoreactivities were determined using the avidin–biotin-peroxidase method. Sections of 3 µm thickness were taken on slides with polylysine. After deparaffinization, it was boiled in citrate buffer solution for 10 min. After treatment with H_2O_2 (TA-060-HP, Lab Vision Corporation) and Ultra V Block (TA-125-UB, Thermo Scientific), primary antibody (caspase-3, -8, -9, KISS1, HSP-70, CPT1C) was incubated 60 min. It was incubated with secondary antibody (TP-125-BN, Thermo Scientific) for 30 min in a humid environment at room temperature. HRP (TS-125-HR, Thermo Scientific) and PBS were moved within 30 min of room temperature incubation in humid environment. DAB (SK-4100, Vector) for caspase-3, -8, -9, HSP-70, and CPT1C antibodies, and AEC (TA-060-HA, Lab Vision Corporation) solution for KISS1 antibody were dripped, and the preparations prepared were evaluated and photographed. Abdel-Wahhab et al.'s [20] scoring system was used to analyze the structural alterations observed in the testicular tissue sections of the experimental and control groups (Table 2).

Statistical Analysis

The Statistical Package for the Social Sciences was used to examine the data (Version 23, Chicago, IL, USA). Number, percentage, mean, and standard deviation were used to express the measurement values in the experimental and control groups. Kurtosis and skewness values, along with the Shapiro–Wilk test, were used to determine whether the data had a normal distribution. The data correspond with a normal distribution when the values for kurtosis and skewness fall within the range of ± 2 [21]. If the sample size is smaller than 35, the Shapiro–Wilk test [22] can be utilized. Since the data were normally distributed, the independent samples *T*-test was used to compare two independent groups, and one-way ANOVA analysis was performed to analyze three or more groups. A $p < 0.05$ value was deemed statistically significant. The difference between the mean scores was found to be statistically significant between the groups ($p < 0.05$). In order to determine which group caused this difference, the "post hoc" Bonferroni test, which is one of the multiple comparison tests, was applied.

Results

Biochemical Results

When the body weights of the rats were compared at the end of the experiment, a significant increase ($p < 0.05$) was detected in the control and RES groups, while a significant decrease ($p < 0.05$) was observed in the MTX and MTX + RES groups (Table 3).

Table 3 Weight comparisons in the control, RES, MTX, and MTX + RES groups

Group	Weight	Number	Mean	Standard deviation	p^*
Control	First weight	8	372.625	26.821	0.000
	Last weight	8	374.250	26.617	
MTX	First weight	8	380.875	35.898	0.003
	Last weight	8	336.375	36.461	
RES	First weight	8	360.875	33.875	0.005
	Last weight	8	366.625	34.400	
MTX + RES	First weight	8	381.000	36.331	0.001
	Last weight	8	376.875	37.391	

*The dependent t-test

Table 2 Scoring table of histopathological and immunohistochemical evaluations

(−) score	Negative score	It means the absence of any structural changes
(+) score	1 positive score	It refers to a slight structural change
(++) score	2 positive scores	It represents moderate structural change
(+++) score	3 positive scores	It represents a serious structural change

Table 4 Comparison of TAS, TOS, and OSI values using in the control, RES, MTX, and MTX + RES groups (mean SD)

Group	TAS (mmol H_2O_2 Equiv./L)	TOS (μmol Trolox Equiv./L)	OSI
Control[a]	1.493 ± 0.253	19.413 ± 4.589	1.333 ± 0.386
RES[b]	1.618 ± 0.336	15.356 ± 4.442	1.012 ± 0.526
MTX[c]	1.103 ± 0.230	25.358 ± 6.824	2.431 ± 1.004
RES + MTX[d]	1.238 ± 0.299	21.378 ± 3.60	1.859 ± 0.726
Bonferroni test[†]	$b * c < 0.05$	$b * c < 0.05$	$a * c$, $b * c < 0.05$
F	5.515	5.523	6.308
p	0.004	0.004	0.002

TAS, total antioxidant status; *TOS*, total oxidant status; *OSI*, oxidative stress index

[†]: Only statistically significant values were indicated

TAS was highest in the RES group ($p < 0.05$), while TAS was higher in the MTX + RES group compared to MTX ($p < 0.05$). TOS levels were highest in the MTX group ($p < 0.05$), while TOS levels were lowest in the MTX + RES group ($p < 0.05$). The MTX group had the greatest OSI level ($p < 0.05$). The MTX + RES group was considerably lower than the MTX group ($p < 0.05$) (Table 4).

The RES group had the greatest TT and NT levels, while the MTX group had the lowest ($p < 0.05$). TT and NT levels were low in the MTX + RES group but significantly higher than in the MTX group ($p < 0.05$). The MTX group had the highest DS level, and the RES group had the lowest ($p < 0.05$). The DS level in the MTX + RES group was significantly lower than in the MTX group ($p < 0.05$). The IMA level was significantly higher in the MTX group and significantly lower in the MTX + RES group ($p < 0.05$) (Table 5).

Light Microscopic Results

Light microscopic staining differences are shown in the figure (Fig. 2).

Immunohistochemical Results

Immunohistochemical staining differences are shown in the figure (Fig. 3). Caspase-3, caspase-8, caspase-9, HSP70, CPT1C, and KISS1 immunoreactivity scores of testicular tissues are given in the table (Table 6).

Discussion

In this study, it was shown with histochemical (MSB, HE, PAS), immunohistochemical (caspase-3, -8, -9, HSP70, CPT1C, and Kiss1), and biochemical (TAS, TOS, OSI, TT, NT, DS, and IMA) methods (Fig. 1) by using different parameters that MTX caused testicular toxicity, oxidative stress, apoptotic effects, and resveratrol largely prevented acute testicular damage.

MTX has been documented to have toxic effects on numerous organs of the body, including the central nervous system [23] and gonads [24], when used at high dosages in the treatment of cancer or at low levels over long periods of time in the treatment of autoimmune diseases. In mouse testis, MTX injection has been shown to reduce the diameter of seminiferous tubules, increase interstitial space, and alter the morphology of Leydig cells [25]. In our study, the MTX group stained with MSB showed irregularity, separation, and disruption in the tunica albuginea, vascular congestion, and disruption in the seminiferous tubule and interstitial area. These findings are consistent with those of Shrestha et al. Separation, disruption, and deletion in the seminiferous tubule, congestion in the vessel, irregular contours, and atrophic seminiferous tubules were observed in the MTX group in HE-stained tissues (Fig. 2). Our findings supported those of Padmanabhan et al. [26]. The impact of RES on male fertility is still controversial. RES prevented MTX-induced testicular damage in our study by restoring the normal histologic process. The mechanism of action of

Table 5 Comparison of TT, NT, DS, and IMA values in the control, RES, MTX, and MTX + RES groups (mean ± SD)

Group	TT	NT	DS	IMA
Control[a]	361.719 ± 43.214	278.625 ± 38.340	41.547 ± 10.807	256.025 ± 13.508
RES[b]	403.001 ± 40.584	329.529 ± 28.722	36.736 ± 7.765	252.475 ± 10.319
MTX[c]	266.660 ± 33.523	150.160 ± 21.364	58.250 ± 8.399	279.137 ± 12.107
RES + MTX[d]	318.140 ± 46.390	232.626 ± 36.061	42.757 ± 7.879	243.237 ± 13.196
Bonferroni test[†]	$a * c$, $a * d$, $b * c$, $b * d$, $c * d < 0.05$	$a * c$, $a * d$, $b * c$, $b * d$, $c * d < 0.05$	$a * b$, $b * c$, $b * d < 0.05$	$a * b$, $a * d$, $b * c$, $b * d < 0.05$
F	16.132	14.902	8.977	12.231
p	0.000	0.000	0.000	0.000

TT, total thiol; *NT*, native thiol; *DS*, disulfide; *IMA*, ischemia modified albumin

[†]: Only statistically significant values were indicated

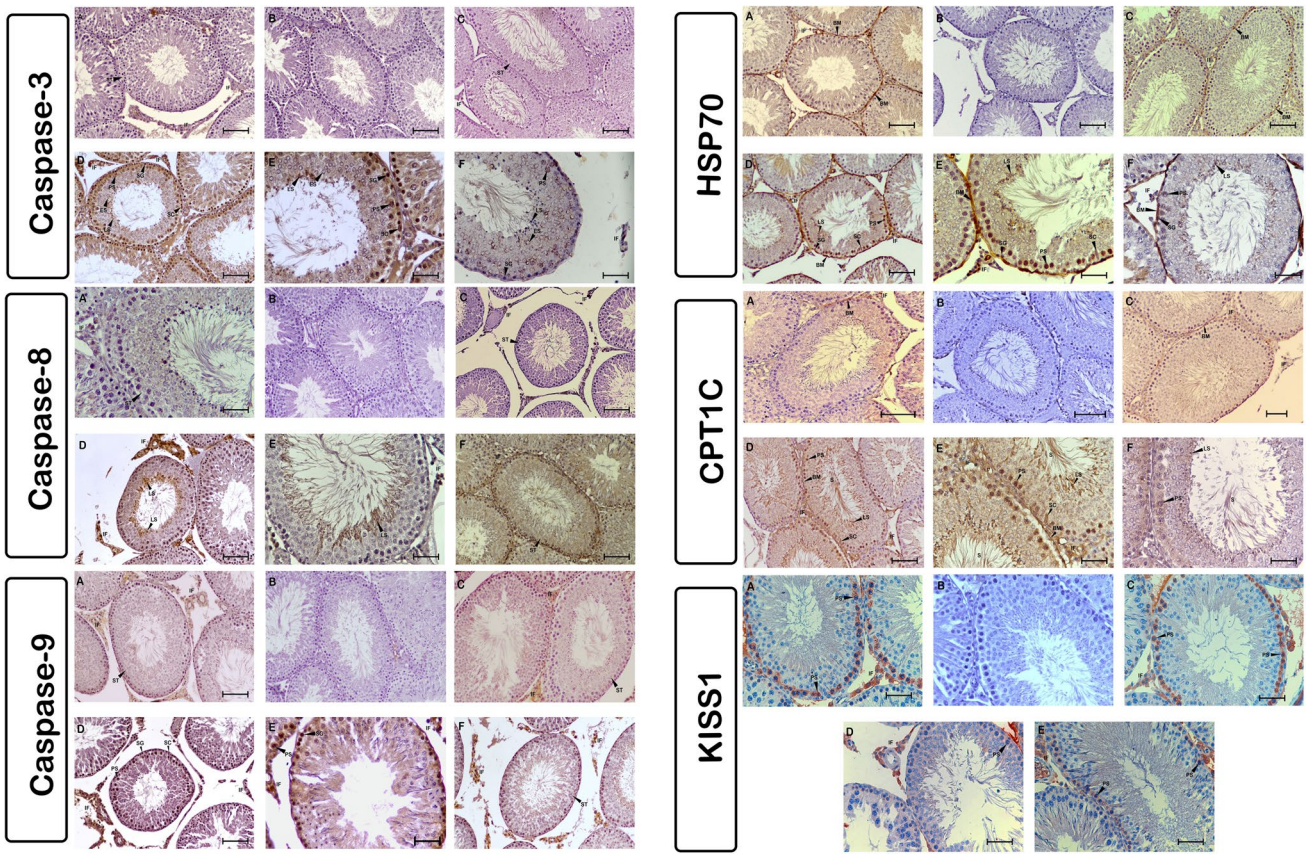

Fig. 3 Control (**A**), RES (**C**). (−) caspase-3 in spermatogenic cells (ST) in seminiferous tubule epithelium, interstitial space, and Leydig cell (IF) (DAB × 20, scale bar = 100 µm). Negative control (**B**). (DAB × 20, scale bar = 100 µm). MTX (**D**, **E**). + + + caspase-3 in spermatogonium (SG), primary spermatocyte (PS), early-stage spermatid (ES), late-stage spermatid (LS), Sertoli cell (SC) and IF (DAB × 20, scale bar = 100 µm), (DAB × 40, scale bar = 50 µm). MTX + RES (**F**). (−) caspase-3 in SG, ES, and LS, + caspase-3 in PS and IF (DAB × 40, scale bar = 50 µm). Control (**A**), RES (**C**). ST (−) caspase-8, + caspase-8 in IF (DAB × 40, scale bar = 50 µm) (DAB × 20, scale bar = 100 µm). Negative control (**B**). (DAB × 20, scale bar = 100 µm). MTX (**D**, **E**). + + + caspase-8 (DAB × 20, scale bar = 100 µm) in LS and IF (DAB × 40, scale bar = 50 µm). MTX + RES (**F**). + caspase-8 in ST and IF (DAB × 20, scale bar = 100 µm). Control (**A**), RES (**C**). (−) caspase-9 in ST, + caspase-9 in IF (DAB × 20, scale bar = 100 µm). Negative control (**B**) (DAB × 20, scale bar = 100 µm). MTX (**D, E**). + + + caspase-9 in SG, PS, SC, and IF (DAB × 20, scale bar = 100 µm) DAB × 40, scale bar = 50 µm). MTX + RES (**F**). (−) caspase-9 in ST, + caspase-9 in IF (DAB × 20, scale bar = 100 µm). Control (**A**), RES (**C**). + HSP 70 in BM and IF (DAB × 20, scale bar = 100 µm). Negative control (**B**) (DAB × 20, scale bar = 100 µm). MTX (**D**, **E**). + + + HSP 70 in BM, SG, PS, LS, SC, and IF (DAB × 20, scale bar = 100 µm), (DAB × 40, scale bar = 50 µm). MTX + RES (**F**). + HSP 70 in BM, SG, PS, LS, and IF (DAB × 40, scale bar = 50 µm). Control (**A**), RES (**C**). BM, IF + CPT1C (DAB × 20, scale bar = 100 µm). Negative control (**B**) (DAB × 20, scale bar = 100 µm). MTX (**D**, **E**). BM, PS, LS, SC, spermium (S) + + + CPT1C (DAB × 20, scale bar = 100 µm) (DAB × 40, scale bar = 50 µm). MTX + RES (**F**). + CPT1C in PS, LS, and S (DAB × 40, scale bar = 50 µm). Control (**A**), RES (**C**). PS, IF +++ KISS1 (AECx40, scale bar=50 µm). Negative Control (**B**) (AECx20, scale bar=100 µm). MTX (**D**). + KISS1 in PS and IF (AECx40, scale bar=50 µm). MTX+RES (**E**). ++ KISS1 immunoreactivity in PS and IF (AECx40, scale bar=50 µm)

RES on spermatogenesis is believed to be linked to the two factors listed below. Firstly, RES, which has a polyphenol structure similar to estrogen, can increase FSH and T concentrations via feedback regulation of the hypothalamic-pituitary–gonadal axis [14]. As a result, RES may function as an agonist in the relevant area, triggering spermatogenesis. On the other hand, RES may mediate a variety of oxidative stress effects within the seminiferous tubules.

Our findings explain the observed positive relationship between apoptosis and DNA damage in testicular cells. The RES group has the highest TAS. When compared to MTX, TOS was highest in the MTX group and lowest in the MTX + RES group. OSI levels were highest in the MTX group and significantly lower in the MTX + RES group when compared to the MTX group. MTX increased the level of TOS in testicular tissue (Table 4). Our findings are similar to those of Daggulli et al. [27] TT and NT were highest in the RES group, while TT and NT were lowest in the MTX + RES group but significantly higher than in the MTX group. The DS level was highest in the MTX group and significantly lower in the MTX + RES group when compared to the MTX group. The MTX group had

Table 6 Immunoreactivity scores of caspase-3, caspase-8, caspase-9, HSP70, CPT1C, KISS1 of testis tissues

	Control				MTX				RES				MTX + RES			
Parameters/scores	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
Caspase-3	ST IF	*	*	*	*	*	*	SG PS ES LS SC IF	ST IF	*	*	*	SG ES LS	PS IF	*	*
Caspase-8	ST	IF	*	*	*	*	*	LS IF	ST	IF	*	*	*	ST IF	*	*
Caspase-9	ST	IF	*	*	*	*	*	SG PS SC IF	ST	IF	*	*	ST	IF	*	*
HSP70	*	BM IF	*	*	*	*	*	BM SG PS LS SC IF	*	BM IF	*	*	*	BM SG PS LS IF	*	*
CPT1C	*	BM IF	*	*	*	*	*	BM PS LS SC S	*	BM IF	*	*	*	PS LS S	*	*
KISS1	*	*	*	PS IF	*	PS IF	*	*	*	*	*	PS IF	*	*	PS IF	*

"*" Indicates that there is no immunoreactivity

significantly higher IMA (Table 5). According to studies in the literature, the levels of TT, NT, DS, and IMA were compared for the first time in relation to MTX-induced testicular damage [28]. Histopathologic examination results back up our biochemical parameters. Immature germinal cells were discovered in the seminiferous tubular lumen of the MTX group. This indicates that the next stage of spermatogenesis is incomplete and impaired [10]. Invagination in the seminiferous tubule and cells from the spermatogenic series shed into the lumen without maturation were observed in the MTX group stained with MSB and PAS. These changes are thought to be the result of MTX-induced oxidative damage. Cell damage is caused by an increase in reactive oxygen species, which causes lipid peroxidation, oxidative damage to DNA and proteins, and apoptotic cell death [29]. Significant caspase-3 immunoreactivity was found in cells from the spermatogenic series (spermatogonium, primary spermatocyte, early-stage spermatid, late-stage spermatid) and Leydig cells from the MTX group in our study. This supports the DNA damage observed in the MTX group. We found that the antioxidant effect of RES significantly reduced the damage caused by MTX in the MTX + RES group. In our study, caspase-8 immunoreactivity was observed in late-stage spermatids and Leydig cells treated with MTX. In the MTX group, there was significant caspase-9 immunoreactivity in spermatagonium, primary spermatocytes, and Leydig cells, negative caspase-9 immunoreactivity in spermatogenic series cells in the MTX + RES group, and decreased caspase-9 immunoreactivity in Leydig cells (Fig. 3), (Table 6). When we evaluate our findings, we can conclude that apoptosis occurred in the experimental groups (MTX). These findings revealed that both intrinsic and extrinsic pathways converge in caspase-3, and they are both employed.

It was found that MTX-treated rats lost weight and showed signs of diarrhea [10]. When the body weights of the rats were compared in our study, decrease was significantly preserved in the MTX group. The MTX + RES group also showed a significant decrease, but this decrease was significantly preserved when compared to the MTX group (Table 3).

HSP70 promotes polypeptide chain folding and activates ATP-dependent mechanisms. Environmental stresses reduce ATP levels, and cell integrity is impaired [30]. According to Kim et al., HSPs play an important role in aging and male fertility [31]. HSP70 was found in high concentrations in spermatogenic series cells, Sertoli, and Leydig cells in the

MTX group in our study (Fig. 3; Table 6). This validated the presence of MTX-induced oxidative stress. Furthermore, the presence of HSP70 in Sertoli and Leydig cells may affect sperm cell development and cause infertility, implying that administering different doses of HSP70 prior to MTX administration may reduce MTX-induced oxidative stress.

KISS1 is a protein that has important effects in the reproductive system. Güvenc et al. reported that co-administration of kisspeptin-10 and MTX alleviated the adverse effects caused by MTX [32]. In our study, KISS1 was markedly labeled in testicular tissues, primary spermatocytes, and Leydig cells of the control and RES groups. In the MTX group, we found a decrease in the severity of KISS1 immunoreactivity in parallel with the formation of structural changes in cells belonging to the spermatogenic series and Leydig cells. In the MTX + RES group, KISS1 immunoreactivity was stronger in cells belonging to the spermatogenic series and Leydig cells than in the MTX group (Fig. 3; Table 6). This suggests that administration of KISS1 in combination with RES at different doses and durations may yield favorable results in male infertility.

CPT1C has been shown to play a key role in cancer cell senescence regulation. CPT1C was found to be significantly reduced in senescent cells based on mRNA and protein analyses. CPT1C was described as a novel biomarker and key regulator of cancer cell senescence via mitochondria-dependent metabolic reprogramming, and it may represent a therapeutic strategy in cancer treatment [33]. We detected CPT1C prominently in spermatogenic cells, Sertoli cells, and Leydig cells in parallel with the occurrence of structural changes in the MTX group. CPT1C levels in spermatogenic cells, Sertoli cells, and Leydig cells were lower in the MTX + RES group than in the MTX group (Fig. 3; Table 6). This recalled the significance of mitochondria in sperm motility. Numerous studies should be conducted to support the effects of different forms of CPT1C on sperm motility, male infertility, and the senescence process. According to our literature review, this is the first study to present CPT1C immunoreactivity on testicular tissue.

Conclusion

When examined in terms of histochemical, immunohistochemical, and biochemical examinations, our study revealed that resveratrol has positive effects on methotrexate-induced acute testicular damage, oxidative stress, and apoptosis. We suggest that various applications of CPT1C may have significant effects on mitochondrial dysfunction and the aging process, as well as on sperm motility, that administration of HSP70 prior to administration may reduce oxidative stress, and that KISS1 may have an antioxidant effect and reduce the side effects of chemotherapeutic drugs. We think that applying it at different doses and times can provide a clearer perspective.

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Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethical Approval In this study, an experimental animal (rat) was used. The necessary permissions were obtained from the Animal Experiments Local Ethics Committee of Suleyman Demirel University's (SDU) Faculty of Medicine (Ethics Committee No. 13–2/2019).

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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