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The Antioxidative effect of Carvacrol on Methotrexate induced testicular damage in rats

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ABSTRACT

Methotrexate (MTX) is an antiproliferative drug used widely in chemotherapy, rheumatoid arthritis, and other autoimmune diseases. Despite this, its long-term administration causes testicular adverse effects. Carvacrol is a phytochemical derived from aromatic plants of the genera Oregano. It is known to have anti-inflammatory and antioxidant effects and to inhibit the growth of a variety of cancer cells. The present study was designed to evaluate the antioxidant effect of carvacrol on MTX-induced testicular damage. Twenty-four adult rats were randomly divided into four groups, six rats each, as follows: Control, Carvacrol (40mg/kg b.w. daily orally), MTX (25 mg/kg b.w single shot I/P) and Carvacrol and MTX. Testicular biomarkers, redox status and Nrf2 gene expression tests were assessed after 15 days. MTX caused alterations in testosterone hormone and total and prostatic acid phosphatases. Furthermore, testicular 8-OHdG and MDA were increased while reduced GSH was decreased indicating testicular oxidative damage. Concomitant administration of carvacrol was able to restore the adverse effects of MTX. This was observed in the revised levels of the mentioned parameters nearly to normal in comparison to the control and MTX groups. In the present study, we report that carvacrol ameliorates MTX-induced testicular damage by protection from oxidative stress, decrease in DNA damage, and enhancement of serum testicular biomarkers

1. INTRODUCTION

Methotrexate is a folic acid antagonist firstly used in children with acute leukemia and is now commonly used in the treatment of a wide range of malignant and non-malignant diseases (Conway et al .2017). However, the drug has several side effects. Cells with a high turnover rate are highly susceptible such as bone marrow, gastrointestinal mucosa, and hair. Also, renal toxicity associated with high dose MTX occurs due to the inverse proportion relation between MTX concentration and renal clearance (Gaies et al.,2012). Oxidative stress has been contributed to the pathogenesis of MTX-induced testicular damage. MTX shows toxic effects on oogenesis, spermatogenesis, and fertility due to oxidative stress. It damages the testis and the structure of the germ cells particularly. It causes a decrease in sperm count and damage to sperm DNA and seminiferous tubules (Koc et al. 2018). Investigations were done on the use of antioxidants as to reduce the side effects resulting from MTX administration.

Antioxidant systems include enzymatic and nonenzymatic antioxidants that are usually effective in blocking harmful effects of ROS (Birben et al. 2012). Plant and essential oil extracts also are used as antioxidants. Carvacrol and thymol are the main components of the essential oils of the Lamiaceae family of plants (Ru´A et al.2019). Carvacrol (CRV) is a phenolic compound found in the essential oil of different aromatic herbs and spices such as thyme (Thymus vulgaris L.), and marjoram (Origanum majorana L.). It has various pharmaceutical properties, such as antioxidant, anti-apoptotic, anti-cancer, and anti-microbial effects (Shoorei et al. 2019). Carvacrol significantly promotes the glutathione (GSH) level due to the removal of ROS through its radical scavenging effects. It also raises total antioxidant capacity levels in cell cultures and animals. So, the administration of CAR might prevent chronic stress-induced tissue damage through protection against oxidative stress (Samarghandian et al. 2016).

This study was carried out to investigate the antioxidative effect of carvacrol following testicular damage induced by MTX in rats. Biochemical tests were done to detect adverse changes in rat testes after MTX exposure and possible protective role of carvacrol.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Methotrexate and Carvacrol were obtained from Cayman Chemicals, USA. A diagnostic kit for Gene Jet Genomic DNA purification kit was obtained from (Thermo Scientific, USA) and a Commercial 8-hydroxy 2-deoxyguanosine (8-OH-dG) ELISA kit was obtained from (Abcam, Cambridge, UK). Total RNAs Extraction kit using RNeasy Mini Kit, miScript II RT Kit for Reverse transcription, and Rotor-Gene SYBR Green PCR Kit were obtained from (Qiagen, Germany). Serum Testosterone, acid phosphatase, prostatic acid phosphatase ELISA kits, GSH and MDA kits were obtained from Sigma Aldrich, USA.

2.2. Animals, Experimental design and Sampling

Twenty-four male albino rats weighing 130 ± 20 g (obtained from medical research institute, Alexandria University). The animals were housed under standard conditions of temperature and light, fed on a standard diet, and given water *ad libitum* for 2 weeks to acclimatize to laboratory conditions before the start of the experiment. The rats were randomly divided into four groups each consisting of six animals as the following design:

Group (1): Control rats fed on basal diet, distilled water *ad libitum* daily for 15 days.

Group (2): Carvacrol treated group, the rats were treated orally with carvacrol at dose 40mg/kg. (Dagli Gul, 2013) daily for 15 days with exception at day 8 (40ml carvacrol is dissolved in 1ml tween).

Group (3): MTX treated group, the rats were fed on basal diet, distilled water ad libitum daily for 15 days and treated with MTX I/P at dose 25mg/kg (STORM, 1985) (25mg is dissolved in 25 ml PBS and added distilled water until 100 ml) a single shot at day 8.

Group (4): Carvacrol and MTX group, the rats were treated orally with carvacrol at dose 40mg/kg. daily for 15 days with exception at day 8 and treated with MTX I/P at dose 25mg/kg a single shot at day 8.

The day following the last doses, rats were fastened, and blood samples were withdrawn from the plexus of eye of each rat. The animals then were sacrificed by cervical dislocation and the testis were dissected quickly and weighed. The left testis was kept at -80°C and used for analysis of gene expression and oxidant/antioxidant indices.

2.3. Biochemical analysis

2.3.1. Assay for serum T, ACP, and PAP

After blood collection, coagulation occurred and then centrifugation at 3000 rpm for 10 min and kept at -20°C until analysis. Serum concentration of testosterone, acid phosphatase and prostatic acid phosphatase activities were determined using enzyme-linked immunosorbent (ELISA) test kits according to the manufacturer's instructions.

2.3.2. Oxidative stress assessment

The excised testis tissues were rinsed with saline and then homogenized in phosphate buffer saline (PBS) pH 7.4 in the ratio of 1:10 (0.25 gm of tissue in 2.25 ml PBS). After homogenization, the homogenates were centrifuged at 10000 rpm, at 4°C for 20 minutes. The supernatants were divided into aliquots and stored at -20°C for subsequent determination of GSH, MDA (as markers for LPO) and 8-OHdG. The enzymatic method described by (Griffith et al,1980) was used to measure the total glutathione and GSSG content.

Malondialdehyde in whole homogenate was determined according to the method (Draper and Hadley, 1990). 8-OH-dG, as indicator for oxidative DNA damage, was measured in DNA samples using a commercial 8-OH-dG ELISA kit following the manufacturer's protocol.

2.3.3. Gene expression analysis using RT-PCR.

Quantitative analysis of NRF2 in the testicular tissues was performed using quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR). First, the total RNA was isolated from the tissues using RNeasy Mini Kit according to the manufacturer instructions, then the isolated RNA was reverse transcribed by reverse transcriptase enzyme into complementary DNA (cDNA) using miScript II RT Kit, then amplified and detected using specific primers by real-time PCR using Rotor-Gene SYBR Green PCR Kit. A normalizer or reference gene (GAPDH) was used as internal control for experimental variability in this type of quantification. The specific primer sets for each gene is represented in Table 1.

Table 1. Primer sequences of NRF2 and GAPDH

Gene	Accession No.	primer sequence	
NRF2	NM_031789.2	F:	5'-CAAATCCCACCTTGAACACA-3'
		R:	5'-CGACTGACTAATGGCAGCAG -3'
GAPDH	NM_017008.4	F:	5'-GGGTGTGAACCACGAGAAATA-3'
		R:	5'-AGTTGTCATGGATGACCTTGG-3'

2.4. Statistical analysis

Data were statistically analyzed General Linear Model's procedures of SAS GLM (SAS, 2004). Duncan's multiple range tests has been used for multiple comparison between means at P<0.05 (Duncan, 1955). Kolmogorov-Smirnov's test has been used to test the normal distribution of data.

3. RESULTS

3.1. Serum T, ACP, and PAP

Figure 1 showed a decrease in testosterone levels in MTX group (1.60±0.14 ng/ml) in compare with the control group, while it was increased again in group cotreated with carvacrol with MTX (1.86±0.17 ng/ml) in compared to MTX group. Also, an increase in ACP (40.00±2.66 units/ml) and PAP (1.375±0.08 units/ml) was observed in MTX group compared to the control and decreased in carvacrol with MTX group compared to MTX group.

3.2. Testis weight

In the present study, data represented in Figure 2 showed that MTX administration caused decrease in testis weight (1.92±0.18 gm) when compared to control ones. Moreover, treatment of MTX-intoxicated rats with carvacrol showed improvement in testis weight (2.32±0.14 gm) in comparison to MTX ones.

3.3. Oxidative/Antioxidant status

In figure 3, MTX caused an increase in MDA (9.55±0.33 nmol/g Tissue) and 8-OHdG (5.98±0.19 pg/µg DNA) in compare with control group indicating its testicular damage effect. Notably, levels of 8-OH-dG (4.00±0.18 pg/µg DNA) and MDA (6.86±0.17 nmol/g Tissue) were significantly decreased in MTX and carvcarol group compared to MTX treated group.

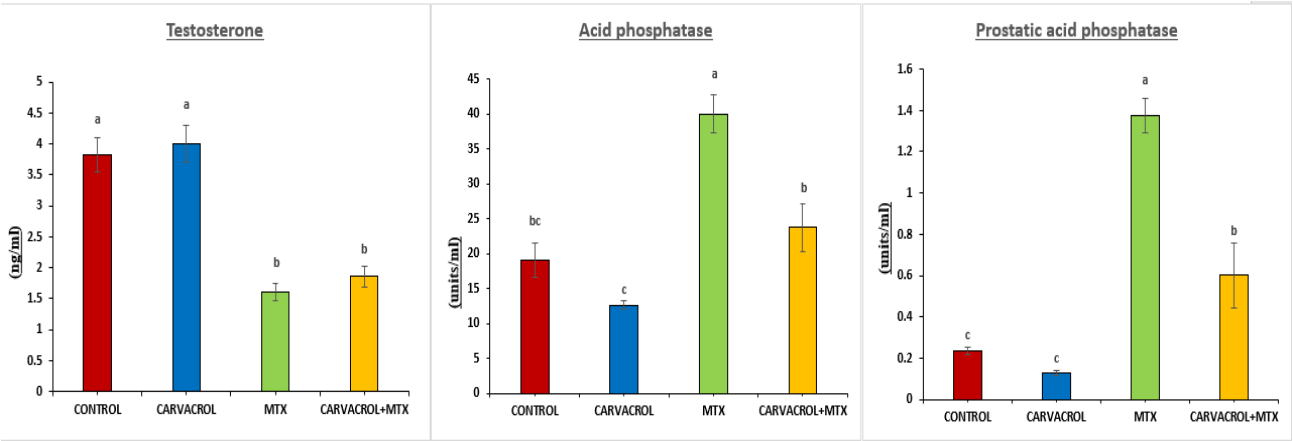

Fig 1: Effect of carvcrol and MTX on testicular damage
Means denoted within the same row with different superscripts are significantly (P<0.05).

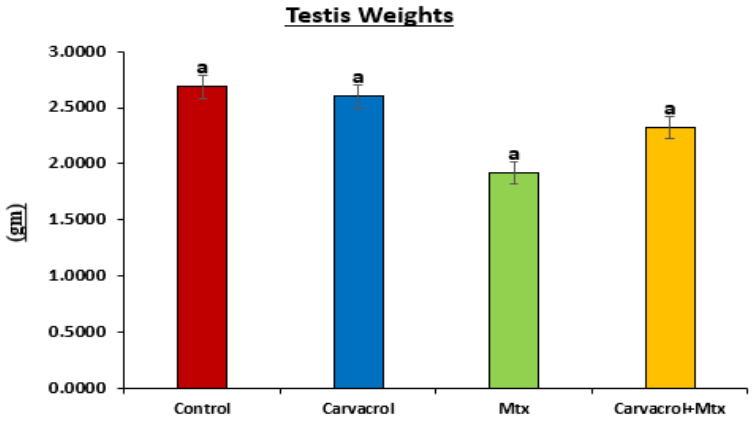

Fig 2. Effect of carvacrol and MTX on testicular weight. Means denoted within the same row with different superscripts are significant (P<0.05).

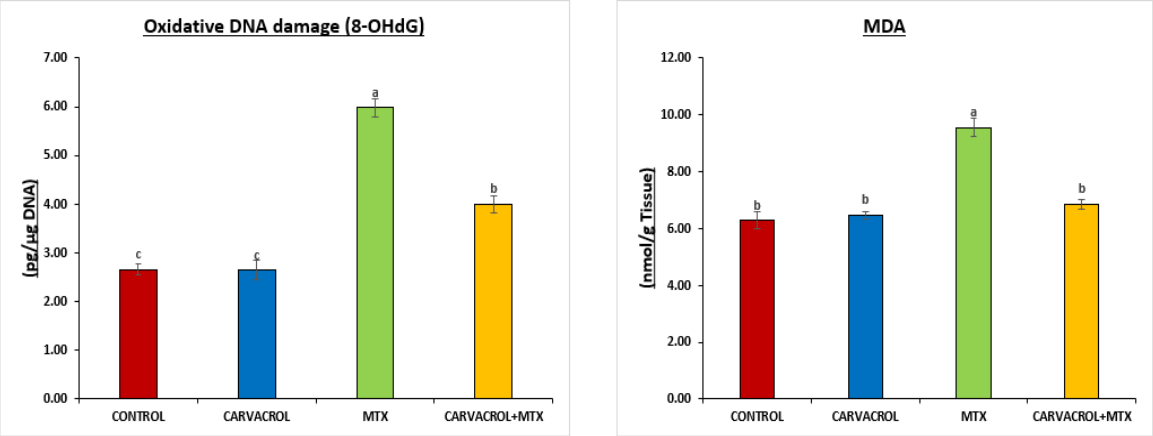

Fig 3: Effect of carvacrol and MTX on oxidative stress. Means denoted within the same row with different superscripts are significantly (P<0.05).

Data represented in figure 4 showed that MTX also there was an increase in GSSG (0.24±0.01 umol/g Tissue), a decrease in total GSH (1.98±0.05 umol/g Tissue) and reduced GSH (1.50±0.03 nmol/g Tissue) with a significant decrease in GSH/GSSG ratio (6.37±0.29) in MTX group as compared to control ones. Interestingly, MTX-intoxicated rats co-treated with carvacrol showed increase in levels of total GSH (2.16±0.09 umol/g Tissue), reduced GSH (1.85±0.08 nmol/g Tissue) and GSH/GSSG ratio (11.87±0.45) with decrease in GSSG (0.16±0.01 umol/g Tissue) compared to MTX group.

3.4. Nrf2 gene expression

In figure 5, gene expression of Nrf2 in MTX treated group was significantly decreased in testis tissue (0.45±0.04-fold change) as compared to control group. Moreover, treatment of rats with carvacrol non-significantly changed (1.08±0.12-fold change) the gene expression of Nrf2 compared to control ones. While treatment of MTX-intoxicated rats with carvacrol showed increase in Nrf2 levels (0.85±0.10 fold change) upon compare to the MTX group.

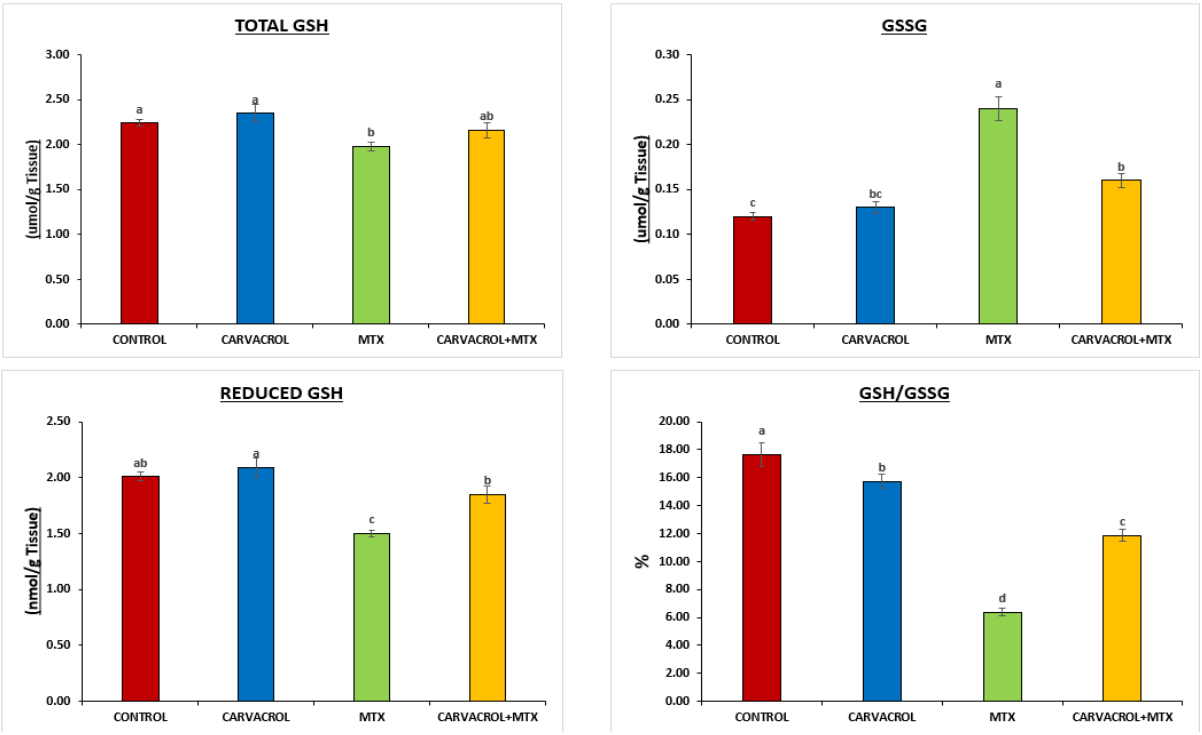

Fig 4: Effect of carvacrol and MTX on oxidative stress
Means denoted within the same row with different superscripts are significantly (P<0.05).

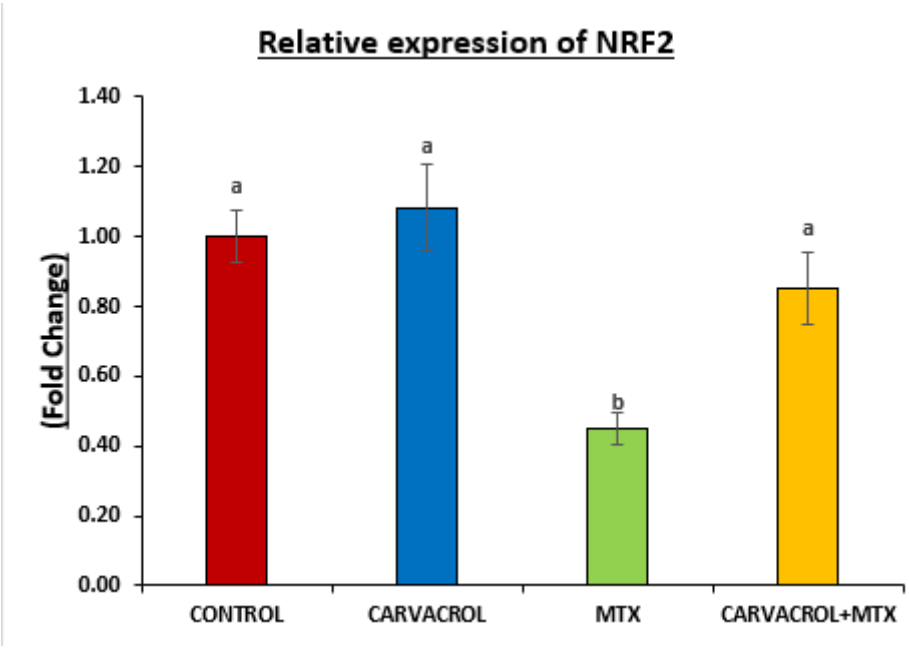

Fig. 5: Effect of carvacrol and MTX on Nrf2 gene expression
Means denoted within the same row with different superscripts are significantly (P<0.05).

4. DISCUSSION

Methotrexate (MTX) is an anticancer drug used in treatment of other diseases such as psoriasis and other inflammatory diseases by interfering with cellular replication (Ojo et al., 2019). Testicular adverse effects are important side effects of MTX. MTX was found to damage the testicular tissue and germ cells by reducing the antioxidant factors of the cells and causing the harmful effects of free oxygen radicals. The imbalance between the ROS and antioxidant system leads to oxidative stress (Pınar et al., 2018). Carvacrol is a monoterpenic phenol produced by an abundant number of aromatic plants

as thyme and oregano (Suntres et al., 2013). It can decrease oxidative stress-related damages because of its radical scavenging capabilities as it promotes the activity of the enzymatic antioxidants such as catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and, glutathione s-transferase (GST) and increasing the levels of nonenzymatic antioxidants including vitamin E, vitamin C, and the reduced form of glutathione (GSH) (Evazalipour et al., 2020). The current study considered MTX reverse effects on testicular biochemical markers, oxidative change and gene expression changes and the protective effect of carvacrol.

The current data revealed that testosterone levels were decreased due to MTX administration. This was attributed to the oxidative stress condition which causes damage to the Leyding cells and decreases the levels of testosterone. Also, MTX is known to reduce testosterone biosynthesis secondary to its suppressive effects on LH levels (Vanishree et al., 2020) due to downregulation of its receptors on Leydig cells (Mansour et al., 2021). This is parallel with Akacha et al. (2020) and Felemban et al. (2020) who showed that MTX causes decrease in testosterone level. On the other hand, testosterone levels in carvacrol treated group is nearly the same as in the control group and slightly enhanced in the group treated with MTX and carvacrol together. Shoorei et al. (2020) and Araghi et al. (2017) also showed the same effect of carvacrol on testosterone and how it enhances its level. This is due to the antioxidative effect of carvacrol.

Total acid phosphatase showed an increase after MTX administration in our study. Al-Motabagani (2006) stated the increase in acid phosphatase in degenerated areas of the liver was due to increased lysosomal activity and subsequent increase in their enzymes as ACP. Also, an increase in acid phosphatase after methotrexate injection was shown in liver by Soliman (2009). Interestingly, carvacrol ameliorated the effect of MTX on ACP levels, as there was decrease in the group treated with MTX and carvacrol than the group treated with MTX only. The same was stated by Mohammed (2017) and Mokrane et al. (2019).

Current results showed that MTX caused reduction in testicular weight compared to the control group. This was also showed by Vanishree et al. (2021) and Akacha et al. (2020). This may be referred to decrease in the seminiferous tubules and lowered number of germ cells, alongside with spermatogenesis inhibition and decreased steroidogenic enzyme activity (Felemban et al., 2020). Administration of carvacrol with MTX showed improvement in testicular weight compared to the MTX group. Arkali et al. (2020) showed recovery in testicular weight in carvacrol treated diabetic rats compared to diabetic ones without carvacrol administration.

ROS can damage the DNA structure when they react with guanine bases forming 8-hydroxy-2′-deoxyguanosine (8-OHdG) (Andrés et al., 2020), which is a biomarker used in oxidative stress to express the severity of DNA damage in testicular destruction (Belhan et al., 2017). Increased oxidative stress causes shape and structural changes of the nucleus by causing DNA fragmentation and denaturation, which play a significant role in the initiation of apoptosis (Vardi et al. 2010). Our study revealed that MTX increased significantly 8-OHdG levels in testicular tissue. This is similar to those reported by Belhan et al. (2017) and Kanpalta et al. (2021). Additionally, Akdemir et al. (2018) recorded that MTX (20 mg/kg single dose, IP) causes high levels in 8-OHdG in liver tissue. Concerning carvacrol in our study, its administration improved the level of 8-OHdG after MTX treatment compared to the group administrated MTX only with no significant difference compared to the control group. Yeliz Kılıc et al. (2015) showed that treatment by carvacrol at different dosages 50, 100 and 200 mg/kg (single I/P shot) decreased 8-OHdG level that were increased by cerulin induced acute pancreatitis. Moreover, Kandemir et al. (2021) reported that carvacrol treatment significantly decreased 8-OHdG levels in liver and kidney as comparison with the cadmium group.

In the current study, MTX causes decrease in levels of total and reduced GSH, and GSH/GSSG ratio and causes increase in levels of GSSG (oxidized glutathione) and MDA in the MTX group in compared to the control group. Previous studies (Wang et al., 2018 and Belhan et al., 2018) showed that MTX increases MDA level with decrease in GSH levels, the same as our results. Also, Öktem et al. (2006) confirmed that MTX, at dose 20 mg/kg I/P, causes increase in MDA levels and decrease in GSH levels in kidneys. Moreover, MTX elevated liver MDA levels assured by Vardi et al. (2010). Considering carvacrol, our results support the idea that carvacrol protects the cells against oxidative stress through considerable decrease in MDA levels with increase of total and reduced GSH levels compared to MTX-treated group and nearly the

same level compared to the control group. These results have been previously reported by Banji et al. (2013) in liver and Selimoğlu Şen et al. (2014) in lungs where carvacrol administration decreased the oxidative stress compared to MTX-treated group. Also, Araghi et al. (2017) confirmed the antioxidant effect of carvacrol against ketamine induced testicular damage by decreasing MDA levels and increasing the antioxidant enzymes levels.

Moreover, a decline in the gene expression of Nrf2 was observed in our study. Nrf2 is an emerging regulator of cellular resistance to oxidants. Ebrahimi et al. (2019) stated that MTX causes downregulation to Nrf2 in hepatocytes due to intensive ROS formation. Also, Mansour et al., 2021 showed a decline in the gene expression of Nrf2 in rat testicles after MTX administration which affects the antioxidant mechanism and exposing the germ cells to oxidative stress. On the other hand, a marked recovery was observed in carvacrol treated group as Nrf2 expression level was improved, supporting that carvacrol possess free-radical scavenging and antioxidant property. This is parallel with Arkali et al. (2020) and Naeem et al., (2021) who stated a significant increase and considerable upregulation in Nrf2 expression levels.

Conclusion

From the previous results, it could be concluded that carvacrol showed promising antioxidative properties as it successfully improved biochemical oxidative alterations caused by MTX and provided some degree of protection against MTX testicular damage.

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