Investigation of the effects of mesenchymal stem cell administration on liver recovery in experimental hepatotoxicity model

ABSTRACT

Hepatotoxicity refers to liver dysfunction associated with certain medical drugs and chemicals. Studies have shown that mesenchymal stem cells have a positive effect on the improvement of liver diseases. This study aimed to investigate the potential protective effects of fetal kidney-induced mesenchymal stem cells on Doxorubicin-induced hepatotoxicity in rats. Sprague dawley rats were divided into three groups: Control, sham, and treatment group. Intraperitoneally administered mesenchymal stem cells were treated with BrdU before transplantation so that they could be followed up after invivo transplantation. After completion of the experimental steps, the groups were monitored for 5 weeks. Then the rats were terminated and liver tissues were taken for histopathological and immunohistochemical evaluation. In immunohistochemical examinations performed with TNF-α, Caspase-3, and COX-2 primary antibodies, the most severe positivity was in the sham group, followed by the control and treatment groups. While the control and sham groups were found to be statistically similar in immunohistochemical staining with anti-BrdU antibody, the treatment group was found to be significantly different from the other groups ($p<0.05$). As a result, it has been revealed that intraperitoneally administered mesenchymal stem cells prevent degeneration and necrosis in hepatocytes, significantly decreasing TNF-α, COX-2, and Caspase-3 levels thus increasing liver regeneration in rats with Doxorubicin-induced hepatotoxicity.

Keywords: Doxorubicin, Hepatotoxicity, Mesenchymal Stem Cell, Rat

INTRODUCTION

Doxorubicin (DOX) is an anthracycline and an anti-neoplastic agent with a wide range of activity in human cancers (Alegra et al., 2006). Detailed pharmacokinetic studies have been performed in humans and animals. In humans, plasma clearance of DOX is rapid and has substantial tissue binding. DOX is predominantly metabolized in the liver and accumulates mainly in the kidney, but it is also found in the heart, liver, and small intestine (Singh et al., 2015). The application of DOX as a therapeutic drug is limited by its adverse effects on the bone marrow, intestinal epithelium, heart, liver, and kidney, as well as causing cancer cell resistance (Jacevic et al., 2017).

Recently, DOX has been widely used for the treatment of different types of cancer, such as hepatocellular carcinoma, due to its ability to kill transformed liver cells. During the treatment of other cancers, the most common side effect of this drug is DOX-related liver damage. Several experimental studies reported DOX-induced hepatotoxicity.

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Research Article

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Hepatotoxicity refers to the dysfunction of the liver caused by drugs and chemicals or chemotherapeutic drugs. The mechanism of DOX-induced toxicity includes an oxidative stress state characterized by excessive production of reactive oxygen species (ROS) and/or a decrease in antioxidant defenses leading to an imbalance in normal oxygen metabolism (Mansouri et al., 2017).

Once the oxidative stress begins irreversible changes occur, causing hepatocyte apoptosis or tissue necrosis and resulting in increased hepatic enzyme levels in the blood, particularly alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Jacevic et al., 2017). Hepatotoxicity is mainly related to the formation of free radicals, the presence of inflammatory markers, and environmental factors. All these factors contribute to the pathological abnormalities in hepatocytes and result in acute liver disease which, if left undiagnosed, develops into chronic liver disease (CHD). CHD is characterized by the regular destruction and regeneration of hepatic parenchymal cells leading to fibrosis and cirrhosis. CHD is often associated with portal hypertension and liver failure. There is always a high probability for the development of hepatocellular carcinoma (HSC), due to fibrosis and cirrhosis (Iqubala et al., 2016).

Liver transplantation is accepted as the most effective treatment for advanced liver fibrosis. However, the chance of transplantation is limited due to insufficiency of donor organs, surgical complications, immunological rejection, and high medical costs. Recently, it has been suggested that mesenchymal stem cell (MSC) application is an effective alternative for treatment. MSCs have the potential to differentiate into hepatocytes. Its immunomodulatory properties gain therapeutic value with the secretion of trophic factors such as growth factors and cytokines. In addition, MSCs can suppress inflammatory responses, decrease hepatocyte apoptosis, increase regeneration, help regress liver fibrosis, and restore liver function (Kim et al, 2015).

Autologous bone marrow-derived MSC transplantations have been reported for the treatment of liver fibrosis in humans, and improvements in serum albumin levels and liver histology 3-4 months after transplantation have been reported (Amin et al., 2013; Jang et al., 2014). In experimental studies, it has been reported that MSCs obtained from different tissues treat liver failure, increase regeneration, and can be an alternative treatment method to organ transplantation (Kuo et al., 2008; Ramanathan et al., 2017; Gazdic et al., 2018; Luo et al., 2018). No specific and effective therapeutic agent for DOX-related hepatotoxicity is known. This study aimed to investigate the effect of intraperitoneally transplanted fetal kidney-derived MSCs (FKD-MSCs) on the improvement of hepatotoxicity by histopathological and immunohistochemical methods.

MATERIAL and METHOD

Preparation of the FKD – MSCs

To obtain FKD-MSCs, 19-day pregnant rats were subjected to hysterectomy under sterile conditions and the fetuses were obtained. Fetuses were euthanized by providing general anesthesia with ether. The kidneys were removed immediately and the tissues were transferred to the laboratory fresh. The tissues were stored in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Belgium). Fetal kidney tissues were mechanically dissected into small pieces using a sterile scalpel. Tissues were dissected, obtained by explant culture method, then placed in T25 flasks and incubated at 37°C, 5% CO_2 humidified atmosphere. Medium consisting of 77% DMEM (Lonza, Belgium), 20% fetal bovine serum-FBS (Lonza, Belgium), 2% L-Glutamine (Lonza, Belgium), 1% Penicillin, Streptomycin,

Amphotericin (Biological Industries, Israel) added. Non-adherent cells were removed by changing the medium once every 2-3 days. When there was approximately 70% adhesion, the adherent MSCs were passaged and aliquoted 1:2 with 0.25% Trypsin in PBS. The cells were grown to the 3rd passage. Obtained FKD-MSCs were labeled with BrdU before the transplantation.

Animal model

Thirty Sprague Dawley rats (males, 10 weeks old) were randomly divided into 3 groups: control, sham, and treatment. 10 mg/kg DOX dissolved in 0.9% NaCl was administered as a single dose injection through the tail vein in the sham and treatment groups to induce hepatotoxicity. All rats were kept under standard laboratory conditions (21 ± 2°C, 65% humidity, and 12 h light / 12 h dark). The animals were fed ad libitum with standard rat chow and allowed access to water continuously.

Control group (n=10): Did not receive DOX and/or FKD-MSCs. 0.9% NaCl (the same volume as MSC) was administered to the rats intraperitoneally 3 times at one-week intervals.

Sham group (n=10): Received DOX only, no treatment. 0.9% NaCl (the same volume as MSC) was administered to the rats intraperitoneally 3 times at one-week intervals, 7 days after DOX injection.

Treatment group (n=10): Received DOX and FKD-MSCs. 2 x 10^6 MSC injections were administered to the rats intraperitoneally 3 times at one-week intervals, 7 days after DOX injection.

The animals were followed for 5 weeks from the end of the treatment and they were euthanized under general anesthesia with xylazine/ketamine. The experimental protocol was approved by the Local Animal Ethics Committee (Approval number: 2014/55).

Histological study

The liver tissues obtained from the rats were fixed in 10% buffered formol. Paraffin blocks were obtained by embedding in paraffin after dehydration and transparency processes. Sections of 5-micron thickness from the prepared paraffin blocks were stained with Hematoxylin and Eosin (H&E) (Luna, 1968), examined under binocular head light microscopes (Olympus CX23, Tokyo, Japan), and photographed.

In the histopathological examination, H&E-stained sections were evaluated and scored as reported by Öz et al., (2020). In the histopathological evaluation of liver tissues, degeneration and necrosis in hepatocytes, mononuclear cell infiltrations in portal areas, bile duct hyperplasia, and fibrosis in parenchyma were evaluated. The histopathological changes were evaluated as follows:

0: No lesion.

+1: “Mild” if the relevant changes are only localized in one region.

+2: “Moderate” if the relevant changes were multifocal but limited.

+3: If the pathological change was diffusely distributed, it was considered as "severe".

Immunohistochemical study

A polymer-based indirect-immunoperoxidase method was used for immunohistochemical studies. For this purpose, the sections were taken on 5-micron thick poly-lysine slides in the microtome, deparaffinized in xylol, and rehydrated in grade alcohols. Then an antigen retrieval procedure was performed with Proteinase K enzyme (RE7330-K, Novocastra) for 15 minutes at room temperature. After washing with deionized water, endogenous peroxidase activity was removed by dripping 3% hydrogen peroxide solution on the sections. It was washed 3 times with TBS for 5 minutes, then Protein Block (Novocastra RE7102) for 10

minutes, 3 times with TBS for 5 minutes, Primary antibody [Caspase 3 Recombinant Rabbit Monoclonal Antibody (9H19L2), TNF alpha Monoclonal Antibody (MP6-XT22), PE, eBioscience™, COX2 Monoclonal Antibody (COX 229)] at room temperature for 1 hour, washing 3 times with TBS for 5 minutes, in post-primary block solution (NovoLinkTM Max Polymer Detection System RE7280-K, Novocastra) 30 min, 3 washes with TBS, 30 min, 3 washes with TBS in Novalink polymer solution (NovoLinkTM Max Polymer Detection System (RE7280-K, Novocastra), and DAB (3,3'-Diaminobenzidine) (NovoLinkTM Max Polymer Detection System RE7280-) K, Novocastra) at room temperature for 3-5 minutes. After washing with distilled water, counterstaining with Hematoxylin was performed, after 2 changes in alcohol and xylol, and covered with entellan. Negative controls used in each staining were also stained according to the same procedure, but primary TBS was used instead of antibodies. All sections were evaluated under a light microscope (Olympus CX23, Tokyo, Japan).

Immunohistochemical scoring method

The Allred system was used for immunohistochemical scoring of the cases. The method used by Qureshi and Pervez (2010) was modified and applied to the study. Similar to standard scoring systems, staining intensity and staining prevalence were evaluated by this scoring system. Staining intensity (darkness) score was determined as: 0 (no staining), 1 (weak), 2 (medium), 3 (intense/dark). The extent of staining was determined based on the ratio of stained cells to all cells in the examined area as follows: 0 (no staining), 1 (> 0 - 1/100), 2 (> 1/100 - 1/10), 3 (> 1/ 10 - 1/3), 4 (> 1/3 - 2/3), 5 (> 2/3 - 1). Allred score between 0 and 8 was determined for each case by summing the staining intensity score and the staining prevalence value. 10 different microscope fields were examined randomly at 40 magnification and the average of the obtained scores was accepted as the score of the relevant case.

Statistical analysis

Statistical analyzes in the study were obtained with the IBM SPSS Statistics 22 program. Kruskal-Wallis and Tukey tests were used for statistical evaluation of histopathological findings; Kruskal-Wallis and Duncan tests were used for evaluation of immunohistochemical findings. The $p<0.05$ criteria were used for all statistical evaluations.

RESULTS

Histopathological findings

Histopathological results and statistical values are given in Table-1 and Table-2. Accordingly, hepatocytes in the control group had a normal histological appearance. The lumens of the vena centralis in the liver tissues in this group were empty (Figure 1A). In addition, no inflammatory cell infiltrates were found in the portal areas. Degeneration of hepatocytes was found only in 3 cases. The most severe lesions were found in the sham group. Severe hydropic and vacuolar degenerations were observed in hepatocytes of 3 cases (Figure 1B). It was determined that these degenerative hepatocytes were mostly located in the centrilobular regions. Statistically, the sham group was found to be significantly different from the other groups ($p<0.05$).

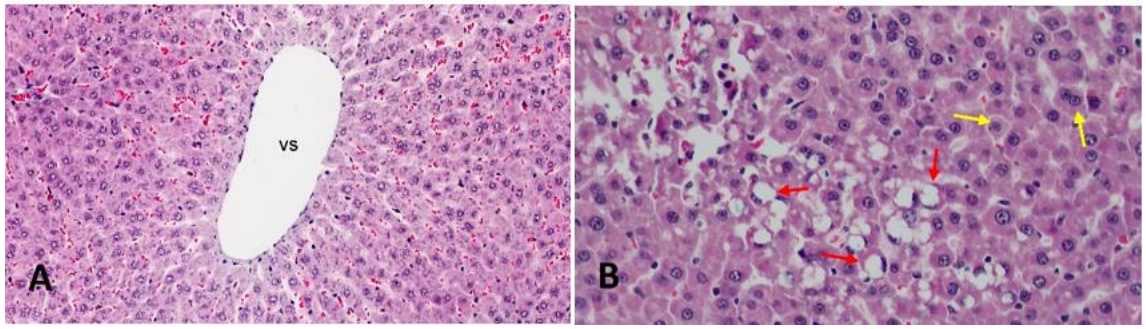

Figure 1. A. Empty lumens of the vena centralis (VS) and normal histological appearance in the liver of rats in the control group. X200. B. Hydropic and vacuolar degenerations in hepatocytes (red arrows) and normal hepatocytes (yellow arrows) of rats in the sham group. X400, H&E.

Table 1. Number of histopathological changes observed in the cases

n=10	Control Group				Sham Group				Treatment Group			
Lesion score	0	+1	+2	+3	0	+1	+2	+3	0	+1	+2	+3
Degeneration and necrosis in hepatocytes	7	3	-	-	1	1	5	3	5	4	1	-
Mononuclear cell in the portal area and bile duct hyperplasia	10	-	-	-	4	4	2	-	3	4	1	2
Parenchymal fibrosis	10	-	-	-	10	-	-	-	10	-	-	-

Table 2. Statistical values of histopathological results

	Control Group (Mean ± Std Error)	Sham Group (Mean ± Std Error)	Treatment Group (Mean ± Std Error)
Degeneration and necrosis in hepatocytes	0.300 ± 0.152^{b}	2.000 ± 0.298^{a}	0.600 ± 0.221^{b}
Mononuclear cell in the portal area and bile duct hyperplasia	0^{b}	0.800 ± 0.249^{ab}	1.200 ± 0.359^{a}
Parenchymal fibrosis	0	0	0

Values in rows that do not contain a-b common superscripts are significantly different according to the Kruskal Wallis – Tukey test ($p<0.05$).

Moderate mononuclear cell infiltrations and bile duct hyperplasia were observed in portal areas of 2 cases. When the treatment group was examined, it was determined that the degenerations in hepatocytes were moderately severe only in one case. On the other hand, severely enlarged bile ducts in the portal areas of 2 cases were observed (Figure 2). Hepatic necrosis and/or parenchymal fibrosis were not observed in any of the groups.

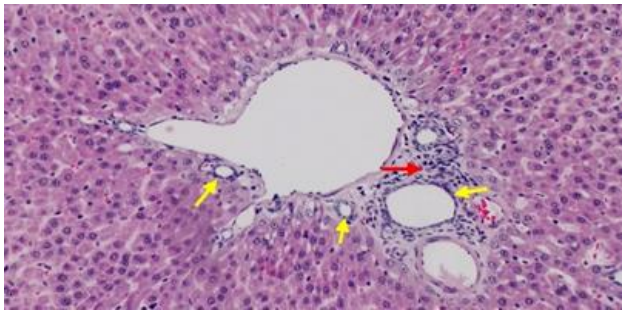

Figure 2. Mononuclear cell infiltrations in portal areas (red arrow) and bile duct hyperplasia (yellow arrows) of rats in the treatment group, X200, H&E.

Immunohistochemical findings

The statistical results of the Allred scoring method, in which the staining intensity and the extent of staining were evaluated together, are given in Table-3. According to this, while all groups were found to be statistically different in immunohistochemical staining with TNF-α, the sham group had the most severe positivity. This was followed by the control and treatment groups, respectively. It was noted that positive staining was mostly randomly distributed throughout the lobe and intracytoplasmic immunoreactions were observed in hepatocytes (Figure 3). No positive reactions were found in other cell groups in the liver tissue.

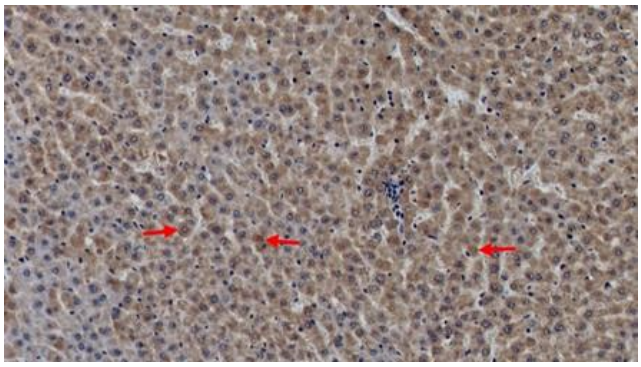

Figure 3. Anti-TNF-α intracytoplasmic immunopositive reactions were randomly distributed in hepatocytes (arrows) in the sham group, X200, DAB

In immunohistochemical analyzes performed with Caspase-3 primary antibody, the most intense positive staining was observed in the sham group. Treatment and control groups were statistically significantly different from the sham group ($p<0.05$). Positive staining was observed mostly in the hepatocytes and bile duct epithelium in portal areas (Figure 4). Positive immunoreactions were found in the cytoplasm of the cells, similar to those in TNF-α.

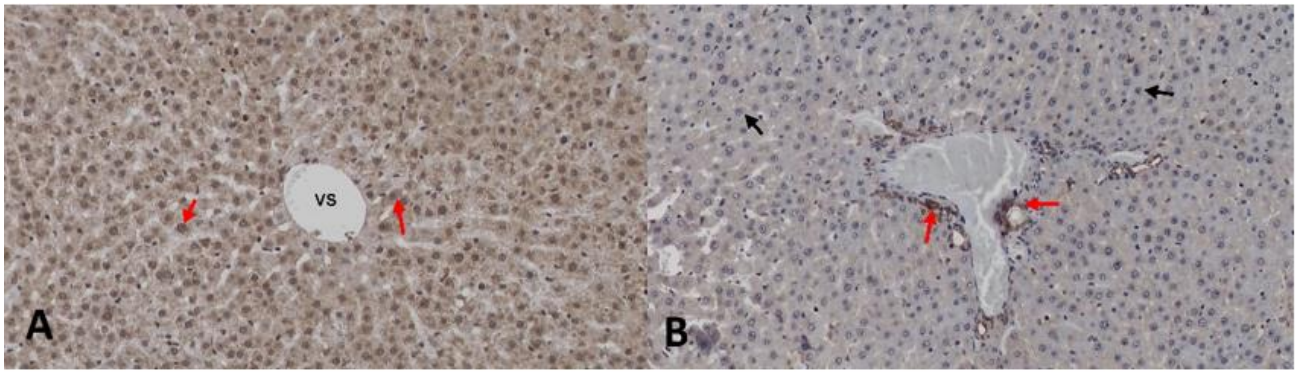

Figure 4. A. Anti-Caspase3 positive immunohistochemical stains (arrows) randomly scattered among hepatocytes around the vena centralis (VS), X200, DAB. B. Anti-Caspase3 positive immunoreactions (red arrows) in bile duct epithelium of portal areas and immunonegative hepatocytes (black arrows), X200, DAB.

Similarly, as a result of the immunohistochemical staining of COX-2 primary antibody, the most severe reactions were obtained in the sham group, followed by the treatment and control groups, respectively. While these two groups were statistically similar ($p>0.05$), the sham group was different ($p<0.05$). Although intranuclear staining was observed more intensely in hepatocytes, intracytoplasmic staining was also observed (Figure 5).

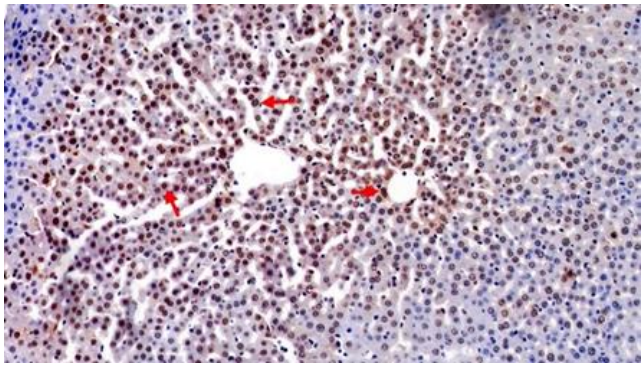

Figure 5. Anti-COX-2 intranuclear positive immunohistochemical staining with centrilobular distribution (arrows) in the sham group, X100, DAB.

The most severe reactions were observed in the treatment group in immunohistochemical staining with anti-BrdU antibody. In these cases, immunopositive staining was noted especially in the nuclei of hepatocytes (Figure 6A). Weak positive and negative immunoreactions were observed in the control and sham groups (Figure 6B). According to these findings, the control and sham groups were found to be statistically similar, while the treatment group was significantly different from the other two groups ($p<0.05$).

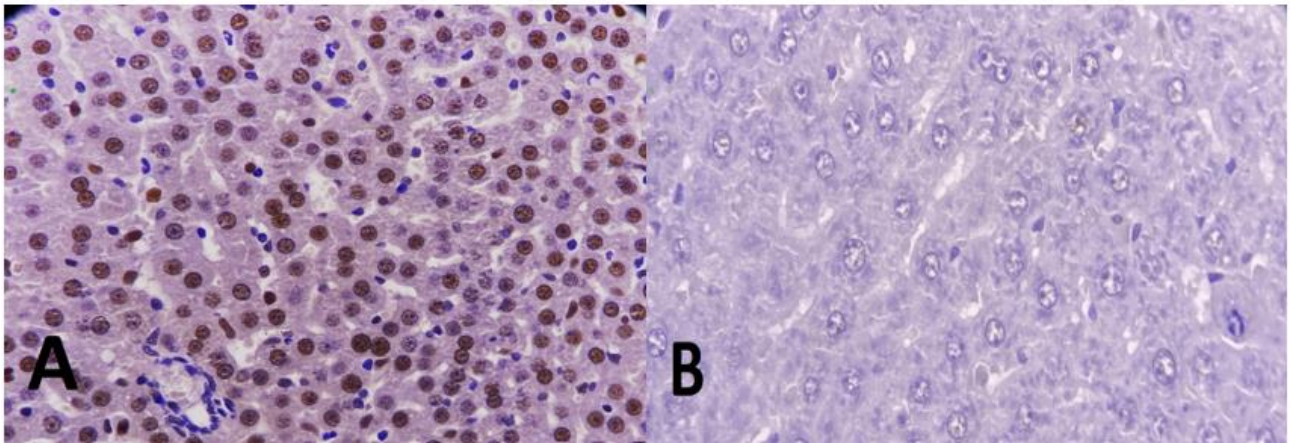

Figure 6. A. Intranuclear Anti-BrdU immunopositive reactions in hepatocytes, treatment group X630, DAB. B. Negative Anti-BrdU immunoreactions in hepatocytes, sham group X630, DAB.

Table 3. Statistical values of immunohistochemical results

	Control Group (Mean ± Std Error)	Sham Group (Mean ± Std Error)	Treatment Group (Mean ± Std Error)
TNF-α	4.860 ± 0.172^{b}	6.040 ± 0.394^{a}	3.890 ± 0.355^{c}
Caspase 3	2.610 ± 0.302^{b}	6.420 ± 0.226^{a}	3.520 ± 0.512^{b}
COX-2	3.300 ± 0.222^{b}	4.850 ± .0290^{a}	3.660 ± 0.302^{b}
BrdU	0.280 ± 0.035^{b}	1.220 ± 0.213^{b}	3.370 ± 0.800^{a}

Values in rows that do not contain a-c common superscripts are significantly different according to the Kruskal Wallis – Duncan test ($p<0.05$).

DISCUSSION

Various results have been reported based on structural examination of liver tissue extracted from animal models treated with DOX. Marked hyperplasia in the bile duct, dilatation of the sinusoidal space and central vein congestion, vacuolization in hepatocytes, dilatation of sinusoids, condensation in nuclei and degeneration of hepatocyte cords, cellular edema, focal necrosis, and irregularity of the hepatic trabecular necrosis, proximal trabeculae ducts, dilatation and vacuolization and swelling of mitochondria, lymphocyte infiltration were observed in the intercellular spaces as a result of the histopathological examination of hepatic tissue after DOX administration (Prasanna et al., 2020).

Previous studies reported that DOX has toxic effects on organs such as the heart, kidneys, and testicles as well as the liver (Pugazhendhi et al., 2018). Shivakumar et al. (2012) reported that they encountered degenerative changes in the liver after exposure to DOX in Wistar albino rats in an experimental study. In another study,

It was reported that DOX at different concentrations caused hydropic and vacuolar degenerations in hepatocytes (Jacevic et al., 2017). In our study, in accordance with the literature, it was revealed that both hydropic and vacuolar degenerations in hepatocytes occurred mostly in the sham group, and it was determined that the number and severity of these findings decreased in the treatment group. Thus, it was thought that the transplantation of MCSs might have a histopathological preventive effect on DOX-related degenerative changes in the liver.

Wang et al. (2016) revealed that the use of anthracycline group antitumoral drugs caused a systemic inflammation in the patient. In our study, however, it was noticed that inflammatory reactions were mostly seen in the sham and treatment groups. In addition, the more frequent observation of bile duct hyperplasia in the treatment group was considered as a response of the liver to

degenerative events, and it was interpreted that the use of stem cells led the liver to regenerate.

A previous study reported that DOX did not cause liver fibrosis and did not cause histological changes (Di Stefano et al., 2006). Similarly, the absence of fibrosis findings in any of the groups in our study shows that the results obtained are compatible with the literature. In general terms, it can be stated that stem cell therapy has a positive effect on the histological changes in the liver.

In a previous study revealed that apoptosis occur in DOX-induced hepatotoxicity (El Sayyad et al., 2009). In another study, it was determined that stem cells have a strong anti-apoptotic and mitogenic effect on the liver (Yu et al., 2007). In our study, it was thought that Caspase-3 activity is more effective in the sham group and doxorubicin administration triggers apoptosis in animals. It was noted that Caspase-3 expressions were decreased in the treatment group compared to the sham group, and this decrease was statistically similar to the control group. It was concluded that stem cell therapy prevents programmed cell death. However, it can be said that the expression of other cytokines such as Caspase-8 and Caspase-9 should be investigated to determine whether apoptosis is triggered internally or externally.

As stated in many previous studies, TNF-α, the proinflammatory cytokine of DOX, is expressed more in cells (Supriya et al., 2016; Cengiz et al., 2021). In our study, it was interpreted that TNF-α was expressed significantly less in the treatment group compared to the sham group, this could also prevent inflammatory reactions. In addition to these findings, the histologically lower severity of inflammatory reactions in the treatment group also proves the accuracy of the aforementioned immunohistochemical findings.

Yu et al. (2007) reported that after the transplantation of stem cells, they encountered “hepatocyte-like” cell formations in ischemic damaged rat livers. It has been revealed that while it takes 5-7 days for stem cells to reach the sinusoids in the liver tissue, it takes at least 10-14 days for them to transform into the above-mentioned cells. In the same study, it was reported that BrdU positive reactions in the liver were observed in hepatocytes 24 hours after the transplantation of stem cells. In our study, BrdU positive immunoreactions were observed in the treatment group, especially in the nuclei of hepatocytes. This shows that FKD-MSCs have reached the liver tissue after being transplanted intraperitoneally.

CONCLUSION

As a result, it can be stated that intraperitoneally administered FKD-MSCs to rats with DOX-induced hepatotoxicity, prevent degeneration and necrosis in hepatocytes. It was determined that TNF-α, COX-2, and Caspase-3 levels were significantly reduced in the treatment group and this shows that FKD-MSCs provided regeneration in the liver. In addition, strong BrdU positive reactions in the treatment group was interpreted as intraperitoneally transplanted stem cells being able to reach the liver tissue and helping reduce tissue damage. However, further studies are required to investigate the effects of stem cells on some other parameters such as liver enzymes, angiogenesis, and oxidative stress.

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Ethical approval:

This study was approved by Dışkapı Yıldırım Beyazıt Education and Research Hospital Local Animal Ethics Committee (Approval number: 2014/55).

Conflict of interest: The authors declared that there is no conflict of interest.

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