**Araştırma Makalesi / Research Article**

**DOI: https://doi.org/10.35414/akufemubid.1424423**

AKÜ FEMÜBİD **24** (2024) 061003 (1275-1284) AKU J. Sci. Eng. **24** (2024) 061003 (1275-1284)

***\*Makale Bilgisi / Article Info***

Alındı/Received: 23.01.2024

Kabul/Accepted: 11.08.2024

Yayımlandı/Published: xx.xx.xxxx

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| **Immunotherapeutic and Cell-Protective Effects of Probiotic Kefir on Cyclophosphamide‐induced Nephrotoxicity and Urotoxicity in Rats** |
| **Probiyotik Kefirin Sıçanlarda Siklofosfamid Kaynaklı Nefrotoksisite ve Ürotoksisite Üzerine İmmünoterapötik ve Hücre Koruyucu Etkileri** |

**Songül ÇETİK YILDIZ1[](http://orcid.org/0000-0002-7855-5343) , Cemil DEMİR1[](https://orcid.org/0000-0002-6365-0196), Mustafa CENGİZ2\*,[](http://orcid.org/0000-0002-6925-8371), Halit IRMAK3[](https://orcid.org/0000-0002-8184-9377) , Betül PEKER CENGİZ4[](https://orcid.org/0000-0002-2503-7446) ,**

**Adnan AYHANCİ5[](http://orcid.org/0000-0003-4866-9814)**

*1 Mardin Artuklu University, Health Services Vocational School, Department of Medical Services and Techniques, Mardin, Türkiye*

*2 Siirt University, Faculty of Education, Department of Elementary Education, Siirt, Türkiye*

*3 Mardin Artuklu University, Faculty of Engineering And Architecture, Department of Computer Sciences, Mardin, Türkiye*

*4 Eskisehir Yunus Emre State Hospital, Department of Pathology, Eskişehir, Türkiye*

*5 Eskişehir Osmangazi University, Faculty of Science, Department of Biology, Eskişehir, Türkiye*

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**Abstract**

To evaluate kefir, a naturally occurring fermented dairy product, with pharmacological and therapeutic qualities including antioxidant, anti-apoptotic, and anti-inflammatory effects against cyclophosphamide (CP)-induced hemorrhagic cystitis and nephrotoxicity in rats. For this purpose, experimental rats were divided into 6 groups; control (Group 1), 150 mg/kg CP (Group 2), 5 mg/kg kefir (Group 3), l0 mg/kg kefir (Group 4), 5 mg/kg kefir+150 CP (Group 5), l0 mg/kg kefir+150 CP (Group 6). Since there was no difference in kefirs fermented on different days, kefirs from the 1st, 2nd, and 3rd days were mixed and given to the rats for 12 days, while CP was given as an only dose and i.p. on the 12th day of the experiment. Histologic evaluations revealed that CP caused toxicity in the kidney and bladder. On the other hand, biochemical evaluations showed a significant increase in serum blood urea nitrogen (BUN) and creatinine (Cre) levels, which are tissue toxicity markers, and a significant decrease in catalase (CAT), glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GPx) levels, which are intracellular antioxidant system markers, in the CP-treated experimental group. However, all values were reversed as a result of kefir (5 and 10 mg/kg) treatment. These results showed that kefir is an effective protective agent against CP-induced hemorrhagic cystitis and nephrotoxicity.

**Keywords:** Cyclophosphamide; Hemorrhagic cystitis; Immunotherapy; Kefir; Nephrotoxicity.**Öz**

Doğal olarak oluşan fermente bir süt ürünü olan kefirin, sıçanlarda siklofosfamid (CP) ile indüklenen hemorajik sistit ve nefrotoksisiteye karşı antioksidan, anti-apoptotik ve anti-inflamatuar etkileri gibi farmakolojik ve terapötik niteliklerini değerlendirmek. Bu amaçla, deneysel sıçanlar 6 gruba ayrılmıştır; control (Grup 1), 150 mg/kg CP (Grup 2), 5 mg/kg kefir (Grup 3), l0 mg/kg kefir (Grup 4), 5 mg/kg kefir+150 CP (Grup 5), l0 mg/kg kefir+150 CP (Grup 6). Farklı günlerde fermente edilen kefirlerde farklılık olmadığı için 1., 2. ve 3. gün kefirleri karıştırılarak sıçanlara 12 gün boyunca verilmiş, CP ise deneyin 12. gününde tek doz ve i.p. olarak verilmiştir. Daha sonra elde edilen parametreler değerlendirilmiştir. Histolojik değerledirmeler sonucunda CP'nin böbrek ve mesane de toksisiteye neden olduğu belirlenmiştir. Öte yandan biyokimyasal değerlendirme ile CP uygulanan deney grubunda, doku toksisite belirteçleri olan serum kan üre nitrojen (BUN) ve kreatinin (Cre) seviyelerindeki önemli artış görülğrken hücre içi antioksidan sistem belirteçlerinden olan katalaz (CAT), glutatyon (GSH), superoksit dismutaz (SOD) ve glutatyon peroksidaz (GPx) düzeylerinde ise önemli azalma olduğu belirlenmiştir. Bununla birlikte, kefir (5 ve 10 mg/kg) tedavisi neticesinde meydana gelen tüm değerler tersine dönmüştür. Bu sonuçlar kefirin CP kaynaklı hemorajik sistit ve nefrotoksisiteye karşı etkili bir koruyucu olduğunu göstermiştir.

**Anahtar Kelimeler:** Siklofosfamid; Hemorajik sistit; İmmünoterapi; Kefir; Nefrotoksisite.

**1. Introduction**

An essential tactic in the treatment of cancer is conventional chemotherapy. Anticancer medications interact with the DNA of cancer cells to regulate apoptosis and stop the cell cycle mechanism (Mills *et al.* 2019). Off-target organ damage, however, is a known drawback and catastrophic side effect of anticancer medication therapy. Undesirable developments can occasionally throw off treatment plans and patients' adherence to medicines (Ayhanci *et al.* 2020). Cyclophosphamide (CP) is a common chemotherapeutic and immunosuppressive agent widely used in the treatment of chronic autoimmune disorders and numerous cancer types (Cengiz *et al.* 2020). Bladder toxicity is the main factor restricting the use of CP and can cause potentially fatal hemorrhagic cystitis. Additionally, CP causes multi-organ toxicity in many tissues such as liver, kidney and heart in cancer patients (Cengiz 2018b, Çetik Yıldız *et al.* 2024, Qiu *et al.* 2023, Wróbel *et al.* 2019). Hemorrhagic cystitis is a bladder urotoxicity occurring in approximately 10-40% of patients receiving CP (Akbaş *et al.* 2022, Cengiz *et al.* 2022, Qiu *et al.* 2023, Wróbel *et al.* 2019). Studies have reported that the bladder-toxic hepatic metabolite of CP, acrolein, is linked to CP urotoxicity (Aboulhoda *et al.* 2020). Accumulating research evidence has shown that acrolein disrupts the antioxidant mechanism and bladder epithelium, aggravating the inflammatory responses and production of reactive oxygen species (ROS) (Cengiz *et al.* 2018, Fatima *et al.* 2022, Peng *et al.* 2022). Before utilizing hepatic microsomal cytochrome P450, most especially CYP2B6, to exert its therapeutic benefits, CP must first undergo metabolic activation (Haghi-Aminjan *et al.* 2018). When CP biotransformation occurs, acrolein is produced, and it has a wide range of toxicity. Because the kidneys express CYP2B6, the renal injury could result from the local production of poisonous acrolein (Cengiz 2018, Knights *et al.* 2013, Zanger and Klein 2013). CP-related nephrotoxicity may cause glomerular and tubular dysfunction with a decreased rate of glomerular filtration (Sugumar *et al.* 2007). Lipid peroxidation, cellular defense depletion, and ROS generation have been identified as the primary causes of glomerular injury and renal failure observed after CP treatment (Rehman *et al.* 2012). Many studies have reported a pro-oxidant effect of CP in experimental animals, increasing oxidative stress, inflammatory state, and apoptosis (Alhaithloul *et al.* 2019, Can *et al.* 2022, Cengiz *et al.* 2017, Germoush and Mahmoud 2014). It is noteworthy that the adverse effects of CP can be reduced by natural compounds (Gözüoğlu 2021, Yıldız 2020). Among these functional products, kefir has been found as a prebiotic with health-promoting properties in both experimental and clinical studies.

Kefir is an acidic and slightly alcoholic fermented dairy product originating from the Caucasus mountains (Cetik Yildiz *et al.* 2024, Urdaneta *et al.* 2007). Unlike other fermented milk products, this beverage is produced through fermentation using a mixed microflora contained within a matrix of individual "kefir grains," rather than the metabolic activity of an evenly distributed microflora (Kahraman *et al.* 2021; Marshall and Cole 1985). Grain microbial makeup varies depending on where the grain comes from. The genera that are most frequently reported are acetic acid bacteria, leuconostoc, lactobacilli, lactococci, and homofermentative and heterofermentative yeasts (Angulo *et al.* 1993, Toba 1987). The microorganisms that make up kefir grains include the production of antibiotics, bactericides, and lactic acid which prevent the growth of pathogens and unwanted microorganisms in kefir (Angulo *et al.* 1993). It has been stated that kefir positively affects antioxidant variables and reduces lipid peroxidation in carbon tetrachloride toxicity in mice (Cetik Yildiz *et al.* 2024; Güven *et al.* 2003). Kefir exhibits activities such as antioxidative, hematoptotective, cytoprotective (Yildiz and Gözüoğlu 2021), antimicrobial, and anticarcinogenic properties, and protection against apoptosis (Cetik Yildiz *et al.* 2019). Besides, Kefir protects kidney tissue in diabetic animal renal tissue by dramatically up-regulating Nrf2, modifying CAT and SOD, and significantly lowering NO, superoxide anion, and 3-NT (Pugliero *et al.* 2021). In another study, it was reported that kefir can prevent damage due to its protective properties on tissue and serum functions in ischemia/reperfusion-induced kidney injury (Yener *et al.* 2015). However, no study showing the effect of kefir on the urinary system was found in the literature. This experimental study was designed to demonstrate the adverse side effects of CP on the kidney as well as to demonstrate the possible protective role of kefir against CP-caused hemorrhagic cystitis and nephrotoxicity in rats. To our knowledge, no study has been found in the literature showing the effects of kefir on CP-caused urotoxicity and nephrotoxicity in rats.

**2. Materials and Methods**

***2.1 Fermentation of kefir***

In our study, commercially supplied and freeze-dried kefir yeast and 1 liter of cow's milk were preferred for kefir fermentation. Three groups of kefirs were created, with fermentation at 24-26 °C temperature at intervals of 24, 48, and 72 hours and days 1, 2, and 3. It was kept at +4 °C ready for use. We gave kefir to rats by gavage method for 12 days. Kefirs from the 1st, 2nd, and 3rd days were mixed and given by gavage method for 12 days Yildiz and Gözüoğlu 2021).

***2.2 Chemicals and injections***

Cyclophosphamide (CP) (Sigma-Aldrich) was commercially available. 500 mg CP was dissolved in 25 ml bidistilled water to prepare for injection of 150 mg/kg CP. The injection was performed as a single dose intraperitoneally (i.p.) / body-weight (b.w.) on the 12th day of the experiment, using sterile disposable syringes.

***2.3 Experimental setup***

In our experimental study, healthy, males, 200±20 gr, about 3 months age Wistar albino rats were used. During the experiment, the animals were kept in rooms with 12;12 light/dark lighting, 45-50% humidity, and 22±2 C˚ temperature. And were given tap water and normal pellet feed. The 42 rats used in this study were divided into 6 groups, each group including 7 rats. Kefir gavage method as mg/kg/body weight (b.w.) for 12 days was given. CP is a single dose and i.p. was given on the last day of the experiment (day 12). Group 1 was designated as control (0.5 mL, saline), 150 mg/kg/bw CP to rats in Group 2, 5 mg/kg/bw kefir was given to the rats in Group 3, 10 mg/kg/bw kefir to experimental animals of Group 4, 5 mg/kg/bw kefir + 150 mg/kg/bw CP for Group 5, and finally, 10 mg/kg/bw kefir + 150 mg/kg/bw CP was administered to the rats of Group 6. Following the research study, anesthesia was used to remove tissues (the kidney and bladder) and blood (for biochemical markers).

***2.4 Biochemical analysis***

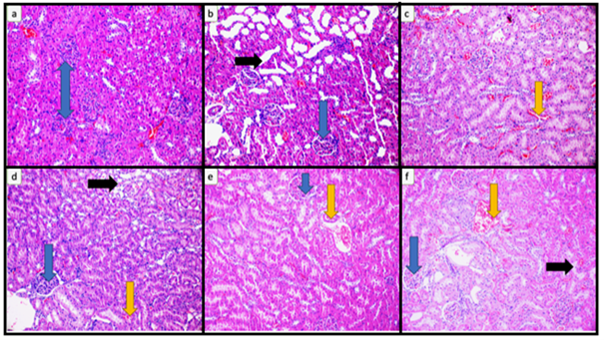
Using the thiobarbituric acid (TBA) reagent method, MDA and GSH levels were measured with a spectrophotometer set at 520 nm and 412 nm respectively (Sedlak and Lindsay 1968). With the support of commercially available kits, the activity of the SOD enzyme was evaluated spectrophotometrically. Sun et al.'s (1988) protocol was followed when measuring the activity of the SOD enzyme (Sun *et al.* 1988). Using the Beers and Sizer method as reported by Usoh et al. (2005) serum CAT activity was measured by detecting the drop in absorbance at 240 nm caused by the breakdown of H2O2 (Usoh *et al.* 2005). GPX was measured by Rotruck et al. and showed no discernible change. Tris-Hcl buffer, hydrogen peroxide, glutathione, DNTB, TCA, Tris-EDTA, a homogenous tissue serum sample, and sodium azide were used in this experiment. Then, using an ELISA reader (AWARENESS STAT FAX-2100, USA), absorption was measured at 420 nm (Valibeik *et al.* 2020). The concentration of serum Cre was measured using the Jaffe technique (Kojima *et al*. 2013), and BUN was measured using the autoanalyzer (BT 3000) UV method.

***2.5 Statistical Analysis***

The quantitative values obtained at the end of the study were evaluated by applying the Duncan test after one-way ANOVA, which is used in the statistical analysis of more than two independent groups, using the SPSS 26.00 statistical data program.

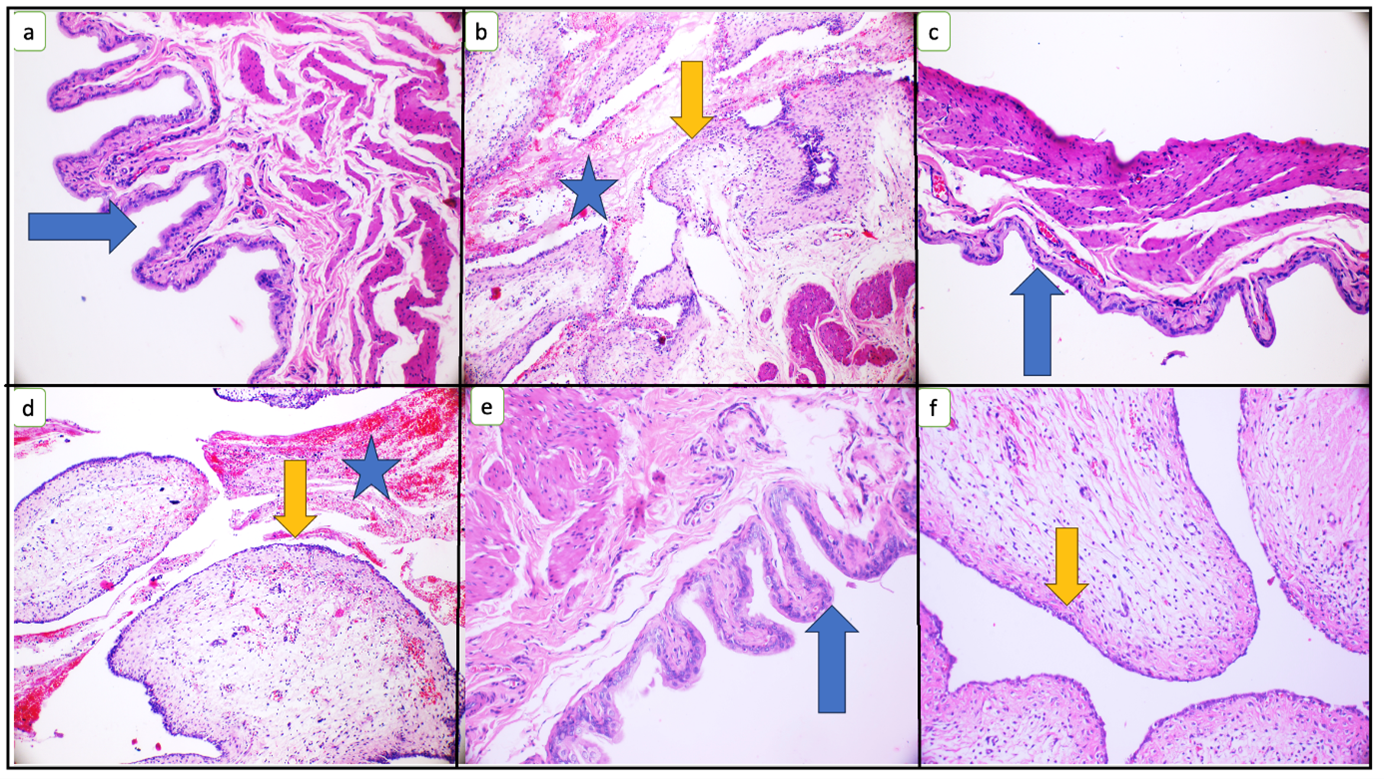
**3. Results**

As seen in Figure 1, the control group’s rats had normal kidney structures. In contrast, the glomerular structure was atrophic and deformed in the CP group (Figure 1b), some cells were shed into the renal microcapsule, and there was a clear infiltration of inflammatory cells on one side. Also, there were discontinuities in the parietal cells and an incomplete renal microcapsule structure. In the proximal convoluted tubule lumen, there were exfoliated cells as well as some denatured cells that developed edema. Moreover, distal convoluted tubules with discrete and irregularly arranged cells were also visible. However, it was observed that the structure of the kidney in the kefir-given groups was importantly improved compared to the CP-induced group’s animals. Especially in the 10mg/kg kefir + CP group (Figure 1e), there was no evidence of inflammatory cell infiltration, and the glomerular structure was largely normal. In the 10 mg/kg kefir + CP group, the proximal convoluted tubules featured prominent brush-like borders and a tiny, irregular lumen. Capillaries in the interstitium were visible, as were the borders of the distal convoluted tubules. In conclusion, the 10 mg/kg kefir dose was more effective than the 5 mg/kg kefir dose in ameliorating CP-induced kidney damage.

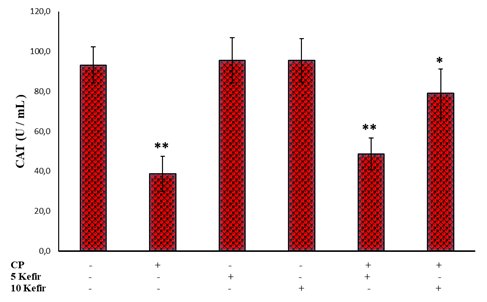
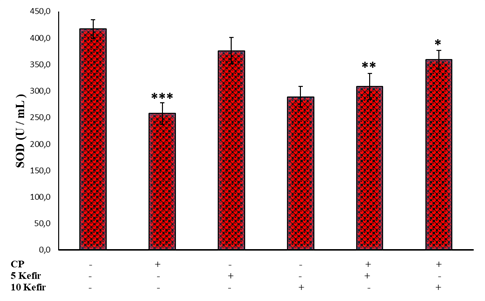


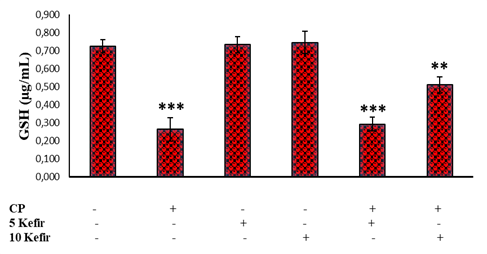
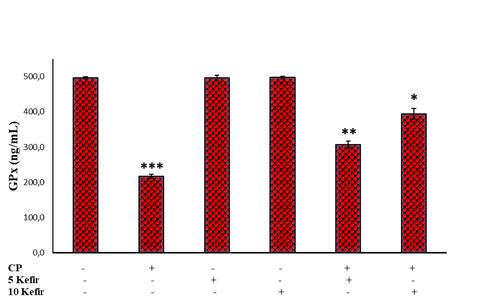
**Figure 1.** (a) Normal-looking tubules and glomeruli (blue arrow) in the kidney parenchyma of rats in the control group, (b) Glomeruli (blue arrow) and thyroidization (black arrow) in the kidney parenchyma of rats administered CP, (c) Congestion (yellow arrow) in the kidney parenchyma of rats given kefir, (d) Glomeruli (blue arrow), congestion (yellow arrow) and focal area thyroidization (black arrow) in the renal parenchyma of rats administered CP and kefir, (e) Congestion (yellow arrow) and glomeruli (blue arrow) in the renal parenchyma of rats administered kefir, (f) CP and congestion (yellow arrow), glomeruli (blue arrow) and thyroidization (black arrow) in the kidney parenchyma of rats administered kefir. (H&E, X200).

As seen in Figure 2, the bladder structure of the rats in the control and kefir groups appeared normal (Figure 2a). However, in the CP group (Figure 2b), widespread ulceration in the bladder epithelium, edema on the wall, inflammation, congestion, and blood and fibrin mass in the lumen were observed. However, it was observed that the bladder structure in the groups given kefir improved significantly compared to the CP group. Especially in the 10 mg/kg kefir + CP group (Figure 2e), regeneration in the bladder epithelium, mild edema on the wall, and mild inflammation were observed. As a result, the 10 mg/kg kefir dose was more effective than the 5 mg/kg kefir dose in improving CP-induced bladder damage.



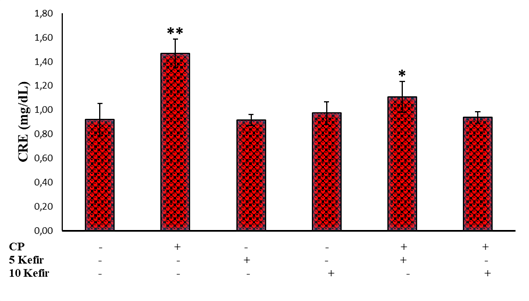
**Figure 2.** (a) Bladder epithelium (blue arrow) and bladder wall within normal limits, (b) Widespread ulceration in the bladder epithelium (yellow arrow), wall edema, inflammation, congestion, and blood and fibrin mass in the lumen (blue star), (c) Bladder epithelium (blue arrow). and bladder wall within normal limits, (d) Bladder epithelium (blue arrow) and bladder wall within normal limits, (e) Widespread erosion of the bladder epithelium (yellow arrow), edema on the wall, mild inflammation, congestion, and blood and fibrin mass in the lumen (blue star), (f) Regeneration in the bladder epithelium (yellow arrow), mild edema and mild inflammation on the wall. (H&E; X200)

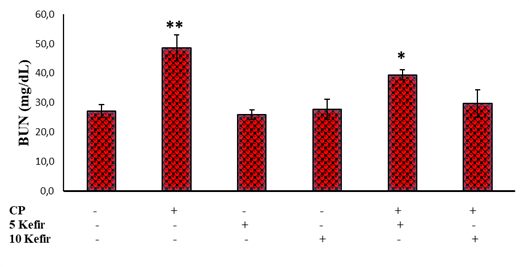


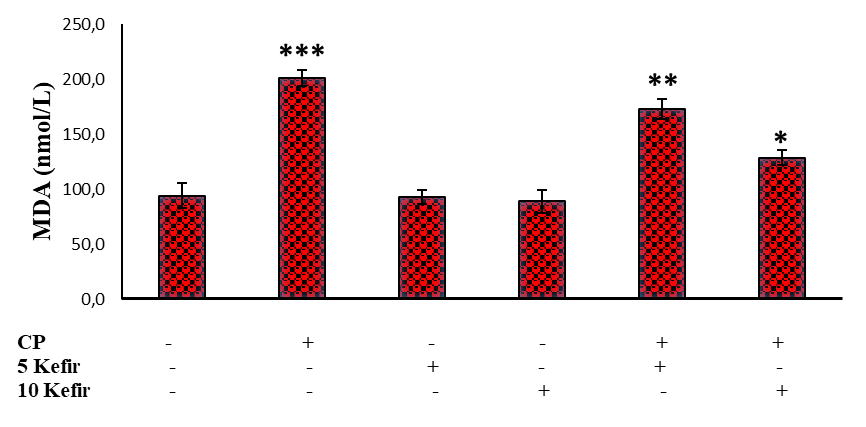
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**Figure 3.** Showing the effect of CP and kefir on the levels of SOD, CAT, GPx, and GSH (*\*\*\* P<0.001; \*\* P<0.05; \* p<0.01*)

CAT, SOD, GPx and GSH parameters have antioxidant effects in CP-induced toxicity. By measuring CAT, GPx, GSH, and SOD activity in the serum of control and CP-induced rats, the impact of kefir on the antioxidant system was observed. Figure 3 illustrates how the CP group's GSH, SOD, CAT, and GPx activities were importantly lower (*P<0.01*) than those of the control group. CAT, GSH-Px, and SOD activity rose in the CP-induced rats when they were given kefir at different dosages (5 and 10 mg/kg) as opposed to the CP group.

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**Figure 4.** Showing the effect of CP and kefir on the levels, CRE (A), BUN (B), and MDA (C) (*\*\*\* P<0.001; \*\* P<0.05; \* P<0.01*).

Serum CRE and BUN levels were measured to assess the preventive impact of kefir against CP-induced nephrotoxicity. As shown in Figure 4, When the CP group was compared to the control and kefir-treated groups, there was a substantial increase in both CRE and BUN levels (*P<0.05*). This indicates that CP may cause cell toxicities. These findings were in line with previous research on kidneys (Lin et al. 2020, Rehman et al. 2012, Sinanoglu et al. 2012). It's interesting to note that CRE and BUN levels were considerably lower in CP-induced rats treated with different kefir dosages than in the CP group (*P<0.01*). This suggests that kefir can effectively ameliorate CP-caused nephrotoxicity in rats. An essential indicator of endogenous lipid peroxidation is MDA. In comparison to the control and kefir groups, the CP group's MDA levels were considerably higher (*P<0.001*). Rats given 5 and 10 mg/kg kefir showed significantly lower MDA levels than those in the CP group (*P<0.05* and *0.01*). This shows that kefir can reduce MDA levels in CP-induced lipid peroxidation in rats (Figure 4).

**4. Discussion and Conclusions**

The kidneys are crucial for controlling blood volume and eliminating medications and poisons from the body. However, because of their great absorptive capacity, kidneys are susceptible to toxicity and damage. Despite the effectiveness of CP, which is widely used in the treatment of neoplastic diseases, its use is associated with nephrotoxicity and urotoxicity (bladder), which are the main limitations of CP-induced therapy (Alshahrani *et al.* 2022). Kefir is an acidic and slightly alcoholic fermented dairy product with antioxidant, antiapoptotic, anti-lipid peroxidation, and anti-inflammatory activities (Can *et al.* 2012, Hadisaputro 2011, Hadisaputro *et al*. 2012). So far, there are no reports on the use of kefir against CP-induced nephrotoxicity and bladder toxicity. Therefore, the purpose of this study was to examine the protective effect of kefir against CP-caused nephrotoxicity and bladder damage through the antioxidant response pathway. Drugs' ability to protect the kidneys is frequently assessed using two key renal function markers, CRE and BUN (Aladaileh *et al*. 2019, Alhaithloul *et al*. 2019). The increased serum CRE and BUN levels after CP administration observed in this study are consistent with reports published in the literature(Abraham and Isaac 2011, AlHaithloul *et al*. 2019, Caglar *et al*. 2002, Temel *et al*. 2020). The increase in the serum levels of these enzymes may be due to the leakage of these cytosolic enzymes into the circulatory system as a result of kidney damage after CP administration. This is indicative of the onset of kidney damage due to renal dysfunction and change in membrane permeability due to impairment in the biosynthesis of these enzymes. With kefir administration, a sharp decrease in serum BUN and CRE levels was seen, preserving renal cellular membrane integrity, and subsequently preventing CP-induced renal toxicity. This is indicative of the possible nephroprotective activity offered by kefir compared to the untreated and CP-treated groups.

Histopathological studies also provided supporting validation for biochemical parameters demonstrated by photomicrographs. In the kidney tissue of CP-treated rats (Figure 1), There was clear inflammatory cell infiltration on one side, the glomerular structure was atrophic and deformed, and some cells were lost into the kidney microcapsule. Additionally, the renal microcapsule lacked some structural integrity, and the parietal cells were irregularly shaped. In the proximal convoluted tubule lumen, there were exfoliated cells as well as some denatured cells that developed edema. Furthermore, distal convoluted tubules with discrete and irregularly arranged cells were also visible. These histopathological findings were compatible with findings in the literature (Caglayan *et al*. 2018, Lin *et al*. 2020, Rehman *et al*. 2012). The main histological finding of this study was that kefir affected the recovery of CP-induced renal structure. One of the most significant side effects of CP chemotherapy is bladder damage (Davis and Kuttan 2000, Manesh and Kuttan 2005, Lin *et al*. 2020). The acute effect of CP is necrosis of the urothelium, with only a few cells surviving after 24 hours. Since the bladder is the place where urine accumulates, the level of toxic metabolites of CP is higher in the bladder than in other organs (Beyer-Boon *et al*. 1978, Valibeik *et al*. 2020). CP-induced bladder damage is mainly associated with renal excretion of acrolein, which is known to be a urotoxic metabolite of CP (Gray *et al*. 1986, Cengiz *et al*. 2018). Urothelial damage has been shown to occur in direct contact with acrolein, causing necrosis, edema, ulceration, bleeding, and leukocyte infiltration. Acrolein causes highly reactive free radicals, consumes cellular thiol, and disrupts the antioxidant defense mechanism of tissues (Mythili *et al*. 2004, Ayhanci *et al*. 2020). In addition to these side effects, it has also been reported that CP application causes oxidative stress by producing free radicals and ROS (McDermott and Powell 1996, Cetik Yildiz *et al*. 2024). These findings are compatible with our study results.

In this study, the level of MDA was examined using it as a key indicator for lipid peroxidation. MDA level, which indicates oxidative stress caused by CP, increased in the CP group compared to the control group. On the other hand, the significant decrease in plasma MDA level with kefir therapy compared to the CP group indicates that its protective effects on kidney and bladder tissues may be due to the antioxidant properties of kefir. This study is also compatible with the literature results (El-Shabrawy *et al*. 2020, Ijaz *et al*. 2022, Jiang *et al*. 2020). Kidney and bladder damage caused by CP is commonly associated with the pro-oxidant qualities of acrolein due to its ability to collect ROS, leading to oxidative stress and depression of the antioxidant functions (Jiang *et al*. 2020, Mahmoud *et al*. 2017). Numerous studies have shown that CP depletes antioxidative enzymes like SOD, GSH, CAT, and GPx and increases lipid peroxidation and ROS in the kidneys and bladder of CP-exposed animals (Jiang *et al*. 2020, Lin *et al*. 2020). Similarly, results from this study showed important decreases in renal activities of GSH, SOD, GPx, and CAT in CP-administered rats indicating oxidative damage. Kefir exhibited significant antioxidant activity through the restoration of antioxidative activities (high SOD, CAT, GPx, and GSH capacities). This result is consistent with previous reports on the antioxidant activity of kefir (El Golli-Bennour *et al.* 2019, Punaro *et al*. 2014).

The results of this study showed that oxidative stress, decreased antioxidant capacity, and lipid peroxidation are closely related to CP-caused bladder and kidney toxicity, and kefir has protective effects on these CP-caused toxicities. The protective effects of kefir are probably thanks to increasing the decreasing antioxidant capacity and reducing oxidative stress and lipid peroxidation.

**Declaration of Ethical Standards**

This study was approved by the Ethics Committee of Eskisehir Osmangazi University Animal Experiments Local Ethics Committee (784-145/2020). The authors declare that they comply with all ethical standards.

**Credit Authorship Contribution Statement**

Author-1: Methodology / Study design, Software, Validation, Formal analysis, Investigation

Author-2: Methodology / Study design, Software, Validation, Formal analysis, Investigation

Author-3: Writing – original draft, Writing – review and editing, Visualization, Supervision

Author-4: Investigation, Resources, Data curation, Writing – original draft

Author-5: Visualization, Project administration, Funding acquisition

Author-6: Project administration, Funding acquisition

**Declaration of Competing Interest**

The authors have no conflicts of interest to declare regarding the content of this article.

**Data Availability**

All data generated or analyzed during this study are included in this published article.

**Acknowledgment**

This experimental research was financed by Mardin Artuklu University / Coordination Unit of Scientific Research Project (MAU.BAP.20. SHMYO.004).

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