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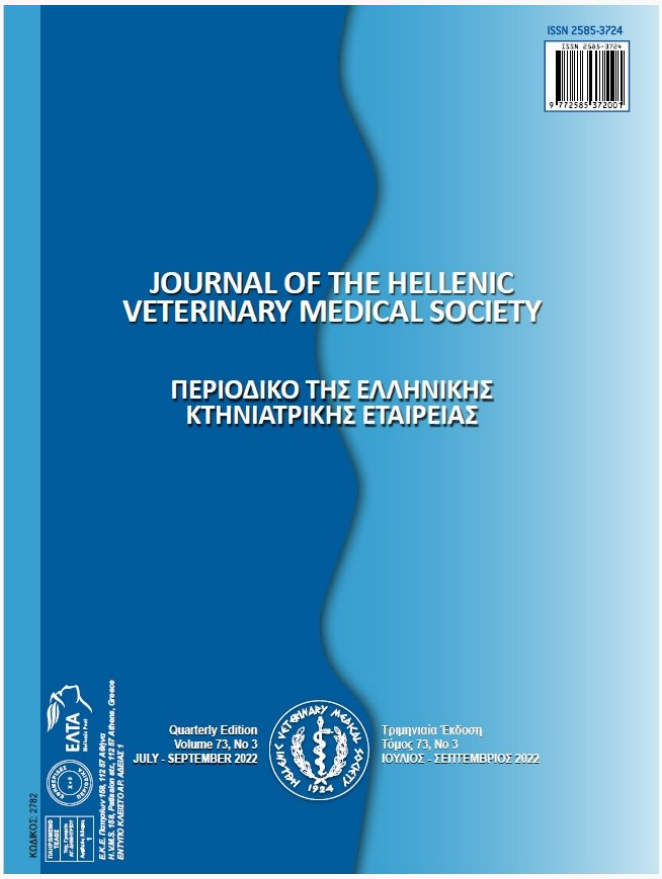

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Doxorubicin-induced oxidative stress injury: The protective effect of kumiss on cardiotoxicity

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ABSTRACT: Doxorubicin (DOX), which is used in cancer treatment, is an effective chemotherapy agent with many side effects. Cardiotoxicity, on the other hand, is the most important side effect, and it has pushed us to work with kumiss, an alcoholic beverage made from mare's milk, rich in fermentates, trace elements, antibiotics, vitamins, ethyl alcohol, lactic acid and carbonic acid.

The aim of this study was to investigate the effect of kumiss on cardiotoxicity caused by DOX. Twenty-eight Wistar-Albino male rats were divided into 4 groups: There was no intervention in the first group (control). The second group received 2 ml/kg/day of kumiss by gavage needle for 7 days, a single dose of 20 mg/kg, intraperitoneal DOX to the third group, and kumiss+DOX to the fourth group. Kumiss application was started 7 days before DOX administration and continued for 7 days. On the 7th day of kumiss application, DOX was administered intraperitoneally. The malondialdehyde (MDA), reduced glutathione (GSH) levels and antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase (G6PD) and glutathione-S-transferase (GST) activities were determined in order to determine their effectiveness in the pathogenesis of cardiotoxicity in cardiac and blood tissues.

When the DOX group was compared with the control group, an increase in MDA ($p<0.001$, $p<0.001$) and GSH ($p<0.001$, $p=0.002$) levels and a decrease in CAT ($p=0.001$, $p<0.001$), GSH-Px ($p<0.001$, $p<0.001$), G6PD ($p<0.001$, $p=0.001$) and GST ($p=0.003$) activities were found, and no statistically significant difference was found in SOD activity. Histopathologically, degeneration, necrosis, hemorrhage and oedema were observed in the DOX administered group. When compared with the group treated with DOX, it was observed that MDA, GSH levels and antioxidant enzyme activities reached the control group values in the group administered kumiss with DOX.

In conclusion, it was determined that an increase in lipid peroxidation products and a decrease in antioxidant enzymes may play a role in the pathogenesis of DOX-induced cardiotoxicity, a potent chemotherapeutic drug, and it has been shown that protects against DOX-induced oxidative damage.

Keywords: Doxorubicin; cardiotoxicity; kumiss; malondialdehyde; antioxidant

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ABBREVIATIONS LIST

1-chlorine-2,4-dinitrobenzene	: CDNB
2,2-Diphenyl-1-picrylhydrazyl radical	: DPPH
5,5'dithiobis 2-nitrobenzoic acid	: DTNB
Catalase	: CAT
Doxorubicin	: DOX
Glucose-6-phosphate dehydrogenase	: G6PD
Glutathione peroxidase	: GSH-Px
Glutathione reductase	: GR
Glutathione-S-transferase	:GST
Hemoglobin	: Hb
Hydrogen peroxide	: H_2O_2
Malondialdehyde	: MDA
Nicotinamide adenine dinucleotide phosphate	:NADP
Nitroblue tetrazolium	: NBT
Oxidized glutathione	: GSSG
Reactive oxygen species	: ROS
Reduced glutathione	: GSH
Superoxide anion	: $O_2^{\cdot-}$
Superoxide dismutase:	: SOD
Thiobarbituric acid	: TBA

INTRODUCTION

Doxorubicin (DOX), which is a broad-spectrum anti-tumor anthracycline, is most commonly used as an effective chemotherapeutic drug for a wide range of cancers, such as leukemia, solid tumors, soft-tissue sarcomas, and breast cancer (Songbo et al., 2019). DOX used in cancer therapy is an effective chemotherapeutic agent with many side effects. The most important side effect of the drug that restricts its use is cardiac toxicity (Narin et al., 2005). Free radicals, membrane lipid peroxidation, and mitochondrial damage are held responsible for DOX-induced cardiotoxicity (Yagmurca et al., 2007; Zare et al., 2019). The chemical structure of DOX increases oxidative stress, and causes a disequilibrium between free oxygen radicals and antioxidants, consequently leading to cellular damage (Narin et al., 2005). This disequilibrium in oxidant-antioxidant systems results in tissue damage, which manifests with lipid peroxidation in the tissue (Kimura et al., 2000). DOX is the active agent in the formation of free oxygen radicals, and one DOX molecule may generate many reactive free oxygen radicals (Gutteridge, 1993). There are two postulated theories of the formation of cardiotoxicity. The first theory is the enzymatic reduction of the quinone ring (Jungsuwadee 2016). In the investigation of cardiotoxic effects, the focus was on quinone and hydroquinone chromophore groups in the tetracyclic ring structure of DOX (Sahna et al., 2003). The quinone ring in its structure is reduced to the semiquinone radical by cytochrome P450 reductase and xanthine oxidase enzymes (Morishima et al., 1999). These radicals cause the formation of superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) by reducing the oxygen molecule. It increases oxidative stress and causes lipid peroxidation by causing a decrease in antioxidant enzyme levels such as endogenous catalase (CAT) and glutathione peroxidase (GSH-Px), which scavenges H_2O_2 and $O_2^{\cdot-}$ free radical (Reiter, 1992). Molecular oxygen is reduced, and semiquinone, hydroquinone, and free oxygen radicals are generated. The substances generated inhibit mitochondrial and microsomal enzymes, lead to lipid peroxidation, and disrupt the electron transport chain (Abd Elbaky et al., 2010; Jungsuwadee, 2016; Arslan et al., 2021). The second theory is the formation of the DOX-metal complex and causes the generation of strong oxidants with the redox cycle (Corna et al., 2004). The organism has the ability to eliminate free oxygen radicals but since the antioxidant enzymes are less abundant in the heart, it cannot protect itself against free oxygen radicals damages. This explains the mechanism of cardiac damage caused by DOX administration.

In recent studies, it was shown that DOX caused an increase in lipid peroxidation products such as malondialdehyde (MDA) and free radical formation, as well as leading to cardiotoxicity by reducing antioxidant enzymes (CAT, GSH-Px, superoxide dismutase (SOD), glutathione reductase (GR), glutathione-S-transferase (GST)) (Elberry et al., 2010; Kwatra et al., 2016).

In the experimental studies of DOX, it was shown that the survival rate was higher in those receiving antioxidants together with cytostatic agents such as DOX than those receiving chemotherapy alone (Narin et al., 2005).

Kumiss is a traditional drink obtained by the fermentation of mare milk. Kumiss, an alcoholic drink made of fermented mare milk, is rich in fermentates, trace elements, antibiotics, vitamins (A, B1, B2, B12, D, E, C), ethyl alcohol, lactic acid, and carbonic acid (Solaroli et al., 1993; Abdel-Salam et al., 2010). Approximately 50% of the mare milk protein is casein, and the other half contains lactate and lactoglobulin (Gulmez and Atakisi, 2020). The high amount of lactoglobulin and lactalbumin in total protein gives the milk easy digestibility, liquid quality, and high nutritional value (Danova et al., 2005). While the amount of fat, protein, and inorganic salts in mare milk is lower than cow's milk, its lactose content is higher than

that of cow's milk and closer to that of human breast milk (Pieszka et al., 2016). Moreover, it also contains high-molecular-weight unsaturated fatty acids, including linolenic acid, rich in high physiological values (Osorio et al., 2011). Many researchers have reported that kumiss provided positive outcomes in the treatment of diseases of endocrine glands, urinary, circulatory, nervous, and gastrointestinal systems, respiratory tract, tuberculosis, dysentery, typhoid fever, paratyphoid fever, ulcer, and hepatitis, and strengthens the immune system (Kurmann et al., 1992). Moreover, since kumiss is rich in amino acids that prevent atherosclerosis such as lysine, tyrosine, tryptophan, and glutamic acid, and contains the appropriate amounts and ratios of these, it can be used as a drug in the treatment of this disease (Kurmann et al., 1992; Danova et al., 2005; Tegin and Gönülalan, 2014; Rajoka et al., 2020; Yigit, 2020).

Although there are some studies in the literature on the chemical structure, properties, and health effects of kumiss, there is a very limited number of studies on its antioxidant effects (Abdel-Salam et al., 2010). Due to the lack of numerous studies on the antioxidant activities of kumiss, investigating whether kumiss has a protective effect on cardiac damage caused by DOX used in chemotherapy was considered a useful approach in terms of scientific resources.

MATERIALS AND METHODS

This study was performed with the approval of the Firat University Animal Experiments Local Ethics Committee (Protocol No: 2012/03-43). In the study, 3-month-old male Wistar-Albino rats weighing 250-300 g, obtained from the Firat University Laboratory Animals Breeding Unit, were used. The rats were kept in air-conditioned rooms with a fixed temperature of 25±2ºC and 60-65% humidity, with a 12/12h light/dark cycle, under standard conditions, and were fed on standard rat food (pellet) and tap water *ad libitum* throughout the experimental practices (Yamauchi et al., 1981). Experimental practices on rats were performed in the Firat University Experimental Research Center.

In the study, rats were divided into 4 groups with 7 rats in each group: 1st group: control group, 2nd group: the group that received 2 ml/kg/day kumiss (It was brought from Kyrgyzstan without breaking the cold chain and applied while fresh) by gavage for 7 days, 3rd group: the group that received single dose 20 mg/kg body weight DOX (Fresenius Kabi Oncology Ltd. 19 Industrial Area, Baddi, Distt. Solan-India) intraperitoneally, and 4th group: the group that received kumiss (2 ml/kg/day by gavage, 7 days) + DOX (20 mg/kg body weight, intraperitoneal single dose). Kumiss was administered 7 days before DOX administration and continued for 7 days. On the 7th day of kumiss administration, DOX was administered intraperitoneally. The amount of DOX used in the study was determined based on the previous studies (Iqbal et al., 2008; Kaya and Yılmaz, 2019). Kumiss was administered via a 2 ml gavage tube. Rats were sacrificed by decapitation method 3 days after DOX administration in DOX treated group, rats in the control and kumiss groups 7 days after the start of the experiment were sacrificed, in the kumiss+DOX group, kumiss pre-treatment was performed for 4 days, then DOX administrated, were sacrificed by decapitation method 3 days after DOX administration.The levels of MDA, GSH, and the activities of antioxidant enzymes such as CAT, GSH-Px, SOD, GST, and glucose-6-phosphate dehydrogenase (G6PD) in the cardiac tissue were determined spectrophotometrically (Thermo Scientific, Genesys 10S UV-VIS Spectrophotometer, USA).

Cardiac tissue samples were stored at -80 °C until biochemical analysis. Cardiac tissue samples were washed with physiological saline solution and then diluted with distilled water at a ratio of 1:10 (weight/volume) and homogenized using Potter-elvehjem homogenizer (CAT R50D, Germany). Homogenates were centrifuged (NUVE NF800R, Turkey) at +4 °C for 15 minutes at 3500 rpm for MDA, GSH, CAT, SOD, GST, and G6PD analysis, and for 55 minutes at 13500 rpm for GSH-Px analysis (Kaya and Yılmaz, 2019).

To blood samples in EDTA tubes (BD Vacutainer, K_2E 5.4 mg, BD-Plymouth, PL67BP, UK) were centrifuged at 3000 rpm for 15 minutes and plasma was obtained. After centrifugation, the plasma accumulated in the upper part of the tube was carefully taken with the help of a pipette and transferred to clean eppendorf tubes (Kaya and Yılmaz, 2019). Plasma was used to measure MDA level as a marker of lipid peroxidation. Whole blood was used for GSH and GSH-Px determination. Plasma separated EDTA blood samples were washed 3 times with saline (0,9% NaCl). After that, CAT, SOD and G6PD activities and hemoglobin (Hb) levels were determined in erythrocytes.

The changes in MDA levels in tissue samples were measured spectrophotometrically using the method modified from Placer et al. (1966). This method

is based on the reaction between MDA, one of the products of lipid peroxidation, and thiobarbituric acid (TBA). The GSH level was determined by the method reported by Ellman et al. (1961). This method is based on spectrophotometric measurement of the yellow color formed when 5,5'dithiobis 2-nitrobenzoic acid (DTNB) is added to sulfhydryl groups. CAT activity was determined by the method of Aebi (1974). The rate of hydrogen peroxide (H_2O_2) degradation by CAT enzyme was determined spectrophotometrically using the H_2O_2's ability to absorb light at 240 nm. GSH-Px and G6PD activities were determined by the Beutler method (1984). GSH-Px catalyzes the oxidation of GSH to oxidized glutathione (GSSG) using H_2O_2. The rate of GSSG formation is measured using GR reaction. SOD activity was determined according to the method modified by Sun et al. (1988). SOD activity is measured using the method based on the measurement of color development upon the reduction of nitroblue tetrazolium (NBT) by the superoxide anion ($O_2^{\cdot-}$) produced by the xanthine-xanthine oxidase system. GST activity was determined by the spectrophotometric measurement of the product (1-(S-glutathionyl)-2,4 dinitrobenzene) formed when GSH was combined with 1-chlorine-2,4-dinitrobenzene (CDNB) compound at 340 nm (Habig et. al. 1974). G6PD activity was measured by the Beutler method (1984). Hemoglobin concentrations were determined according to the method developed with Drabkin' ssolution (Frankel et al., 1970). Protein levels in tissue homogenate were determined according to the method of Lowry et al. (1951).

Histopathological examination

At the end of the experiment, necropsy of the rats was performed and myocardial tissue samples were fixed in 10% neutral buffered formalin. Paraffin embedded blocks were routinely processed and 5 μm thick sections were stained with haematoxylin-eosin and examined under a microscope and randomly 10 microscopic fields were examined in X40 magnification. The histopathological findings in sections were graded as 0 (none), 1 (mild), 2 (moderate), 3 (severe) (Luna, 1968).

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS statistical software (SPSS for Windows, version 22) and R 3.6.2 (https://www.r-project.org/). Continuous variables were presented in mean (±) standard error (S.E.) and also medians and interquartile range (IQR). The variables were investigated using Kolmogorov-Smirnov/ Shapiro-Wilk's test to determine whether or not they are normal distributions. Differences in measured parameters among the four groups were analyzed with a nonparametric test (Kruskal-Wallis test). The Mann-Whitney U test was performed to test the significance of pairwise differences using Bonferroni correction to adjust for multiple comparisons. The correlation coefficients and their significance were calculated using the Spearman test for investigating the associations between non-normally distributed variables. Ap <0.05 was considered statistically significant for all analyses.

RESULTS

Table 1 shows the MDA and GSH levels in the cardiac and blood tissue of rats in the control and experimental groups. Table 2 shows the CAT, GSH-Px, SOD, GST, and G6PD enzyme activities. In the group receiving DOX, an increase in MDA ($p<0.001$, $p<0.001$) and GSH ($P<0.001$, $p=0.002$) levels, and a statistically significant decrease in CAT ($p=0.001$, $p<0.001$), GSH-Px ($p<0.001$, $p<0.001$), G6PD ($p<0.001$, $p=0.001$), and GST ($p=0.003$) activities were detected compared with the control group. When compared with the control group receiving kumiss alone, no statistically significant difference could be detected. No statistically significant difference was detected in SOD activity in the group receiving DOX. When compared with the group receiving DOX, it was found that MDA and GSH levels and CAT, GSH-Px, G6PD, and GST activities in the group receiving kumiss and DOX were closer to those of the control group.

Figures 2-5 show the relationship between MDA levels and antioxidant activities in the groups receiving DOX, kumiss and DOX in the cardiac and blood tissue. There was a negative correlation between MDA levels, an indicator of oxidative stress, and antioxidant activities in the group receiving DOX in the cardiac and blood tissues. As MDA levels increase, antioxidant activities decrease.There was a negative correlation between MDA levels and antioxidants activities in the group receiving kumiss and DOX in the cardiac and blood tissues. As MDA levels decrease, antioxidant activities increase. Antioxidants have a positive correlation among themselves. As the activity of one antioxidant increases, the other also increases in the group receiving kumiss and DOX in the cardiac and blood tissues. No correlation was found between SOD activities and CAT and GSH-Px activities in the group receiving kumiss and DOX in the blood tissue.

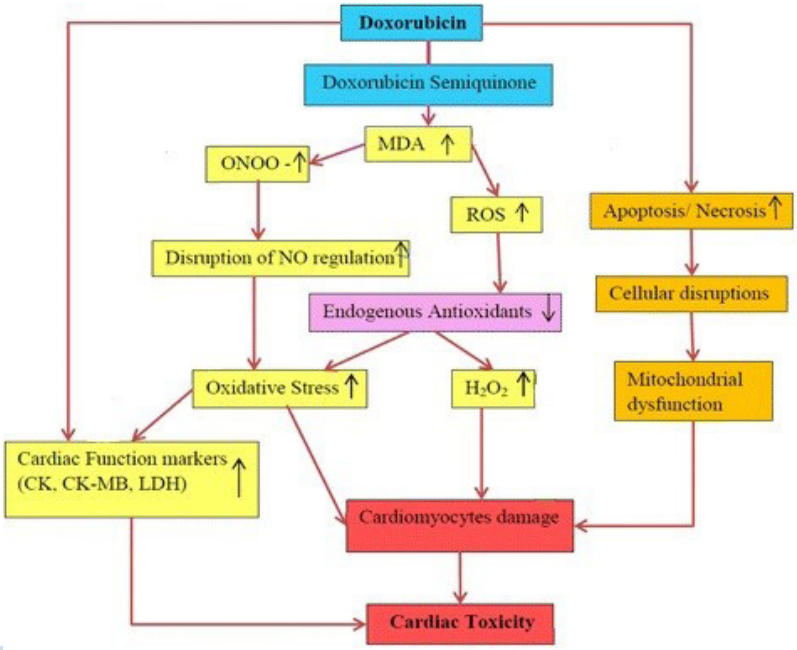


Figure 1. Possible underlying mechanism of DOX-induced cardiotoxicity

Table 1. Effects of kumiss on the levels of MDA and GSH in heart and blood tissues of DOX treated rats

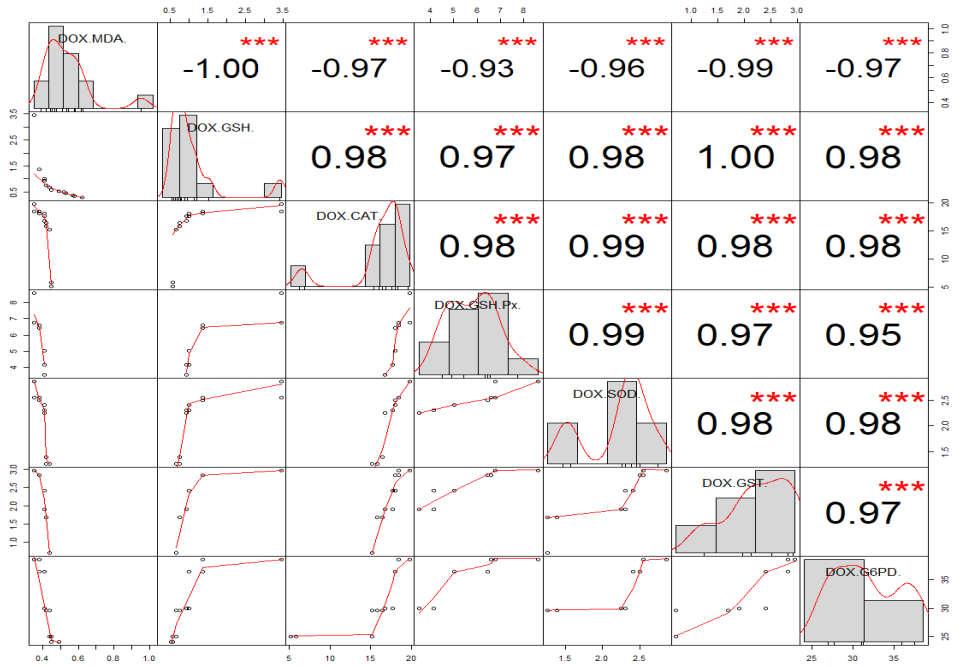
		Control	Kumiss	DOX	Kumiss+DOX	p
Heart	MDA (nmol/g tissue)	0,30±0,01 [a]	0,24±0,0 [a]	0,52±0,02 [b]	0,37±0,0 [a]	p<0,001
	GSH (µmol/ml)	0,25±0,0 [a]	0,33±0,0 [a]	0,86±0,13 [b]	0,31±0,01 [a]	p<0,001
Blood	MDA (nmol/ml)	8,56±0,96 [a]	10,77±1,51 [a]	16,10±0,75 [b]	11,76±1,30 [a]	p<0,001
	GSH (µmol/ml)	73,13±2,93 [a]	82,15±3,47 [a]	58,23±3,18 [b]	72,55±2,10 [a]	p=0,002

Data are expressed as mean ± SEM. Different superscript letters (a, b) show significant differences between the groups

Table 2. Effects of kumiss on the activities of CAT, GSH-Px, SOD, G6PD and GST in heart and blood tissues of DOX treated rats

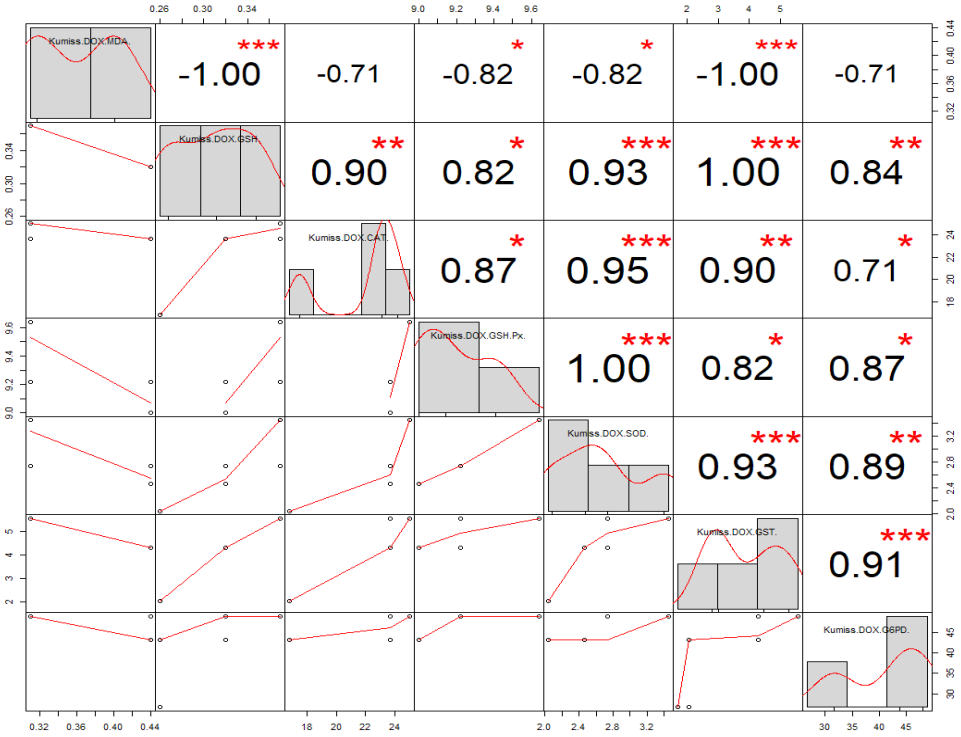
		Control	Kumiss	DOX	Kumiss+DOX	p
Heart	CAT (k/g Prot.)	23,21±1,22 [a]	21,20±1,53 [a]	16,12±0,87 [b]	22,29±1,21 [a]	p=0,001
	GSH-Px (U/g Prot.)	10,70±0,53 [ab]	11,21±0,32 [a]	5,59±0,46 [c]	9,28±0,12 [b]	p<0,001
	SOD (U/mg Prot.)	2,05±0,17	2,58±0,13	2,19±0,14	2,67±0,19	p>0,05
	G6PD (U/g Prot.)	44,76±2,17 [a]	41,68±0,80 [a]	30,60±1,11 [b]	39,59±2,84 [a]	p<0,001
	GST (U/mg Prot.)	3,51±0,29 [a]	3,79±0,25 [a]	2,08±0,18 [b]	3,40±0,48 [a]	p=0,003
Blood	CAT (k/g Hb)	71,06±5,21 [a]	62,32±3,69 [a]	42,27±2,38 [b]	63,47±3,52 [a]	p<0,001
	GSH-Px (U/g Hb)	3,67±0,24 [ab]	4,25±0,34 [a]	1,49±0,09 [c]	3,20±0,18 [b]	p<0,001
	SOD (U/g Hb)	10,69±2,25	14,41±1,20	9,14±1,87	15,69±1,79	p>0,05
	G6PD (U/g Hb)	1,39±0,11 [ab]	1,86±0,22 [a]	0,69±0,04 [c]	1,29±0,15 [b]	p=0,001

Data are expressed as mean ± SEM. Different superscript letters (a, b, c) show significant differences between the groups



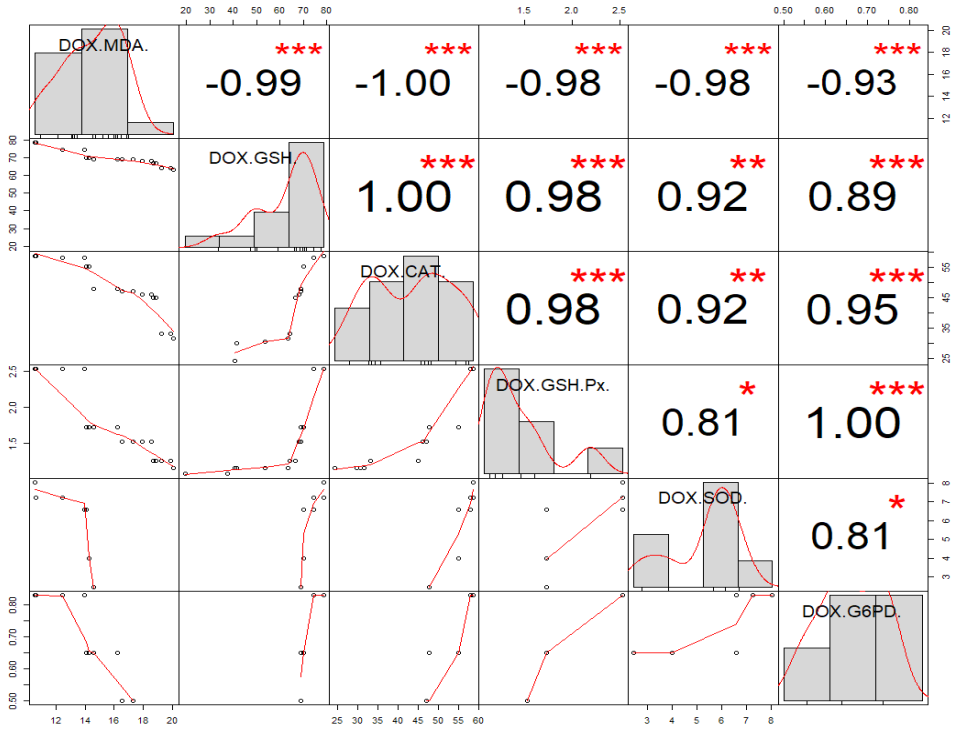
***Correlation is significant at the 0.001 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

Figure 2. Relationship between MDA levels and antioxidant activities in the group receiving DOX in the cardiac tissue



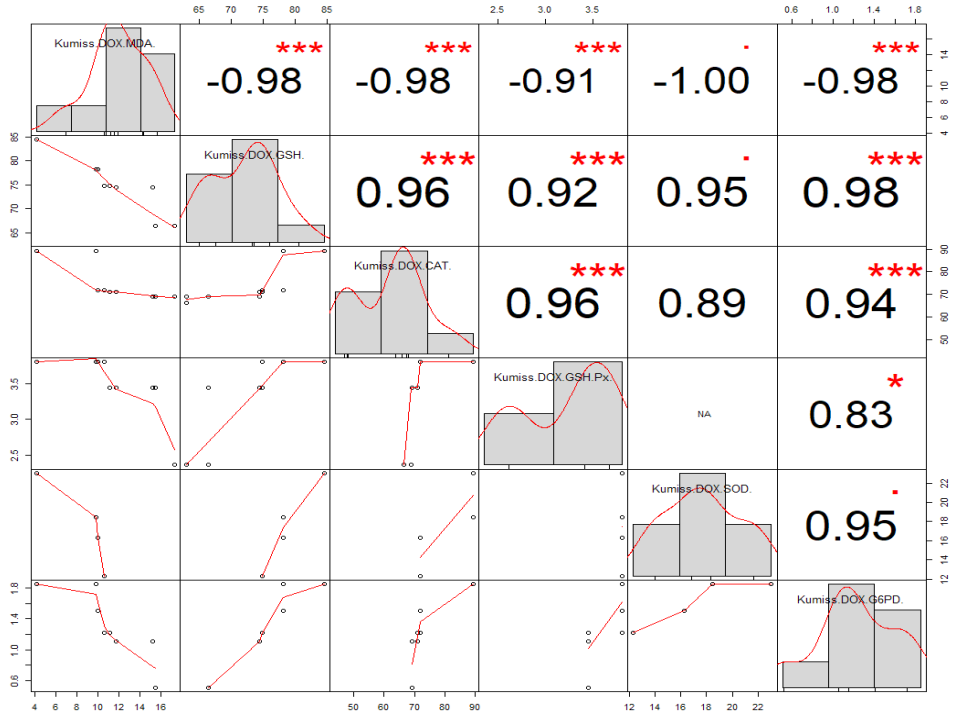
***Correlation is significant at the 0.001 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

Figure 3. Relationship between MDA levels and antioxidant activities in the group receiving kumiss and DOX in the cardiac tissue



***Correlation is significant at the 0.001 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

Figure 4. Relationship between MDA levels and antioxidant activities in the group receiving DOX in the blood tissue.



***Correlation is significant at the 0.001 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

Figure 5. Relationship between MDA levels and antioxidant activities in the group receiving kumiss and DOX in the blood tissue

After the experiments, no pathological signs were detected in the cardiac samples of the rats in the control and kumiss groups and were found to have a normal histological structure. (Figure 6 and Table 3). When DOX and kumiss + DOX groups were examined in terms of degeneration and necrosis, hemorrhage, oedema, and inflammatory cell infiltration, statistically significant differences were detected. It was found that the histopathological signs such as degeneration and necrosis ($p<0.01$), hemorrhage ($p<0.05$), oedema ($p<0.05$), and inflammatory cell filtration was observed in the DOX group was severer compared with the kumiss+ DOX group (Table 3). It was found that the histopathological signs observed in a severe form in the DOX group decreased in the group receiving kumiss and DOX (Figure 6).

DISCUSSION

DOX is an antitumor agent and has widespread use in the treatment of many cancer types in both human and veterinary medicine, but its toxic side effects restrict its therapeutic use (Barton 2001; Hohenhaus et al., 2002; Alshabanah et al., 2010). Dose-dependent and long-term use of DOX has toxic effects on the liver, kidney, and cardiac tissue and disrupt basal metabolism (Kalender et al., 2005; Rawat et al., 2021). It has been proposed that the main reason behind DOX-induced toxicities is oxidative stress (Chen et al., 2007). It has been stated that reactive oxygen species (ROS) that are generated after DOX use, such as $O_2^{\cdot-}$, hydroxyl radicals, and H_2O_2 may cause organ damage (Karim et al., 2001; Yarmohmmadi et al., 2017).

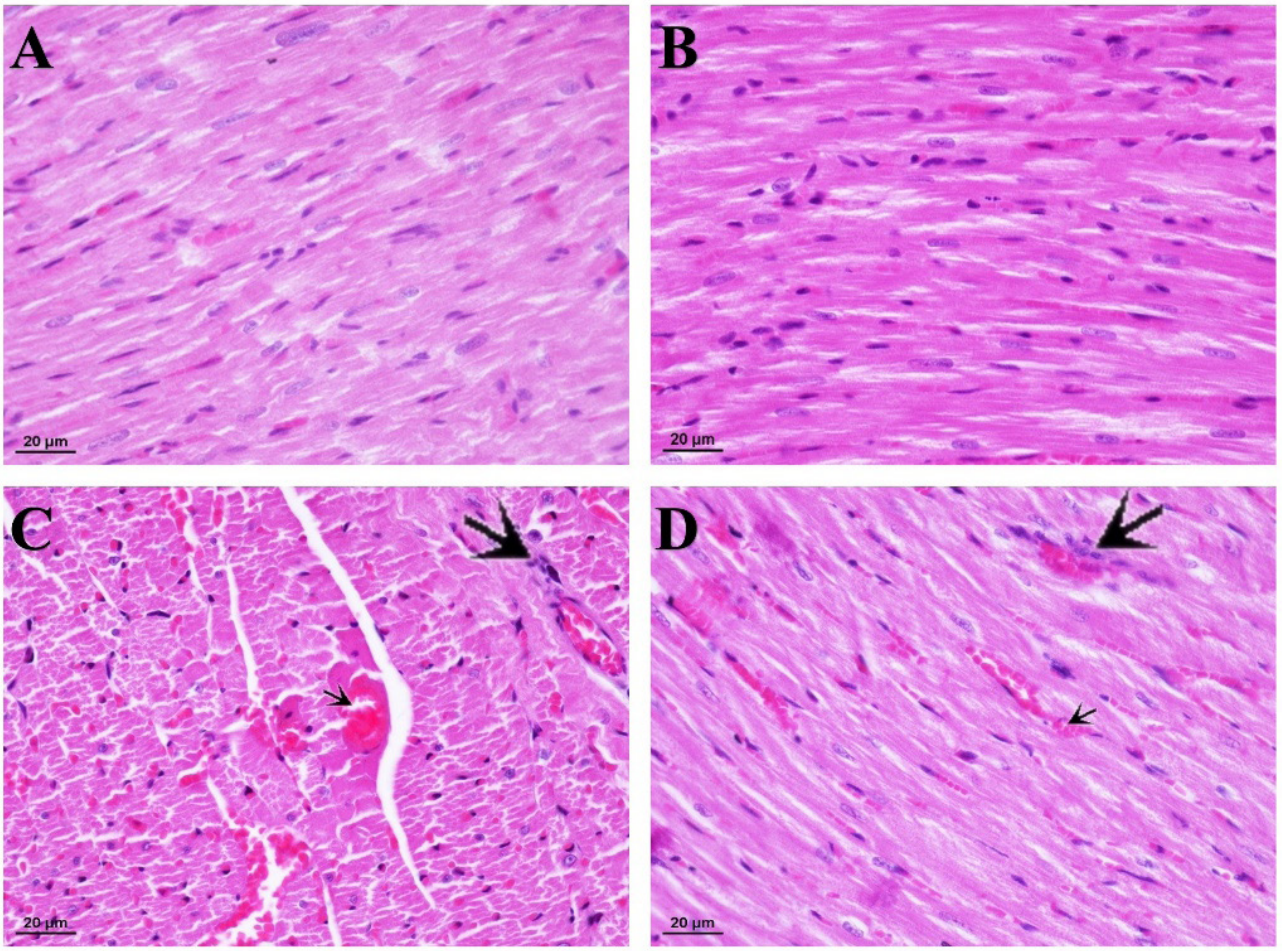


Figure 6. Control group: Normal histological structure (A). Kumiss applied group: Normal histological structure (B). DOX applied group: Inflammatory cell infiltrates (large arrow) and edematous appearance with hemorrhagic areas (small arrow) (C). Kumiss+DOX applied group: Inflammatory cell infiltrations (large arrow) and milder hemorrhagic areas (small arrow) (D)

Table 3. Effects of kumiss on the some pathological change in heart tissue of DOX treated rats

	Control	Kumiss	DOX	Kumiss+DOX	p
Degeneration and necrosis	0.00±0.00[a]	0.00±0.00[a]	1.42±0.29[b]	0.42±0.20[a]	p<0.01
Inflamatory cell infiltration	0.00±0.00[a]	0.00±0.00[a]	0.71±0.28[b]	0.42±0.20[a]	p<0.05
Hemorragie	0.00±0.00[a]	0.00±0.00[a]	1.57±0.36[b]	0.57±0.20[a]	p<0.05
Oedema	0.00±0.00[a]	0.00±0.00[a]	1.14±0.34[c]	0.28±0.18[b]	p<0.05

Data are expressed as mean ± SEM. Different superscript letters (a, b, c) show significant differences between the groups

Restricted use of the antitumor effect of DOX, an effective antitumor agent, due to its cardiotoxic side effect, brings the studies on the prevention of its cardiotoxicity to the fore (Narin et al., 2005). It has been thought that free radical formation, increase in lipid peroxidation, and decrease in antioxidant enzymes play a role in the development of DOX-induced cardiotoxicity (Kalender et al., 2005; Chen et al., 2007; Karim et al., 2001). In the study by Fadillioğlu et al., (2003) DOX-induced cardiac tissue damage was considered biochemical, and it was attributed to DOX-induced protein oxidation and lipid peroxidation. The most critical region for free radical damage is the plasma membrane. Free radicals generated in the extracellular compartments have to pass through the plasma membrane in order to react with intracellular compartments. Thus, free radicals initiate their harmful effects firstly in the membranes (Tanriverdi, 2005). Unsaturated bonds of the cholesterol and fatty acids at the cell membrane easily react with free radicals and generate peroxidation products. This oxidative degradation of polyunsaturated fatty acids is referred to as lipid peroxidation (Cheeseman and Slater, 1993; Tanriverdi, 2005). MDA is an important oxidation product of polyunsaturated fatty acids, and increased MDA content is an important indicator of lipid peroxidation. Almost all biomolecules can be sequestered by free radicals. But it is the lipids that are most exposed. Cell membranes are rich in unsaturated fatty acids, which are easily trapped by oxidizing radicals. Lipid peroxidation, the oxidative breakdown of unsaturated fatty acids, is damaging. Because self-propagating chain reactions continue. MDA, one of the lipid peroxidation products, causes cross-linking and polymerization in membrane components and has carcinogenic properties by reacting with the nitrogen bases of DNA (Cheeseman and Slater, 1993; Acaroz et al., 2018). MDA is not a one-to-one indicator of fatty acid oxidation, but shows a good correlation with the degree of lipid peroxidation. For this reason, the measurement of MDA levels is a frequently used method to measure the level of lipid peroxidation formed in the organism (Uysal, 1998; Sheibani et al., 2020).There are many studies in which elevated cardiac tissue MDA levels are held responsible for the pathophysiology of DOX-induced cardiotoxicity (Shaker et al., 2018; Kaya and Yılmaz, 2019). Luo et al.(1997) showed that MDA levels increased in the cardiac tissue of rats that received single-dose 10 mg/kg DOX, stating that DOX induced the release of MDA or other similar cytotoxic compounds and initiated cardiotoxicity. Again, in a study by Sacco et al., (2001) lipid peroxidation and protein oxidation were listed as the two important outcomes of oxidative damage in the cellular structure, and it was stated that increased free oxygen radicals and $O_2^{\cdot-}$in DOX-induced cardiotoxicity may lead to cellular damage. Iliskovic et al. (1999) reported that they detected a significant increase in MDA levels and a significant decrease in GSH-Px activity in cardiac tissue after DOX administration. Alyane et al. (2008) observed that, 24 hours after 20 mg/kg DOX administration, MDA and $O_2^{\cdot-}$ levels in the cardiac tissue of rats were high. In our study, a significant increase in MDA levels was observed in the group that received DOX compared with the control group.

The effects of oxidative stress can be evidenced by cellular accumulation of lipid peroxides. Cardiomyocytes are rich in mitochondria, a major subcellular target of DOX. Compared with the number in other tissues, the number of mitochondria in cardiomyocytes was increased by 35-40%, which may be one of the reasons why the cardiac cell is prone to injury. In the course of DOX treatment, a large amount of ROS are produced by reduction of the redox cycle at complex I of the electron transport chain, leading to the disruption in ATP synthesis (Alexieva et al., 2014). In our study, a significant increase in MDA levels was observed in the group that received DOX compared

with the control group. The increase in lipid peroxidation might be attributed to free radicals formed either by the reaction of drug toxic radicals with oxygen or by the interaction of $O_2^{\cdot-}$ with H_2O_2 seemed to initiate lipid peroxidation suggesting that increased lipid peroxidation might be associated with cellular damage. The increase in MDA levels especially due to DOX's reaction with polyunsaturated fatty acids at the cell membrane corroborates the view that the increase in lipid peroxidation products also plays a role in the pathogenesis of DOX-induced cardiotoxicity. Kumiss was found to decrease cardiac tissue MDA levels. This result suggests that kumiss exerts its antioxidant effect by preventing lipid peroxidation.

It was shown that DOX led to toxicity by causing free radical formation as well as reducing the activities of antioxidant enzymes such as GSH, GSH-Px, and CAT. In general, antioxidant storage in cardiac tissue is lower compared with the other organs in the body, which makes the heart more sensitive to the damage caused by DOX-induced free radicals (Kaya and Yılmaz, 2019).

The role of these antioxidants is to prevent the lipids in membrane structure from peroxidation by preventing peroxidation chain reactions and collecting ROS (Sangomla et al., 2018; Kaya and Yılmaz, 2019). The main antioxidant enzyme that protects against free oxygen radicals is GSH-Px (Yilmaz and Yilmaz, 2006). In our study, GSH-Px activity was found to decrease in the group that received DOX alone, this finding corroborated the view that the decrease in GSH-Px plays the primary role in the pathogenesis of DOX cardiotoxicity. Li and Singal (2000) reported that they detected a decrease in GSH-Px enzyme activity from the second hour after the last dose of DOX in rats that received DOX up to a cumulative dose of 15 mg/kg, whereas Yin et al. (1998) reported that GSH-Px activity did not change when DOX was administered as a single dose of 15 mg/kg. In the study by Yağmurca et al. (2007) a decrease was detected in the activity of antioxidant enzymes such as CAT and SOD. In the kidney damage model generated with DOX used by Malarkodi et al., (2003) it was shown that the enzymes such as CAT, GSH, and GST decreased significantly compared with the control group. In the study by Chopra et al. (1995) in which the effects of propolis administration on cardiotoxicity caused by DOX administration (10 mg/kg) in rats, it was reported that blood and tissue GSH levels increased after DOX administration. In their biochemical study in rat cardiac cell culture model, Chularojmontri et al. (2005) investigated the antioxidant capacity of cells in the damage caused by DOX and how this changed with vitamin C and Vitamin E, and found that CAT and SOD activity and GSH level were lower in the DOX group compared with the control group, and higher in vitamin C and vitamin E groups. Bolaman et al. (2005) investigated the protective effect of amifostine in rats with acute cardiotoxicity generated by single-dose 10 mg/kg DOX administration, and found that MDA levels in cardiac tissue increased after DOX administration, and the levels of other antioxidant enzymes decreased significantly, concluding that amifostine can decrease the cardiotoxicity of DOX.

Antioxidant enzymes, i.e. SOD, GSH, GSH-Px, GST, GR, G6PD provides defense against oxidative stress mediated tissue injury. In the study, MDA and GSH levels increased significantly in the cardiac tissue of rats that received DOX, whereas CAT, GSH-Px, and G6PD enzyme activities decreased, which corroborated the hypothesis that free radicals play a major role in DOX cardiotoxicity. The findings in the study suggest that DOX may cause the formation of active free radicals due to the increase in oxidative stress it induces. Based on the results of the study, the increase observed in GSH levels after DOX administration was considered a reaction of tissues against oxidative stress. Moreover, the increase in GSH levels can be interpreted as the decrease in the activity of GSH-Px, which is an antioxidant enzyme that catalyzes the transformation of GSH to GSSG, might have prevented the transformation of GSH into GSSG. GSH is the major cellular -SH compound that acts as a nucleophile and potent reducing agent by interacting with numerous electrophilic and oxidizing compounds. It can act as a non-enzymatic antioxidant via the direct interaction of the -SH group with free oxygen radicals, or it can take part in the enzymatic detoxification reaction for free oxygen radicals as a coenzyme (Yilmaz et al., 2006). While GST causes the elimination of many toxic materials from the body, it also facilitates the transport of nonsubstrate ligands such as isomerization of prostaglandins, bile salts, heme, bilirubin, and fatty acids by binding them to GSH. At the same time, it can prevent the reactive electrophilic compounds from causing harm to the organism by covalently binding the same type of compounds to one another (Harmankaya and Özcan 2017). The decrease in GST activity can be interpreted as a response to the increase in toxic substances transported in the cell by being bound to GSH in order

to fight the generation of ROS during DOX metabolism.

G6PD catalyzes the initial reaction in the Pentose Phosphate Pathway where nicotinamide adenine dinucleotide phosphate (NADP) is reduced to NADPH. The Pentose Phosphate pathway is the only source of NADPH in erythrocytes. NADPH has a vital importance in protecting the cell from oxidative stress caused by free radicals on many molecules such as nucleic acids, proteins and membrane lipids.

GSH, which is involved in the reduction of free radicals, is itself oxidized and GSSG is formed, which must be reduced by NADPH in order to take part in the reduction of oxidized glutathione and again in antioxidant reactions. Production of NADPH is essential for the protection of erythrocytes against peroxides. In addition to the G6PD enzyme in the elimination of ROS, reduction of oxidized glutathione and $NADP^+$; SOD, CAT, GSH-Px, GR enzymes are also involved. If the production of free radicals in the cell exceeds the elimination ability of these enzymes, the antioxidant defense system is impaired and oxidative stress occurs (Matés et al., 1999).

In a study where they investigated the effect of kumiss on mercury toxicity in rats, Abdel-Salam et al. (2010) added 6% soluble fiber and Streptococcus thermophilus, Lactobacillus acidophilus, and Bifidobacterium bifidum probiotic bacteria to the kumiss they obtained by fermenting mare milk, and measured this mixture's capacity to reduce 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) and compared the antioxidant activities. Although the compositions were similar, kumiss containing a high amount of fiber and probiotics showed a higher antioxidant activity than kumiss and mare milk. The antioxidant activity of fresh mare milk was measured as 14.97, kumiss as 16.93, and the kumiss containing fiber + probiotics as 28.43 (μmol Trolox/100 g. dry matter). Statistically significant correlations were detected between all antioxidant activities of kumiss and their soluble protein contents, and total phenolic matter, reducing power, H_2O_2 elimination, and DPPH radical reduction antioxidant activities of all kumiss were determined. In a study in which the effects of kumiss, a drink made of fermented mare milk, on sirtuin deacetylases in the oxidative stress induced by 1,2-dimethyl hydrazine (DMH), male mice received kumiss (2×10^8 cfu / mL), DMH (20 mg / kg), and kumiss + DMH (2×10^8 cfu / mL + 20 mg / kg), and sirtuin (SIRT)2, SIRT3 protein expressions, immunolocalization and inhibitor antioxidant activity analysis were performed in liver, colon, and kidney tissues. It was found that DMH administration reduced SIRT2 and SIRT3 protein expression levels and disrupted the antioxidant system, concluding that this can increase DNA damage and oxidative stress and trigger tumor formation. The ability of kumiss to significantly eradicate the effects of oxidative stress is a result of multidirectional effects of sirtuin proteins on antioxidant system. It can be thought that kumiss exerts this effect that increases or induces SIRT3 expression by regulating ROS levels or protecting the cell from oxidative stress via transcription factors. According to these findings, oral intake of kumiss protects the antioxidant system, as well as reducing oxidative stress (Gulmez and Atakisi, 2020). In our study, in the group that received kumiss together with DOX, cardiac tissue CAT, GSH-Px, and GST activities were maintained. Kumiss was thought to be an effective agent in reducing lipid peroxidation and increasing the activities of decreased antioxidant enzymes in cardiotoxicity induced by DOX.

Animal studies revealed that apoptotic cell death occurred *in vivo* after DOX exposure. Cell culture studies also showed apoptotic and necrotic cell death induced by DOX. In the endomyocardial biopsies of patients treated with DOX, evidence of mitochondrial damage and apoptosis was found (Khan et al., 2005; Deman et al., 2001; Su et al., 2015).

They reported kidneys in toxic group revealed reduction in glomerular space, vacuolation in few glomeruli with disruption and degeneration of tubules. Severe haemorrhages in interstitial spaces and mild infiltration of mononuclear cells were observed. Renal tubular disruption and focal areas of tubular atrophy were observed. The kidneys in the DOX group observed in degeneration, necrosis, hemorrhagia, oedema and inflammatory cell infiltration. The present findings were might be due to DOX induced renal tubular necrosis (Zordoky et al., 2011). In ameliorative groups moderate range of degeneration and disruption of cardiac muscle fibers was observed due to their antioxidant properties.

CONCLUSIONS

In conclusion, it was found that kumiss enhanced the antioxidant defense system by restricting DOX-induced free radical production, and it alleviates DOX-induced cardiotoxicity albeit not being able to prevent it. It was concluded that new studies on the efficacy of kumiss in the prevention of DOX-induced

cardiotoxicity should be carried out.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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