Protective Effect of Proanthocyanidin against Methotrexate-Induced Testicle Damage in Rats

Mehmet Yüncü1, Gülşen Zengin2, Hülya Birinci1, Nuray Bostanciéri1, Sait Polat2

1Department of Histology and Embryology, Gaziantep University, School of Medicine, Gaziantep, Turkey
2Department of Histology and Embryology, Çukurova University, School of Medicine, Adana, Turkey

ABSTRACT
Objective: Methotrexate (MTX) is a widely used chemotherapeutic agent showing side effects such as hepatotoxicity and also testicular damage due to an increase in the production of reactive oxygen species. Proanthocyanidins (PACs) are known as natural antioxidants that have protective effects against oxidative stress. This study investigates the protective effect of PAC against the MTX-induced testicular damage.

Methods: Twenty-six male Wistar albino rats were divided into four groups. Saline (2 cc/kg) was administered to the control group, and PAC (200 mg/kg) was administered to the PAC group for 7 days. The MTX group received a single dose of MTX (20 mg/kg) on Day 3. A total of 200 mg/kg of PAC for 6 days was administered to the PAC+MTX group, and the group also received a single dose of 20 mg/kg of MTX on Day 3. The animals were sacrificed on Day 8. Analyses were performed, in addition to light and electron microscopic examinations on the testicular samples.

Results: Histopathological findings such as thinning of the seminiferous tubule epithelium and decrease in spermatogonial cells were detected in the MTX group. Electron microscopic findings indicated similar damage. The MDA levels increased in testicular tissues, while the SOD and GPX levels decreased significantly. The tissue damage and deterioration in biochemical parameters were decreased in the PAC+MTX group.

Conclusion: The results show that PAC does not completely prevent damage to the testicles caused by MTX but that it has an important protective effect.

Keywords: Methotrexate, proanthocyanidin, testis

INTRODUCTION
Methotrexate (MTX), a folic acid precursor, is widely used in the treatment of malignant tumors and some non-neoplastic diseases. Chemotherapeutic drugs such as MTX act on the S phase of the cell cycle by inhibiting the synthesis of DNA precursors. As a result, organs with high mitochondrial activity, such as the testicular tissue, become targets of these drugs (1). It has been reported that the drugs used in cancer treatment cause a decrease in the diameter of the lumen of the seminiferous tubule, and the degeneration and shedding of germ cells (2). Oxidative stress plays an important role in testicular damage and pathogenesis resulting from the use of MTX (2, 3).

Oxygen-derived free radicals or reactive oxygen species (ROS) are produced during normal metabolism and energy production of the body. However, if ROSs exceed the antioxidant capacity of the biological system, they cause an imbalance, known as oxidative stress, due to metabolic and other environmental factors. This can damage biological molecules, such as lipids, proteins, polysaccharides, and DNA. Scientific interest in antioxidant compounds, especially those from plants, has been increasing over recent years. The discovery of the protective function of phytonutrients against cancer and other neurodegenerative diseases, and the growing concern about synthetic antioxidants, have made natural antioxidants more attractive (4).

Proanthocyanidins (PACs) are natural antioxidants found in the seeds, flowers and shells of fruits, vegetables, nuts, and especially grapes (Vitis vinifera) (5). PACs have biological and pharmacological properties and therapeutic effects against free oxygen radicals and oxidative stress (6, 7). Abundant amounts of grape seed extract containing PACs have been shown to protect against cardiovascular diseases, nephropathy, atherosclerosis, and neuropathy (8-12). In addition, its anti-apoptotic, anti-inflammatory and antimicrobial effects have been demonstrated through animal culture studies, as well as its anticancerogenic effects (13) through cell culture studies (9-11, 14).

In this study, the effects of PAC on the testicles of rats who were administered MTX were investigated through light microscopy, electron microscopy, and on a biochemical level.

ORCID IDs of the authors: M.Y. 0000-0002-2519-6834; G.Z. 0000-0002-0853-1362; H.B. 0000-0002-6749-9665; N.B. 0000-0002-3765-8274; S.P. 0000-0003-1646-8831.
Corresponding Author: Mehmet Yüncü E-mail: yuncu@gantep.edu.tr
Received: 11.09.2018 • Accepted: 09.10.2018
**METHODS**

In our study, 26 young adult Wistar albino rats with a mean age of 5-6 months old were used. The animals were housed in wire cages in rooms where the ambient light was set to 12-hour darkness and 12-hour light, and the ambient temperature was fixed at 21±1°C. During the experiment, all animals were fed ad libitum. All animals were weighed, and the weights were recorded at the beginning of the study and before the sacrifice. All the procedures carried out in our study were approved by Animal Experiments Local Ethics Committee of Kahramanmaraş Sütçü İmam University (decision date 05.05.2015, decision number 07).

All chemicals used in our study were of the analytical grade and were from the Sigma Chemical Company (SigmaAldrich; St. Louis, Missouri, USA).

**Experimental Groups**

The experimental animals were divided into four groups: the control group, PAC group, MTX group, and MTX+PAC group, where the control group had 5, and other groups had seven rats each (Table 1).

**Collecting the Tissues**

All animals were sacrificed 24 hours after the last administration (day 8). The animals’ testicles were removed, cleaned from the surrounding tissues, and weighed. For each animal, the right testicles were placed in 10% neutral formaldehyde for a follow-up with light microscopy. A small fraction of the left testicles was placed in a 5% glutaraldehyde solution for electron microscopy, and the remaining part was stored at −20°C for biochemical procedures.

**Calculation of Testicle Weight Index**

The testicle weight index (TWI) values for each animal were determined by calculating the body weight measured on the last day of the experiment and the sum of the right and left testicle weights of the same animal according to the following formula.

\[
TWI = \frac{\text{[right+left testicle total weight]/body weight}}{100}
\]

**Tissue Biochemical Analysis**

The tissues stored in a cold environment (−20°C) were brought up to +4°C just before the experiment. The dissolving tissue samples were weighed in their frozen form and put in glass tubes. Superoxide dismutase (SOD), glutathione peroxidase (GPX), and malondialdehyde (MDA) analyses were performed on the tissues.

**Determination of MDA**

The MDA of the homogenates was determined spectrophotometrically by measuring the existence of thiobarbituric acid reactive substances (TBARS), as defined by Altintas et al. (15). Phosphoric acid (1%) at 3 mL and 0.6% thiobarbituric acid solution at 1 mL were added to 0.5 mL of homogenate. The mixture was heated in boiling water for 45 min. After the mixture was cooled, the colored part was extracted into 4 mL of n-butanol. The absorbance was measured by a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at 532 nm. The amount of lipid peroxides was calculated as the TBARS of lipid peroxidation.

**Determination of the SOD Activity**

Total SOD activity was determined as defined by Parlaktas et al. (16). The principle of this method is the prevention of nitroblue tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as asperoxide generator. One unit of SOD was defined as the amount of enzyme to cause 50% inhibition in the NBT reduction rate. The results were given in Units per gram of protein.

**Determination of the GPX Activity**

The GPX activity was measured as defined by Parlaktas et al. (16). An enzymatic reaction in a tube containing nicotinamide-adenine dinucleotide phosphate (NADPH), glutathione (GSH), sodium azide, and glutathione reductase was initiated by adding \( \text{H}_2\text{O}_2 \); the change in absorbance at 340 nm was observed using a spectrophotometer. The results were given in Units per milligram of protein.

**Preparation of Tissues for Light Microscopy**

After the 10% formaldehyde fixation, paraffin blocks were prepared by routine procedures on a tissue-monitoring device (TP 1010; Leica, Wetzlar, Germany).

Sections that were 5-6 μ thick were taken from the prepared paraffin blocks with a microtome (RM2245; Leica, Wetzlar, Germany) device. The preparations were stained with hematoxylin-eosin (HE), analyzed under a light microscope, and photographed. For assessing spermatogenesis in testicular tissues, the Johnsen score has determined. A minimum of 50 tubules were evaluated, and each tubule was given a score from 1 to 10.

**Measurement of Tubular Diameter (TD) and Tubular Epithelial Thickness (TET)**

Light microscopic sections were examined with the microimage software (cellSens Analysis Systems; Olympus, Tokyo, Japan) and

---

**Table 1. Experiment schedule**

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, n=5</td>
<td>Sa</td>
<td>Sa</td>
<td>Sa</td>
<td>Sa</td>
<td>Sa</td>
<td>Sa</td>
<td>Sa</td>
</tr>
<tr>
<td>PAC, n=7</td>
<td>PAC</td>
<td>PAC</td>
<td>PAC</td>
<td>PAC</td>
<td>PAC</td>
<td>PAC</td>
<td>PAC</td>
</tr>
<tr>
<td>MTX, n=7</td>
<td>Sa</td>
<td>Sa</td>
<td>MTX+Sa</td>
<td>Sa</td>
<td>Sa</td>
<td>Sa</td>
<td>Sa</td>
</tr>
<tr>
<td>PAC+MTX, n=7</td>
<td>PAC</td>
<td>PAC</td>
<td>MTX+PAC</td>
<td>PAC</td>
<td>PAC</td>
<td>PAC</td>
<td>PAC</td>
</tr>
</tbody>
</table>

PAC: proanthocyanidin, 200 mg/kg (gavage); MTX: methotrexate, 20 mg/kg (intraperitoneal); Sa: saline, 2 cc/kg (gavage)
a light microscope (DM 750; Leica, Wetzlar, Germany). In each group, morphometric analyses were performed by measuring the diameters of ten seminiferous tubules of each animal, two measurements per tubule, and through germinal epithelial thickness measurement from four different regions of the same tubule with 10X magnification and averaging them.

**Preparation of Tissues for Electron Microscopy**
Tissue samples were fixed with 5% glutaraldehyde and 1% osmium tetroxide solutions prepared with Millonig’s phosphate buffer. After dehydration with the alcohol series, the tissues were immersed in the rotator overnight in the embedding material (resin), and the Epon blocks were prepared the next day. The thin sections obtained from the blocks were placed on a copper grid and after being contrasted with uranyl acetate and lead citrate, they were analyzed with Transmission Electron Microscopy (JEM 1400; Jeol, Massachusetts, USA), and photographs were taken.

**Statistical Analysis**
Biochemical data were analyzed using the Statistical Package for the Social Sciences 22.0 package program (SPSS IBM Corp.; Armonk, NY, USA). An analysis of variance (ANOVA) test was used to assess whether there was a significant difference between the groups’ averages. Values with a p-value <0.05 were considered statistically significant. Post-hoc tests were performed to assess the difference between the groups in the parameters that resulted in a significant difference between the group averages. Taking the homogeneity of the variances of the groups into account, the Tukey test was applied for the parameters of SOD and GPX, which had a greater value than p=0.05. For the MDA with a p-value <0.05, the Games Howell test was applied to see among which groups the group averages differ significantly.

The statistical SPSS 22.0 package program was used for the statistical evaluation of tubular diameter and epithelium thickness measurements. The normal distribution of the numerical data in this parameter was tested with the Shapiro-Wilk test. The ANOVA test and the Least Significant Difference (LSD) test were used for comparison of the variables that fit normal distribution.

**RESULTS**

**Testicle Weight Index**
The difference between the TWI values of the groups was not statistically significant (p=0.447; Table 2).

**Biochemical Findings**
The levels of SOD, GPX, and MDA in the testicular tissues of the groups are shown in Table 3.

While SOD significantly decreased (p<0.001) in the MTX group compared with the control group, it increased (p=0.002) again in the PAC+MTX group, and its difference from the control group became insignificant (p=0.096). There was also a significant difference between the MTX group and the PAC group (p<0.001).

The results of GPX were found similar to SOD. The level of GPX in the PAC+MTX group was high (p=0.002), while in the MTX group, there was a decrease in the GPX level compared to both the control group (p=0.015) and the PAC group (p=0.003). The difference between the PAC group and the PAC+MTX group was not significant (p=0.952).

There was no significant MDA difference between the control and PAC groups (p=0.468), but it was significantly higher in the MTX group than the control group (p<0.001). In the PAC+MTX group, it decreased with respect to the MTX group (p<0.001). The difference between the PAC group and the PAC+MTX group was not significant (p=0.050). The increase in the PAC+MTX group was significant compared to the control group (p=0.003).

**Light Microscopic Findings**

**Histological analysis**
Samples from the control group and the PAC group showed normal testicular histology (Figure 1). Thinning and vacuolization in the seminiferous tubule, degeneration in the spermatogonium cells and shrinkage of the nuclei, and interstitial edema and congestion in some vessels were observed in MTX group (Figure 2). In MTX+PAC group, there was reduced tubular damage, the spermatogenic cells were preserved and the spermatogenesis continued, while the change in the spermatogonia was not as severe as in the MTX group, although some tubular damage in several tubules persisted. In the interstitial area, although there was a small amount of edema, no congestion was seen (Figure 3).

**Johnsen score analysis**
According to the score results, in the PAC group, there was no difference according to the control group, statistically (p=0.116). The MTX group showed impaired spermatogenesis, and this difference was statistically significant (p<0.001). The MTX+PAC
group was similar to the control and PAC groups, and there was no difference statistically (p=0.093) (Table 4).

Tubule diameter and epithelium thickness measurements
While the diameter of the seminiferous tubules was significantly lower in the MTX group than in the control group (p=0.001), the PAC+MTX group maintained values close to the control group. Testicular epithelium thickness was again thinner in the MTX group than in the control group (p<0.001) and was normal again in the PAC+MTX group (Table 5).

Electron Microscopic Findings
In the control group, normal testicular structures were observed in general (Figures 4, 5). In MTX group, the membrane propria surrounding the seminiferous tubule showed thickening, an increased amount of collagen fibers, and irregularity in the basal lamina (Figure 6). There were indentations between the spermatogonium cells in the tubule epithelium and degradation of the Sertoli cell nucleus (Figure 7). It was observed that the agranular endoplasmic reticulum cisternae in the Sertoli cells...
expanded, while lysosomes and lipid droplets increased. Corruptions were observed in the structures of primitive spermatocytes in the seminiferous tubule epithelium, and apoptotic cell structures were seen in the mitochondria, along with some shrinking (Figure 8). Fragmented Leydig cells and nuclei in the interstitial area and small lipid droplets were worthy of note (Figure 9). The PAC group images were similar to those in the control group (Figure 10). When MTX and PAC were administered together in the MTX+PAC group, the pathological findings in the MTX group partially disappeared (Figure 11).

DISCUSSION
Medicines used in chemotherapy generally have adverse effects on all cells in division (17). MTX, a folic acid antagonist, is used in the treatment of diseases, such as lymphoma, osteosarcoma, neck tumors, lung and breast cancers, and testicular cancer (1, 18). Despite its therapeutic effects, MTX is known to cause thinning of the testicles in the walls of the seminiferous tubules, permanent azoospermia, damage to the sperm DNA, abnormalities in the sperm head morphology, and infertility (19-22).

Seminiferous tubule atrophy in the MTX treatment and apoptosis in spermatocytes are thought to be related to the increase of reactive oxygen radicals, and a number of studies have been conducted on the use of antioxidant substances to reduce these side effects (17, 19-21). MTX also reduces the NADPH in cells, leading to glutathione depletion, thereby weakening the cell against ROS and increasing oxidative stress (23). MTX can damage intercellular connection complexes and cause cells to spill into the lumen.

In our study, the light and electron microscopy findings for the MTX group were similar to those of previous studies, including...
the damage and thinning of the seminiferous epithelium and interstitial edema and congestion (17, 19, 21, 24). Similar to previous studies, the MDA increase in the MTX group and the decreases in the SOD and GPX levels supported these findings.

It is now known that testicular oxidative stress usually causes male infertility in normal or pathological conditions. It has been reported that about 50% of male infertility is due to oxidative stress (25). For this reason, the use of natural antioxidants has become necessary.

In our study, the PAC treatment prevented histopathological findings caused by MTX and improved biochemical parameters that were deteriorated. The electron microscope findings also support these results. There are numerous studies in the literature that show such effects of PAC (5, 12, 14, 25).

Malondialdehyde is a stable end-product of lipid peroxidation generated by ROS, and grape extracts rich in PAC have been shown to reduce elevated MDA levels (9-12, 26).

CONCLUSION

Our study has shown that MTX significantly damages the testicles and that PAC significantly reduces the severity of such damage, even if it does not completely reverse it.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Kahramanmaras Sütçü İmam University (decision date 05.05.2015, decision number: 07).

Peer-review: Externally peer-reviewed.


Conflict of Interest: The authors have no conflicts of interest to declare.
Financial Disclosure: The authors declared that this study has received no financial support.

REFERENCES
25. Sönmez MF, Tascioglu S. Protective effects of grape seed extract on hepatic tissue injury in diabetic rats. Bratisl Lek Listy 2017; 118: 212-6. [CrossRef]
28. Güvenç M, Aksakal M. Ameliorating effect of kisspeptin-10 on methotrexate-induced sperm damages and testicular oxidative; stress in rats. Andrologia 2018; 50: e13057. [CrossRef]
29. Sönmez MF, Tascioglu S. Protective effects of grape seed extract on cadmium-induced testicular damage, apoptosis, and endothelial nitric oxide synthases expression in rats. Toxicol Ind Health 2016; 32: 1486-94. [CrossRef]

How to cite: