

Effect of Kefir on Increased Apoptosis in Liver and Kidney in Cisplatin Toxicity

Efecto del Kéfir sobre el Aumento de la Apoptosis en el Hígado y los Riñones en la Toxicidad del Cisplatino

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SUMMARY: Cisplatin is a chemotherapeutic agent inducing liver and kidney damage. In this study, we intended to investigate the impact of kefir beverage, an essential probiotic and functional food, on liver and kidney damage induced by cisplatin. Wistar albino rats were divided into four groups: Control, Cisplatin (single dose of 7 mg/kg, intraperitoneal), Kefir (2 ml/d, 7 d, oral gavage), and Cisplatin+Kefir (CK). At the end of day 7, animals were euthanized. Blood, kidney, and liver tissue samples were collected. For both tissues, biochemically ALT, AST, Urea, Creatine; histomorphologically, hematoxylin-eosin, Masson's Trichrome, and immunohistochemical staining of caspase-3, a marker of apoptosis, were performed. Serum urea and creatinine levels of the Cisplatin group were significantly higher than the Control group ($p < 0.05$). In the CK group, kefir consumption decreased urea and creatinine levels approached to Control and Kefir groups. Cisplatin resulted in higher ALT and AST activities, indicating hepatocellular damage, compared to the Control group ($p < 0.05$). Kefir consumption decreased ALT activities approached to both the Control and Kefir group. Histomorphological observations were in agreement biochemical results. In liver and kidney tissues, structural damage was observed with an increase in collagen fibers in the Cisplatin group, and Caspase-3 activity was immunohistochemically higher than in the other groups. In the CK group, collagen fiber increase, structural damage, and Caspase-3 activities were less than in the Cisplatin group. Kefir consumption alleviated liver and kidney damage. However, more research is required to understand such effect of kefir better.

KEY WORDS: Cisplatin; Kefir; Urea; Liver; Kidney.

INTRODUCTION

Cisplatin is applied effectively in many cancer treatments, including testicular, ovarian, head and neck solid cancers (Long & Repta, 1981). The cytotoxic effect of cisplatin begins as soon as it penetrates the cell. It gains the ability to bind to DNA, RNA, and proteins by showing nucleophilic properties in the cell. The binding of cisplatin on purine bases causes structural and functional defects in DNA and programmed cell death is initiated (Gómez-Ruiz *et al.*, 2012; Wang *et al.*, 2019).

Kefir is yellow to white and has a popcorn appearance. The beverage is made by fermenting milk and kefir grains, then removing the grains. Kefir microflora consists of yeasts (*Candida*, *Torulopsis*, *Saccharomyces* and *Kluyveromyces*) and bacteria (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*) (Hecer *et al.*, 2019). Kefir has been reported to have a positive effect on the immune system, digestive system, blood pressure, antimicrobial, anti-inflammatory, anticarcinogenic and antiallergic properties,

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hypercholesterolemia, blood sugar regulator, lactose intolerance reduction (El Golli-Bennour *et al.*, 2019).

Many preservatives that reduce the adverse effects of cisplatin on the liver and kidney tissues have been studied (Abdel-Daim *et al.*, 2020). In view of such studies, we wanted to test the protective effect of kefir on possible tissue and cellular damage caused by cisplatin. So, we aimed to test the protective effect of kefir against cisplatin-induced damage in the rat liver and kidney tissues.

MATERIAL AND METHOD

The study was conducted Experimental Animal Research Center (DEHAM) Near East University's Animal Experiments Ethics Committee approved the experimental protocol (Approval No: 2019/02-60). Animals were kept at a constant temperature (22±1°C) with 12 hour (h) light and dark cycles and housed in plastic cages. The rats were allowed free access to their respective diets and water ad libitum for 7 d.

Preparation of Cisplatin. A commercial drug (Koçak A.S., -Istanbul) containing 50 mg/100 ml cisplatin active substance was used in the study. The dose of cisplatin used for each of the rats was determined as 7 mg/kg as Mostafa *et al.* (2018).

Preparation of kefir. The kefir was prepared at weekly intervals. Standard milk (Ultra-High Temperature (UHT) cow market milk) and kefir grains were used in the study. To make kefir, 100 ml milk was inoculated with 5 g of the grains in a sterile glass jar and incubated at 25 °C until the pH reached to 4,4. After gently shaking the jar content, grains were harvested using a plastic kitchen strainer and a plastic spoon and gently washed using a sterile saline solution (0.9 % NaCl) and kept at 4 °C in UHT sterile milk for further use. Kefir bacterial and yeast counts were determined according to Miller and Wolin protocol (Miller & Wolin, 1974). Every day, 0.5 ml kefir per 1.5 ml drinking water was administered via oral gavage to each rat.

Animals and experimental design. A total of 24 Wistar Albino female rats (200-350 g, each) at their period of sexual maturation (60 days old) were randomly divided into four groups Control, Kefir, Cisplatin and Cisplatin+Kefir (CK) with six individuals per group (n = 6). The Control group was treated with 4 ml of physiological saline solution intraperitoneally on d 1. Kefir group was fed with 0.5 ml kefir after dilution in 1,5 ml of water by oral gavage once a day for 7 days. The Cisplatin group was treated with a 4 ml of cisplatin (7 mg/kg rat live weight) solution intraperitoneally on d 1. The CK group was subjected by kefir (as in Kefir group) and cisplatin (as in the

Cisplatin group). At the end of the experiment, blood samples were collected by the cardiac venipuncture from anesthetized rats. Liver and kidney tissues were collected from all animals for histomorphological examination.

Biochemical Analyses. Biochemical analyses were performed using a clinical chemistry analyser (Architect Plus c8000) Abbott Laboratories, Illinois, USA. ALT, AST, urea and creatinine levels were measured.

Histological Evaluation. The liver and kidney tissue samples were fixed in 10 % neutral formalin for 24 h at room temperature, then embedding in paraffin. The 5 µm sections were taken and stained with Haematoxylin and Eosin. Each liver tissue section was evaluated in terms of hepatocytes with eosinophilic cytoplasm, areas of necrosis, congestion and mononuclear cell infiltration. Kidney tissue was evaluated in terms of tubular damage, glomerular damage and mononuclear cell infiltration. For each criterion, 10 different areas from kidney (Abdel Moneim *et al.*, 2019) and liver (Çiftçi *et al.*, 2017) were calculated as none (0), mild (1), moderate (2) or severe (3).

Masson's trichrome kit (Bio-Optica, 04-010802-Italy) was performed, demonstrating increased collagen fiber in liver and kidney tissues. Five different regions under at x40 magnification for liver and kidney sections were evaluated using ImageJ software.

Mouse and rabbit specific HRP/DAB (ABC) Detection IHC kit (ab64264) and primary antibody caspase-3 (ab4051) diluted 1:100 were used for immunohistochemical staining. The increase in caspase-3 in the tissues was evaluated with the H-score method according to the staining degree as negative (0), mild (1), moderate (2) and severe (3). Histological sections were visualized with the Leica Microsystem Framework integrated digital imaging analysis system (version 3.0 Serial 38132019 Leica ICC50 HD camera and Leica DM500 light microscope).

Statistical Analyses. Statistical analyses were carried out using SPSS (IBM SPSS statistics version 26.0) software (SPSS Inc., Chicago, IL, USA). Groups of data were compared with Kruskal Wallis analysis followed by Mann Whitney-U tests. A difference of p<0.05 was considered statistically significant.

RESULTS

Biochemistry Results. The cisplatin group had the highest ALT value of 91.83±56.39 U/L, and statistical significance was found only in the control group. AST value was again

Table I. The rat blood sample parameters that reflect liver and kidney damages. Results are represented as mean±SD (n=6).

Groups	Liver parameters		Kidney parameters	
	Alanine transferase	Aspartate transferase	Urea (mg/dL)	Creatinine (mg/dL)
Control	40.50±7.39 ^a	90.16±17.66 ^{b,c}	47.50±7.12 ^e	0.538±0.061 ^h
Kefir	48±15.82	106.66±25.07 ^d	37.16±6.91 ^{e,f}	0.530±0.044 ⁱ
Cisplatin	91.83±56.39 ^a	218±165.58 ^b	586.83±357.03 ^{fg}	6.608±4.088 ^{h,i,j}
Cisplatin+kefir	51.66±11.50	176.50±18.28 ^{c,d}	77±47.32 ^g	0.803±0.310 ^j
<i>P Value</i>	0.04*	0.005**	0.021*	0.02*

The same-a-j letters represent statistically significant results between the groups at the level of p<0.05.

highest in the cisplatin group compared to the other groups. In the CK group, both parameter values were lower than the Cisplatin group (Table I). However, these values were not significant (p>0.05). Although the ALT value of the CK group was slightly higher than the Control and Kefir groups, a protective effect of kefir against cisplatin was observed (p>0.05). CK group decreased with an AST value of 176.50±18.28 U/L compared to the Cisplatin group. However, this group had significantly higher AST values (p<0.05) when compared to the Control and Kefir groups (Table I).

The urea and creatinine values of kidney parameters were the highest in the Cisplatin group with 586.83±357.03 mg/dl urea and 6.608±4.088 mg/dl creatinine. CK group had significantly lower values with 77±47.32 mg/dl urea and 0.803±0.310 mg/dl creatinine values compared to the Cisplatin group and a significant difference was found

between both groups (p<0.05). As seen in Table I, kidney damage caused by cisplatin was minimized through the protective action of kefir (p<0.05).

Histomorphological Results. Liver tissues had a typical histological structure in Control and Kefir groups. Eosinophilic cytoplasm in hepatocytes was not found in the Control and Kefir groups. It was highest in the Cisplatin group when compared to the other groups. Cells with eosinophilic cytoplasm were lower in the CK group than in the Cisplatin group. It was found that there was a statistically significant difference between the groups except for the Control and Kefir groups (p<0.05) (Table II). The most intense necrotic area, congestion and mononuclear cell infiltration was in the Cisplatin group when compared to the other groups. A statistical difference was found between the Cisplatin group and all groups according to the score of necrosis and mononuclear cell infiltration in liver tissues (p<0.05) (Table II). Hepatocytes

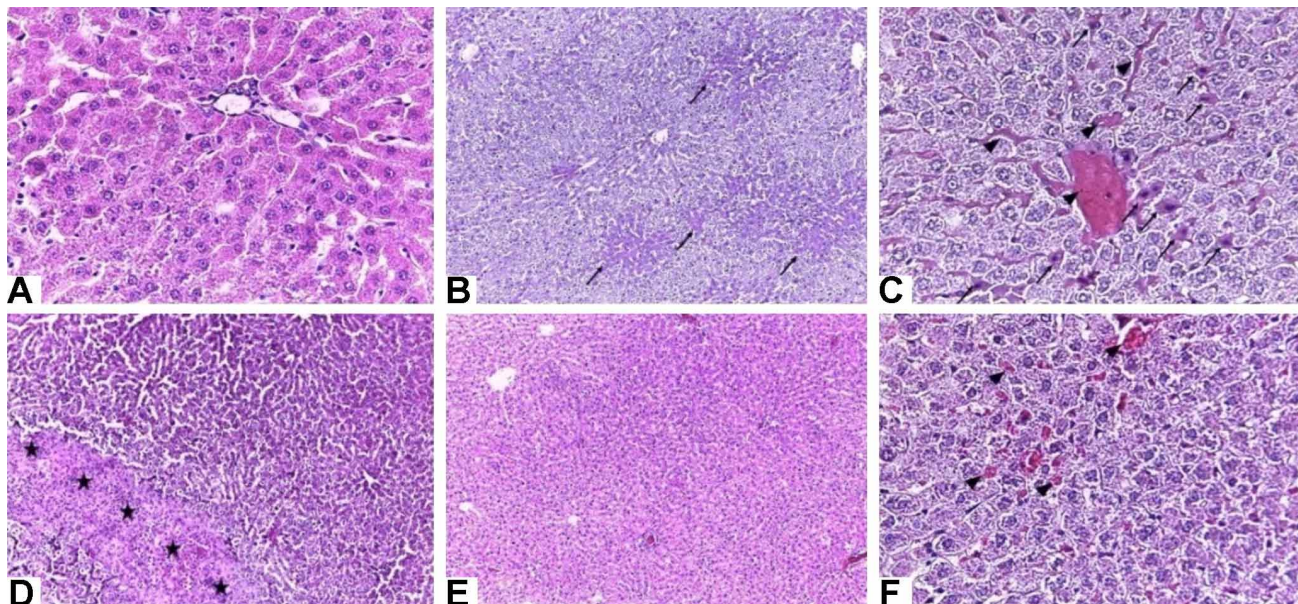


Fig. 1. Light microscopic views of liver tissue belonging to experimental groups. Haematoxylin and Eosin staining. The liver structure of the control group appears to be normal (A, x40). In the group given cisplatin, some of the hepatocytes were lighter colored, some were more eosinophilic colored (→) (B, x10), while eosinophilic cells were evident in large magnification and sinusoid congestion (▶) between hepatocytes was distinguished (C, x40). Common areas of necrosis (★) are observed in liver tissue belonging to the cisplatin group (D, x40). In the group treated with kefir, the liver structure is similar to control, there are no areas of necrosis, hepatocyte staining is similar in all areas (E, x10) and congestion in sinusoids is reduced (F, x40).

with eosinophilic cytoplasm, areas of necrosis, congestion in sinusoids and mononuclear cell infiltration were at reduced levels in the CK group when compared to the Cisplatin group (Fig. 1). The decrease in congestion in the CK group was not statistically significant compared to the cisplatin group ($p>0.05$).

The kidney tissues of the rats in the Control and Kefir groups had a normal histological structure. Tubular,

glomerular damage and mononuclear cell infiltration were found in in the Cisplatin group. There was a decrease in tubular damage in the CK group compared to the cisplatin group, but no statistically significant difference was found. Statistical significance was found between Cisplatin and all other groups in terms of glomerular damage and mononuclear cell infiltration ($p<0.05$) (Table III). It was observed that the damaged areas were significantly reduced in the CK group (Fig. 2).

Table II. Scores of liver histomorphological measurements in the Rat Groups. Results are represented as mean±SD (n=6).

Scores of liver histomorphological measurements.				
Groups	Eosinophilic cytoplasm	Necrose	Congestion	Mononuclear cell infiltrations
Control	0.000±0.000 ^{a,b}	0.000±0.000 ^e	0.016±0.0408 ^h	0.033±0.051 ^j
Kefir	0.000±0.000 ^{c,d}	0.000±0.000 ^f	0.016±0.040 ⁱ	0.033±0.051 ^k
Cisplatin	0.283±0.292 ^{a,c}	0.700±0.819 ^{e,f,g}	0.133±0.136 ^{h,i}	0.266±0.081 ^{j,k,l}
Cisplatin+kefir	0.166±0.121 ^{b,d}	0.050±0.083 ^g	0.066±0.081	0.066±0.051 ^l
P Value	0.002**	0.003**	0.059	0.002**

Table III. Scores of kidney histomorphological measurements in the Rat Groups. Results are represented as mean±SD (n=6).

Scores of kidney histomorphological measurements.			
Groups	Tubular damage	Glomerular damage	Mononuclear cell infiltrations
Control	0.033±0.051 ^{a,b}	0.033±0.051 ^{e,f}	0.016±0.040 ^j
Kefir	0.033±0.051 ^{c,d}	0.033±0.051 ^{g,h}	0.033±0.081 ^k
Cisplatin	0.783±0.440 ^{a,c}	0.633±0.294 ^{e,g,i}	0.366±0.242 ^{j,k,l}
Cisplatin+kefir	0.333±0.196 ^{b,d}	0.200±0.126 ^{f,h,i}	0.083±0.075 ^l
P Value	0.002**	0.001**	0.003**

The same lettera-l represents statistically significant results between the groups at the level of $p<0.05$.

*The degree of statistical significance of the Kruskal Wallis test results.

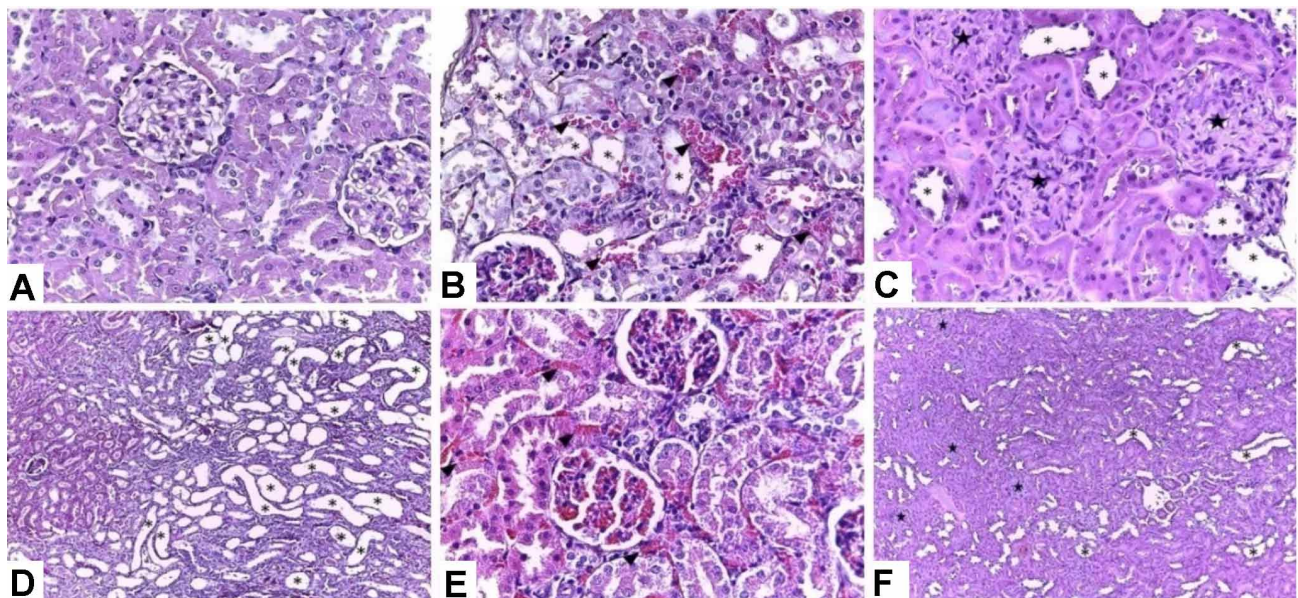


Fig. 2. Light microscopic views of kidney tissue belonging to experimental groups. Haematoxylin and Eosin staining. Glomerular and tubule structures are normal in the renal cortex of the control group (A, x40), in the group given cisplatin, there is significant congestion (▶) in inter-tubule capillaries, degenerated tubules (*), hypertrophic tubule epithelial cells (fi) (B, x40). In the cisplatin group, Bowman's space of some glomerules disappear (★) (C, x40), while in the cisplatin group there are numerous expanded tubules (*) (D, x10). In the kefir-treated group, glomerular and tubule structures are similar to the control group, but Inter-tubule congestion continues, although decreased compared to the cisplatin group (▶) (E, x40) and the number of enlarged tubules (*) is decreased (F, x10).

As a result of Masson's Trichrome staining, it was observed that collagen fiber increase in both the sinusoidal regions of the liver tissues and the intertubular and Bowman's capsule in the kidney was higher in the Cisplatin group than in the other groups. (Fig. 3). A statistically significant difference was found in the increase of collagen fiber in the cisplatin group when compared to all other groups ($p < 0.05$) (Fig. 4). As in liver tissue, intense collagen fiber increase in kidney tissue was in the cisplatin group. Although there was a significant decrease in collagen fiber in the CK group compared to the Cisplatin group, no statistical difference was found between these two groups ($p > 0.05$) (Fig. 4).

According to the immunohistochemistry staining results, the increase in the number of caspase-3 positive cells in the liver and kidney tissues in the Cisplatin group was found to be statistically significant compared to the other groups ($p < 0.05$). While the number of caspase-3 positive cells decreased significantly in the CK group, a statistically significant difference was found compared to the Cisplatin group (Fig. 5) ($p < 0.05$). It was observed that the most caspase-3 positive cells in the kidney tissue were in the Cisplatin group compared to the other groups. In the CK group, the number of positive cells decreased and this decrease was statistically significant compared to cisplatin group ($p < 0.05$) (Fig. 6).

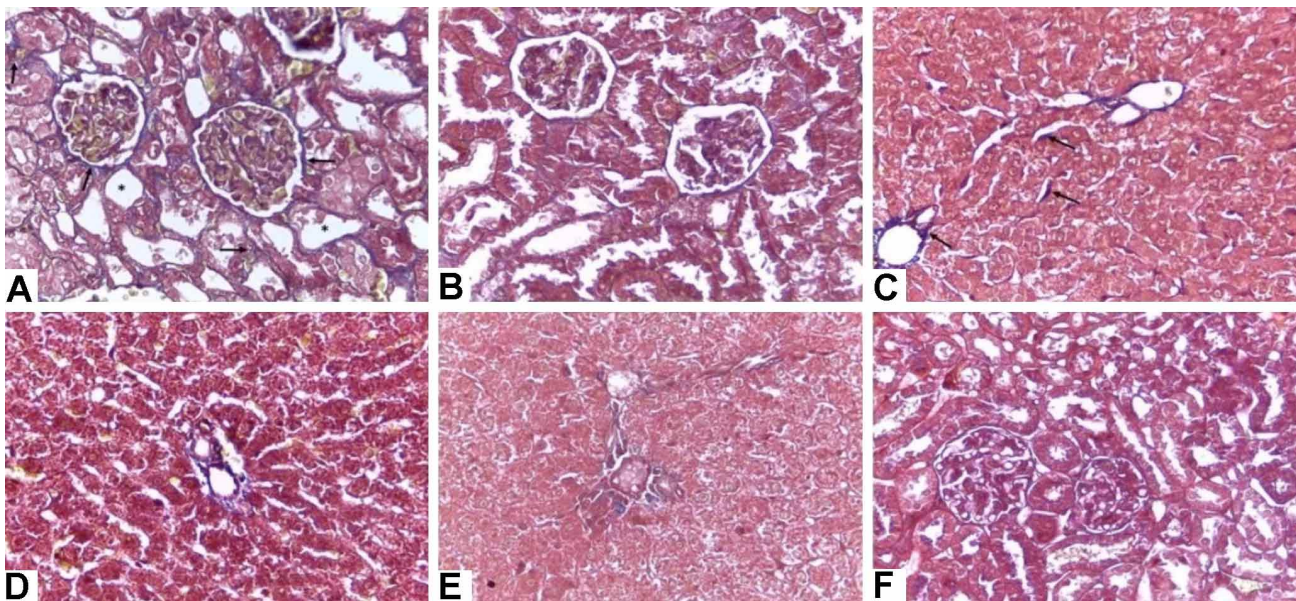


Fig. 3. Enlarged tubules in cisplatin group kidney tissues (*); Collagen fiber increase is evident around the parietal leaf of Bowman's membrane and tubules (\rightarrow) (A). In the group given Kefir after cisplatin, the staining intensity decreased (B). While prominent collagen fiber staining (\rightarrow) was observed in the portal area and sinusoids in the cisplatin-treated group (C), it was not observed in the Kefir-treated group (D). Liver (E) and kidney (F) tissues of Control groups. Masson trichrome staining x40.

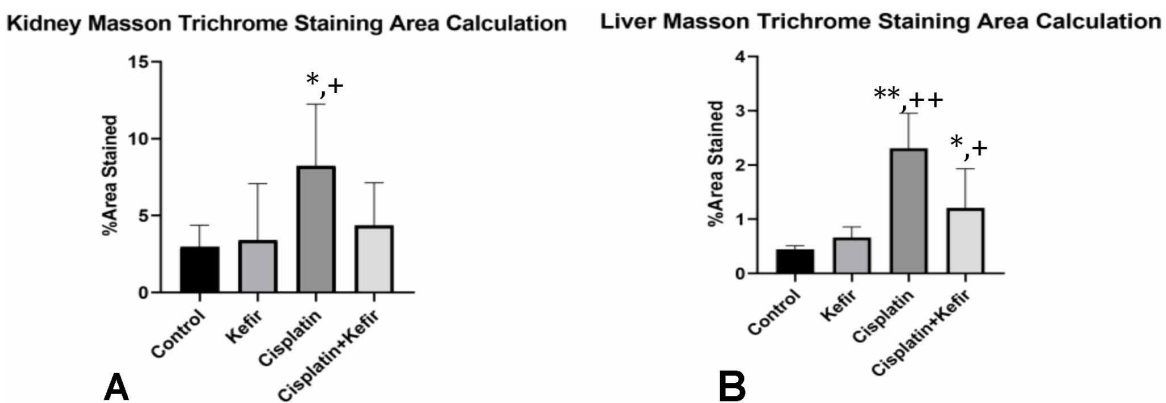


Fig. 4. Evaluated by measuring stained area percentage on image J software. The amount of collagen fiber in kidney (A) and liver (B) tissues by groups. * $p < 0.05$, ** $p < 0.01$ compare to the Control group; + $p < 0.05$, ++ $p < 0.01$ compare to the Kefir group.

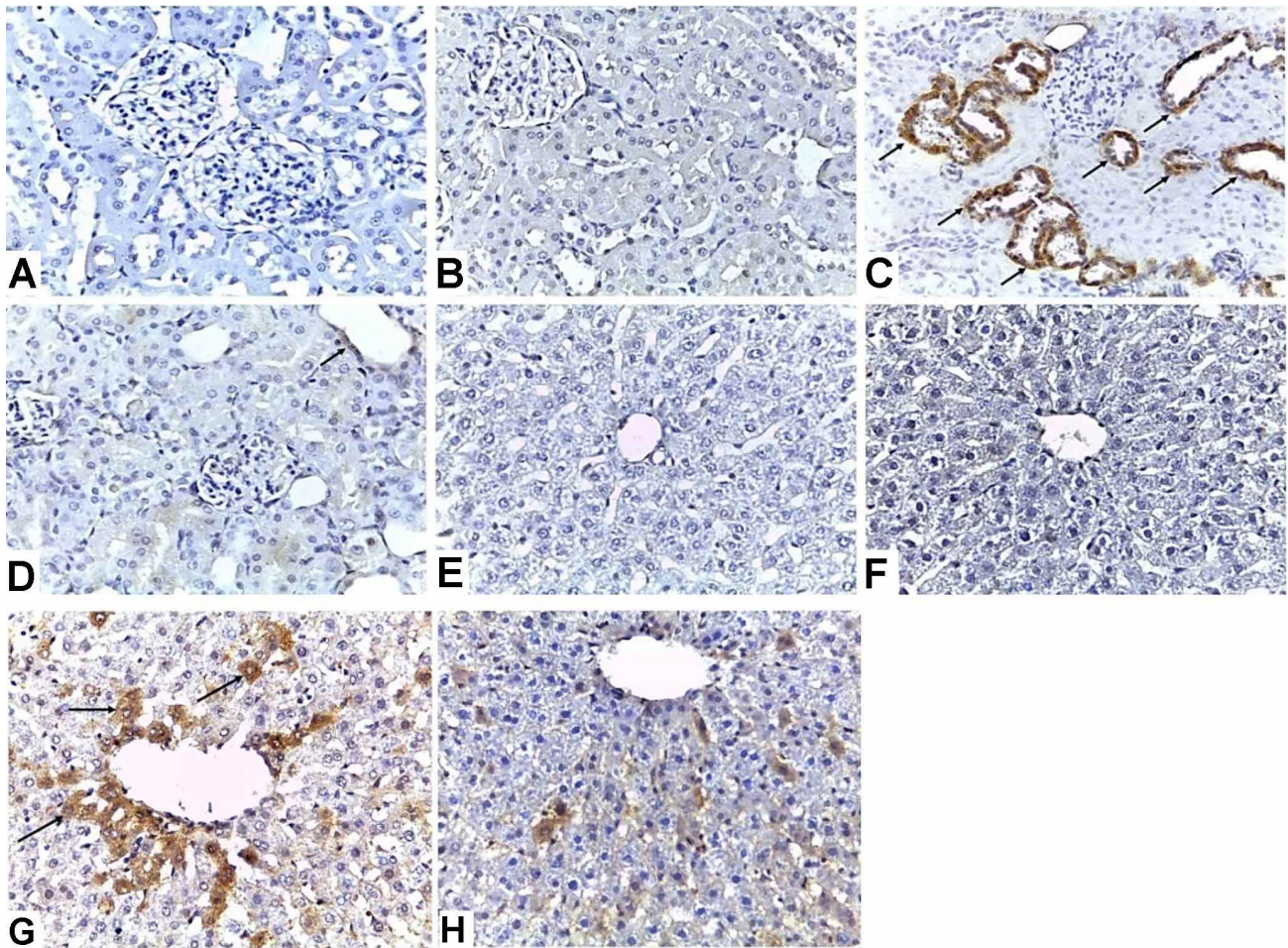


Fig. 5. There appears to be no Caspase-3 immune reaction in glomerular and tubular epithelial cells in control kidney tissue (A). The kidney tissue of the kefir-only group was similar to the control group (B). Caspase-3 staining in enlarged tubule epithelium in kidney tissues treated with cisplatin appears to be quite severe (→) (C). It was observed that caspase-3 (+) staining in tubule epithelial cells was decreased in the group administered cisplatin and given kefir (→) (D). In the liver tissue of the control group, caspase-3 (+) staining is not observed in hepatocytes, endothelial cells, and bile duct epithelial cells around the portal area (E). In the kefir-only group, liver structure was similar to the control group (F). A large number of caspase-3 (+) stained hepatocytes were observed in the cisplatin given group (→) (G). It is seen that the number of caspase-3 (+) stained hepatocytes and the intensity of staining were significantly decreased in the cisplatin administered and kefir given group compared to the cisplatin given group (H). Caspase-3 immunohistochemical staining, X40.

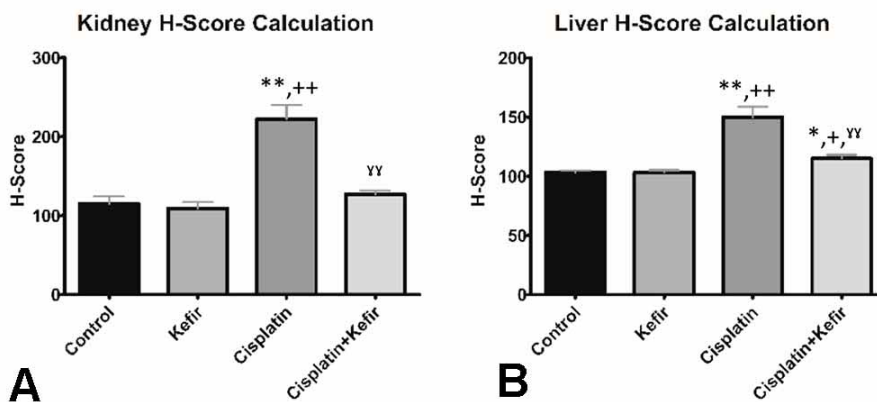


Fig. 6. The amount of caspase-3 positive cells was evaluated by the H-Score method. Amounts of caspase-3 positive cells in kidney (A) and liver (B) tissues by groups. *p<0.05, **p<0.01 compared to the Control group; +p<0.05, ++p<0.01 compared to the Kefir group; ??p<0.01 compared to the Cisplatin group.

DISCUSSION

In this study, the protective effect of oral kefir intake in rats damaged by cisplatin in liver and kidney tissue was determined by biochemical and histomorphological parameters.

The protective effect of various probiotic bacteria on cisplatin-induced liver and kidney damage in rats has been previously investigated (Fang *et al.*, 2014). However, in our study, different doses of cisplatin in different duration were applied. In addition, kefir content includes not only bacteria, but also yeasts, and there is a combined effect of microorganisms. Our findings support Fang *et al.* study and indicate the effect of kefir consumption on both kidney and liver damage.

As a result of the damage of cisplatin on the liver, it causes an increase in serum ALT and AST levels. In the study in which trimetazidine was used against the hepatotoxic effect of cisplatin, it was observed that the ALT value was higher in the Cisplatin group (Ateyya *et al.*, 2016). In our study, higher ALT values were observed in the Cisplatin group compared to the other groups, while this value was found to be approaching to the Control and Kefir groups in the CK group. Ijaz *et al.* (2020) in their study in which they investigated the protective effect of castisin against cisplatin, it was observed that the AST value was the highest in the cisplatin group and lower in the castisin-administered group. In our study, AST values were similarly decreased in the kefir-administered group. This suggests that kefir, a probiotic, may have a protective effect against the hepatotoxic effect of cisplatin.

In the study performed by Abdel-Daim *et al.*, using garlic oil against cisplatin hepatorenal toxicity, it was found that the creatinine values in the cisplatin group were half as much as in our study. It is thought that this may be due to the higher dose of cisplatin administered in our study. In our study, as in the kefir group, was observed that the garlic oil given by Abdel Daim *et al.* (2020) also decreased the creatinine values was found to be approaching to the control group.

Karafakiolu *et al.* (2017) evaluated the protective effect of safaranal against the nephrotoxicity of cisplatin. In this study, urea and creatinine values in the cisplatin group was similar to the values in our study, while urea and creatinine values were observed to be higher in the safaranal + cisplatin group. Our study demonstrated, kefir has a positive effect by reducing urea and creatine values. In rats with acute renal failure due to glycerol-induced rhabdomyolysis, kefir administration did not effectively re-

duce urea and creatinine values (Karabacak *et al.*, 2016). Contrary to these findings, we determined in this study that kefir consumption effectively reduced the urea and creatinine levels in the blood.

As a result of examining liver tissue sections in terms of eosinophilic cytoplasm, necrosis, congestion and mononuclear cell infiltration, it was determined that the most damage was in the Cisplatin group and less damage in the CK group. In other studies, similar histomorphological findings were obtained by using different agents against the hepatotoxic effect of cisplatin (Bhattacharyya & Mehta, 2012; Çiftçi *et al.*).

In the kidney tissue, as in the liver tissue, the most damage was in the Cisplatin group. Compared to the other groups, more mononuclear cell infiltration, tubular and glomerular damage were observed. Abdel Moneim *et al.* and Abdel-Daim *et al.* support our findings.

The increase in collagen fiber in the tissues is due to cellular damage and infiltration in that region (Li *et al.*, 2018). Cisplatin causes an increase in collagen fiber in liver and kidney tissues, as well as histomorphological changes (Alrashed & El-Kordy, 2019; Liang *et al.*, 2021). In our study, mononuclear cell infiltration and the amount of collagen fiber increased in the liver and kidney tissues, especially in the Cisplatin group. Kefir significantly reduced the increase in collagen fiber caused by cisplatin. This finding is Chen *et al.* (2019) is consistent with their study on the effectiveness of kefir peptides against pulmonary inflammation.

On the other hand, antiapoptotic activity of kefir was demonstrated by significantly decreasing caspase-3 marker in the CK group. It is thought that kefir provides this effect thanks to the antioxidants it contains. El Golli-Bennour *et al.* reported that kefir has an antioxidant effect against deltamethrin.

Cisplatin increases free oxygen radicals and induces apoptosis due to DNA damage in cells (Ateyya *et al.*; Li *et al.*). In our study, the activity of caspase-3, one of the apoptosis markers, was higher in both hepatocytes in liver tissues and tubular cells in kidney tissues in the cisplatin group. In the CK group, the number of caspase-3 positive cells in the liver and kidney tissues was significantly reduced. It has been shown that kefir reduces apoptosis by reducing caspase-3 expression in colonic crypt cells damaged by radiation (Matsuu *et al.*, 2003) and spinal cord ischemia damage (Güven *et al.*, 2015). Parallel to these studies, it was observed in our study that kefir decreased caspase-3 expression.

When cisplatin was administered intraperitoneally at a dose of 7 mg/kg, both liver and kidney tissues were damaged. kefir consumption improved the damage in these tissues histomorphologically.

In conclusion, kefir may provide benefits as a strong antioxidant and immune booster for patients with tissue and cell damage due to cisplatin treatment in terms of possible side effects. Nevertheless, it should be considered that the animal model may not be confirmatory for human studies in some circumstances. Therefore, the results need to be confirmed in many other studies.

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RESUMEN: El cisplatino es un agente quimioterapéutico que induce daño hepático y renal. En este estudio, intentamos investigar el efecto del kéfir, un alimento funcional y probiótico esencial, en el daño hepático y renal inducido por el cisplatino. Se dividieron ratas albinas Wistar en cuatro grupos: control, cisplatino (dosis única de 7 mg/kg, intraperitoneal), kéfir (2 ml/día, 7 días, sonda oral) y cisplatino + kéfir (CK). Al final del día 7, los animales fueron sacrificados. Se recolectaron muestras de sangre, riñón y tejido hepático. Se determinó ALT, AST, Urea y Creatina; Para el análisis histomorfológico, se realizaron tinciones con hematoxilina-eosina, tricrómico de Masson y para inmunohistoquímica, caspasa-3, un marcador de apoptosis. Los niveles séricos de urea y creatinina del grupo de cisplatino fueron significativamente más altos que los del grupo de control ($p < 0,05$). En el grupo CK, el consumo de kéfir disminuyó los niveles de urea y creatinina acercándose a los grupos Control y Kéfir. El cisplatino resultó en actividades más altas de ALT y AST, lo que indica daño hepatocelular, en comparación con el grupo Control ($p < 0,05$). El consumo de kéfir disminuyó las actividades de ALT tanto en el grupo Control como en el de Kéfir. Las observaciones histomorfológicas coincidieron con los resultados bioquímicos. En tejidos hepáticos y renales se observó daño estructural con

aumento de fibras colágenas en el grupo de Cisplatino, y la actividad de Caspasa-3 fue inmunohistoquímica-mente mayor que en los otros grupos. En el grupo de CK, el aumento de las fibras colágenas, el daño estructural y las actividades de Caspasa-3 fueron menores que en el grupo Cisplatino. El consumo de kéfir mejoró el daño hepático y renal. Sin embargo, se requiere más investigación para comprender mejor el efecto del kéfir.

PALABRAS CLAVE: Cisplatino; Kefir; Urea; Hígado; Riñón.

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