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Adipose-derived mesenchymal stem cells mitigate methotrexate-induced liver cirrhosis (fibrosis) model

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Abstract. – OBJECTIVE: Hepatic fibrosis is a severe liver condition characterized by abnormal fibroblast activity, excessive extracellular matrix deposition, inflammation, and structural alterations. Methotrexate (MTX), a pharmaceutical agent widely used for its therapeutic properties, is known to induce hepatotoxicity. However, the precise mechanisms underlying MTX-induced liver injury remain elusive. This study investigates the therapeutic potential of Adipose-Derived Mesenchymal Stem Cells (ADMSCs) in alleviating MTX-induced liver injury in a rat model.

MATERIALS AND METHODS: Thirty male Wistar albino rats were employed in this study. Liver injury was induced in twenty rats by a single MTX dose, while ten rats constituted the control group. The MTX group was further subdivided into two cohorts, one receiving ADMSC treatment and the other saline solution. The treatment duration was 14 days. ADMSCs, isolated from adipose tissue, were characterized by CD13, CD29, and CD105 markers. Biomarker analysis, histopathological evaluations, and various measurements were conducted to assess ADMSCs' therapeutic efficacy.

RESULTS: MTX administration significantly increased Transforming Growth Factor-β (TGF-β), Platelet-Derived Growth Factor (PDGF), Plasma Cytokeratin 18, Plasma Malondialdehyde (MDA), and Liver MDA levels, with histopathological liver damage. ADMSC treatment notably lowered TGF-β, PDGF, Plasma Cytokeratin 18, Plasma MDA, and Liver MDA levels, accompanied by reduced liver damage observed histologically. Liver Enzyme ALT levels were also reduced in the MTX and ADMSC groups compared to the MTX and Saline groups.

CONCLUSIONS: ADMSCs exhibit significant potential in ameliorating MTX-induced liver injury, with notable anti-oxidative and anti-apoptotic properties. These findings suggest that ADMSCs may effectively mitigate oxidative stress and inflammation associated with MTX-induced liver damage. Further research is essential to investigate the clinical application of ADMSCs in liver disease management and uncover the underlying therapeutic mechanisms.

Key Words:
Adipose-derived mesenchymal, Stem cells, Methotrexate, Liver injury.

Introduction

Hepatic fibrosis is distinguished by an abnormal buildup of fibroblasts and an excessive deposition of extracellular matrix (ECM), accompanied by noticeable inflammatory lesions and structural changes1. Although the precise pathophysiology of hepatic fibrosis remains uncertain, the timely identification and intervention might mitigate the death rate among affected individuals. Hence, the mitigation or reversal of hepatic fibrosis has emerged as a crucial aspect to be considered in preventing and managing chronic hepatic damage and cirrhosis[1].

Methotrexate (MTX) is a pharmaceutical compound for its anticancer and immunosuppressive attributes. It is an antagonist to folic acid through competitive inhibition of dihydrofolate reductase[2]. MTX is utilized in various clinical contexts, encompassing managing diseases with a progressive inflammatory process[3]. The occurrence of liver toxicity is a significant negative consequence of MTX therapy. The range of manifestations associated with this condition spans from minor hepatic injury to cirrhosis[3].

The precise mechanisms that underlie the hepatotoxic effects of MTX have not been fully elucidated. Scholars[4] have demonstrated that in liver diseases, the administration of MTX induces an elevation of reactive oxygen species (ROS) lipoperoxidation and nitric oxide (NO). On the other hand, it has been noted that MTX can decrease the concentrations of defensive oxidative processes and impede the activities of enzymatic agents accountable for counteracting free radicals[5].

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The clinical utility of MTX is restricted due to its hepatotoxic nature. To address the hepatotoxic effects of MTX, it is beneficial to explore compounds or medications that can function as supplementary therapy alongside MTX treatment[3].

The use of MTX leads to a significant elevation in the blood levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), indicating the hepatotoxic effects associated with this medicine[6].

Antioxidants are essential in reducing oxidative damage by counteracting the harmful impact of free radicals on cellular components[7]. Several antioxidants, including acid chlorogenic, chrysin, vitamin B1, ascorbic acid, and some medicaments, like polyethylene glycol, digoxin, and lactulose, have been employed for ameliorating liver injury induced by MTX[5,6,8-10].

Adipose tissue-derived stem cells (ADSCs) are a type of mesenchymal cell capable of self-renewal and multipotential differentiation[11]. As a result, these cells have been used in clinical trials to address various medical conditions, including diabetes mellitus, liver disease, corneal lesions, and joint and skin lesions[12-15]. Furthermore, stem cells, especially those derived from adipose tissue, have a significant impact as they can facilitate the development of novel therapeutic interventions[16].

In this study, a rat model of acute liver injury induced by methotrexate was employed to evaluate the subsequent hypotheses: mesenchymal stem cells derived from ADMSCs have the potential to improve liver injury associated with methotrexate treatment.

Materials and Methods

Animals

This study utilized a sample of 30 male Wistar albino rats, with an average weight ranging from 150 to 200 grams and an age of 10 to 12 weeks. The experiments conducted in this study followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, adopted by the National Institutes of Health in the United States. The Institutional Animal Care and Ethical Committee of the Demiroğlu Science University, Istanbul, approved the study protocol with the assigned ethical number (Ethical number: 14.01.2022/0723225721). The laboratory rats utilized in the experiment were procured from the Experimental Animal Laboratory at Science University. The rats were provided with unrestricted access to food. They were kept in steel cages under controlled environmental conditions, maintaining a temperature of 22 ± 2°C and a light/dark cycle of 12 hours each.

Experimental Protocol

A total of 30 male rats were selected for the study. A group of twenty rats were administered a single dose of MTX at a concentration of 20 mg/kg to induce liver injury. A total of ten rats were selected to form the normal control group, and these rats were not administered any chemical substances. In the study, 20 rats were allocated into two separate groups.

In this study, Group 1 of rats was administered mesenchymal stem cells (MSC) at a dosage of 2.0 X 10^6 cells/kg *via* intraperitoneal (i.p.) injection twice a week, resulting in a total dose of 8 X 10^6 cells/kg. On the other hand, Group 2 of rats received 1 ml/kg/day of 0.9% NaCl saline solution *via* i.p. injection. All treatments were administered for 14 days.

After the study, all animals underwent euthanasia *via* cervical dislocation, following administration of anesthesia (Ketamine 100 mg/kg, Ketasol, Richterpharma AG Austria) and xylazine 50 mg/kg, Rompun, Bayer, Germany). Subsequently, their blood samples were obtained through cardiac puncture for biochemical analysis. Subsequently, the liver specimen was extracted to conduct histopathological and biochemical analyses.

Isolation of Mesenchymal stem cells (MSC) from Adipose Tissue

Mesenchymal stem cells (MSC) were isolated from the adipose tissues in the rats' flank region. After administering anesthesia (50 mg/kg of Ketasol from Richterpharma AG Austria and 10 mg/kg of Rompun from Bayer, Germany) to the rats, the fat tissue was isolated and promptly placed on ice in a sterile environment. Subsequently, the tissue was transferred to a stem cell laboratory. The adipose tissue was finely chopped into small pieces and treated with 0.2% collagenase type II (Gibco, Waltham, MA, USA) for 40 minutes at 37°C, with continuous agitation. The tissue that had undergone lysis was centrifuged at 1,500 revolutions per minute (rpm) for 5 minutes. The precipitate was resuspended in 2 mL of Dulbecco's Modified Eagles Medium (DMEM; Gibco, Waltham, MA, USA) within culture flasks that contained 3 mL of DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA,

USA), 1% penicillin, 1% streptomycin (Sigma, St. Louis, MO, USA), and two mM L-glutamine (Invitrogen, Thermo Fisher, Netherlands). The culture flasks were then placed in an incubator with 5% CO_2 at a temperature of 37°C and maintained under conditions of saturated humidity.

Following a period of three days, the media was replenished in order to achieve a state of 85% confluence. The cells were subsequently sub-cultured until passage 4 using a 0.25% trypsin solution (Gibco, Waltham, MA, USA). Following this, they were inactivated by adding an equal volume of DMEM. The MSCs isolated in passage four were subsequently cryopreserved for potential future cell transplantation applications. The cryopreservation process involved using a concentration of 2×10^6 viable cells/mL in a medium of 50% DMEM, 40% FBS, and 10% dimethyl sulfoxide (DMSO; MP Bio, Irvine, CA, USA). The cryopreserved MSCs were then stored in sterile cryovials that were appropriately labeled and placed in a nitrogen tank at a temperature of -196ºC. The examination of cell morphology and growth was conducted using an inverted microscope. In order to facilitate cell transplantation, the cells were extracted from a nitrogen tank and subsequently transferred to a water bath maintained at a temperature of 37°C for thawing. The process of centrifugation was conducted for 5 minutes at a rotational speed of 1,500 revolutions per minute (rpm). Subsequently, the resulting cell pellet was resuspended in DMEM and placed within a carbon dioxide (CO_2) incubator set at a temperature of 37°C and maintained at a level of saturated humidity until it was ready for further utilization.

MSC Characterization

This study aims to conduct a comprehensive analysis of cell characteristics. The mesenchymal stem cells underwent characterization by applying immunofluorescence staining targeting CD 13, CD 29, and CD 105 molecules at the second passage. In the immunostaining process, the cells cultivated in culture dishes underwent a washing step using phosphate-buffered saline (PBS). Subsequently, they underwent fixation for 5 minutes in methanol at a temperature of -10°C. Following the fixation process, the methanol was subsequently eliminated and subjected to desiccation. The cells were incubated for 20 minutes with a blocking serum, precisely normal goat serum, to perform the blocking step. Following the application of the blocking serum, the blocking serum was eliminated, and the cells underwent a series of three washes using PBS. The present study describes a straightforward method for isolating, culturing, and characterizing mesenchymal stem cells derived from rat adipose tissue. In this technique, the cells were incubated at 37°C for 1 hour with a primary antibody specific to CD13, CD29, and CD105 molecules. Following a 5-minute wash in PBS, the cells underwent a 45-minute incubation with a secondary antibody. Subsequently, the cells were subjected to three washes in PBS. Following the washing procedure, the cells were affixed using a mounting medium and subsequently observed using a fluorescence microscope. All experimental procedures were conducted under ambient conditions.

Histopathological Evaluation

Formalin-fixed refers to a process in which biological specimens are preserved using a solution containing formal. The liver sections, measuring 4 µm in thickness, were subjected to staining using hematoxylin and eosin. The sections were captured using an Olympus C-5050 digital camera affixed to an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan).

The liver histopathological scoring analysis was conducted following the methodology outlined by Lobenhofer et al[9]. The evaluation involved assigning a numerical grade to each parameter assessed in liver sections, including hepatocyte necrosis, fibrosis, and cellular infiltration. The grading system ranged from 1 (minimal) to 4 (marked), with intermediate grades of 2 (mild) and 3 (moderate) also considered.

Liver Biochemical Analysis

After decapitation, the livers were expeditiously removed and stored at a temperature of -20°C until they were exposed to biochemical examination. To conduct tissue analysis, the brain tissues were homogenized entirely using a glass homogenizer in a volume of PBS (pH 7.4), five times more than the volume of the tissues. The resultant mixture was centrifugated at a centrifugal force of 5,000 times the acceleration due to gravity for 15 minutes. Subsequently, the aqueous component of the amalgamation was collected and denoted as the supernatant. The total protein content in the liver samples was subsequently quantified using Bradford's technique, whereby bovine serum albumin was utilized as the reference standard[17].

The concentrations of TGF-β and PDGF in the liver supernatants were quantified using commercially available Enzyme-Linked Immunosorbent

Assay (ELISA) kits designed for rats. The measurements of each animal's samples were conducted in duplicate, following the guidelines provided by the manufacturer. The Absorbances were measured using a microplate reader (MultiscanGo, Thermo Fisher Scientific Laboratory Equipment, NH, USA).

Measurement of Plasma Cytokeratin 18 Levels

Plasma Cytokeratin 18 levels were measured using a commercially available ELISA kit (Thermo Fisher Scientific Inc, NH, USA).

Determination of Lipid Peroxidation

The quantification of lipid peroxidation was conducted in tissue and plasma samples through the assessment of malondialdehyde (MDA) concentrations as thiobarbituric acid reactive substances (TBARS)11. Concisely, the tissue samples were subjected to adding trichloroacetic acid and TBARS reagent, followed by thorough mixing and subsequent incubation at 100°C for 60 minutes. Following the cooling process on ice, the samples underwent centrifugation at a speed of 3,000 revolutions per minute for 20 minutes. Subsequently, the absorbance of the resulting supernatant was measured at a wavelength of 535 nanometers. The MDA levels in the tissue were determined by utilizing the standard calibration curve with tetraethoxypropane as the reference compound. The MDA levels were then quantified and expressed as nanomoles per gram of protein.

Determination of Plasma ALT Levels

Plasma ALT levels were measured using a commercially available (ELISA) kit (USCN, Life Science Inc., Harrington Oakland, CA, USA).

Statistical Analysis

Data were analyzed with SPSS 26 (IBM Corp., Armonk, NY, USA), percentage, mean, and standard deviation were used to present descriptive data. The conformity of the data to the normal distribution was evaluated with the Shapiro-Wilk test. Continuous variables with normal distribution in univariate analysis were expressed as Mean±SD. One-way Anova was used when comparing more than two groups because the data were normally distributed. When statistical significance was found in multiple group comparisons, the post-hoc Tukey test was used for pairwise comparison to determine where the difference originated. The *p*-values obtained in the post-hoc analysis were corrected with Bonferroni correction. A value of $p<0.05$ was accepted for the *p*-significance level.

Results

Biochemical Analysis

Liver TGF-β Level (pg/g): the Normal group exhibited a baseline TGF-β level of 0.6 ± 0.1 pg/g (Table I). In contrast, the MTX and Saline group showed a significant increase to 1.4 ± 0.08 pg/g ($p < 0.01$), while the MTX and ADMSC group had a level of 0.9 ± 0.1 pg/g ($p < 0.05$), indicating a potential mitigation effect of ADMSCs (Table I).

Liver PDGF Level (pg/mg): the Normal group had a PDGF level of 10.7 ± 1.1 pg/mg. The MTX and Saline group substantially elevated to 30.8 ± 2.4 pg/mg ($p < 0.001$). In contrast, the MTX and ADMSC group had a lower PDGF level at 21.5 ± 1.2 pg/mg ($p < 0.05$), suggesting a beneficial impact of ADMSCs in reducing PDGF levels (Table I).

Table I. Comparaison various parameters among the three groups.

	Normal	MTX and Saline	MTX and ADMSC
Liver TGF beta level (pg/g)	0.6 ± 0.1	1.4 ± 0.08*	0.9 ± 0.1#
Liver PDGF level (pg/mg)	10.7 ± 1.1	30.8 ± 2.4**	21.5 ± 1.2#
Plasma Cytokeratin 18 level (pg/mg)	0.84 ± 0.1	3.62 ± 0.3**	1.5 ± 0.2#
Plasma MDA level (nM)	47.2 ± 5.5	143.9 ± 11.5*	81.5 ± 6.8##
Liver MDA level (nmol/g tissue)	27.3 ± 2.5	92.8 ± 5.1*	45.7 ± 3.7##
ALT (IU/L)	19.8 ± 1.5	61.3 ± 4.7*	30.2 ± 1.8##
Hepatocyte necrosis	0.3 ± 0.1	2.3 ± 0.2*	1.1 ± 0.2#
Fibrosis	0.2 ± 0.1	2.1 ± 0.3**	0.7 ± 0.1##
Cellular infiltration	0.2 ± 0.1	1.9 ± 0.3**	0.5 ± 0.1#

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA. Tissue growth factor (TGF), Platelet-derived growth factor (PDGF), Malondialdehyde (MDA), Alanine transaminase (ALT). *$p < 0.01$ different from normal groups. **$p < 0.001$ different from normal group. #$p < 0.05$ different from MTX and saline group. ##$p < 0.001$ different from MTX and saline group.

Plasma cytokeratin 18 Level (pg/mg): Plasma cytokeratin 18 levels were significantly increased in the MTX and Saline group, reaching 3.62 ± 0.3 pg/mg ($p < 0.001$), compared to the Normal group with 0.84 ± 0.1 pg/mg. The MTX and ADMSC group displayed a lower level of 1.5 ± 0.2 pg/mg ($p < 0.05$), indicating a potential protective effect of ADMSCs against cytokeratin 18 release (Table I).

Plasma Malondialdehyde (MDA) Level (nM): Plasma MDA levels, a marker of lipid peroxidation, were significantly elevated in the MTX and Saline group, reaching 143.9 ± 11.5 nM ($p < 0.01$), compared to the Normal group with 47.2 ± 5.5 nM. In contrast, the MTX and ADMSC group had a lower MDA level at 81.5 ± 6.8 nM ($p < 0.001$), suggesting a potential antioxidant effect of ADMSCs (Table I).

Liver MDA Level (nmol/g tissue): Liver MDA levels followed a similar pattern. The MTX and Saline group exhibited significantly higher levels of MDA at 92.8 ± 5.1 nmol/g tissue ($p < 0.01$) compared to the Normal group with 27.3 ± 2.5 nmol/g tissue. The MTX and ADMSC group had lower MDA levels at 45.7 ± 3.7 nmol/g tissue ($p < 0.001$), indicating a potential reduction in liver lipid peroxidation with ADMSC treatment (Table I).

Liver Enzyme ALT (IU/L)

Liver enzyme ALT levels were significantly elevated in the MTX and Saline group (61.3 ± 4.7 IU/L) compared to the Normal group (19.8 ± 1.5 IU/L, $p < 0.01$). The MTX and ADMSC group exhibited a lower ALT level (30.2 ± 1.8 IU/L, $p < 0.001$), indicating a potential improvement in liver function with ADMSC treatment (Table I).

Liver Histopathology

The liver histopathological analysis (Figure 1) revealed that the Normal group had a healthy liver structure. In contrast, the MTX and Saline group exhibited significant liver damage, including bridging necrosis, fibrosis, and cellular infiltration. The MTX and ADMSC group showed a notable decrease in these pathological features, suggesting a potential therapeutic effect of ADMSCs in mitigating liver injury induced by MTX.

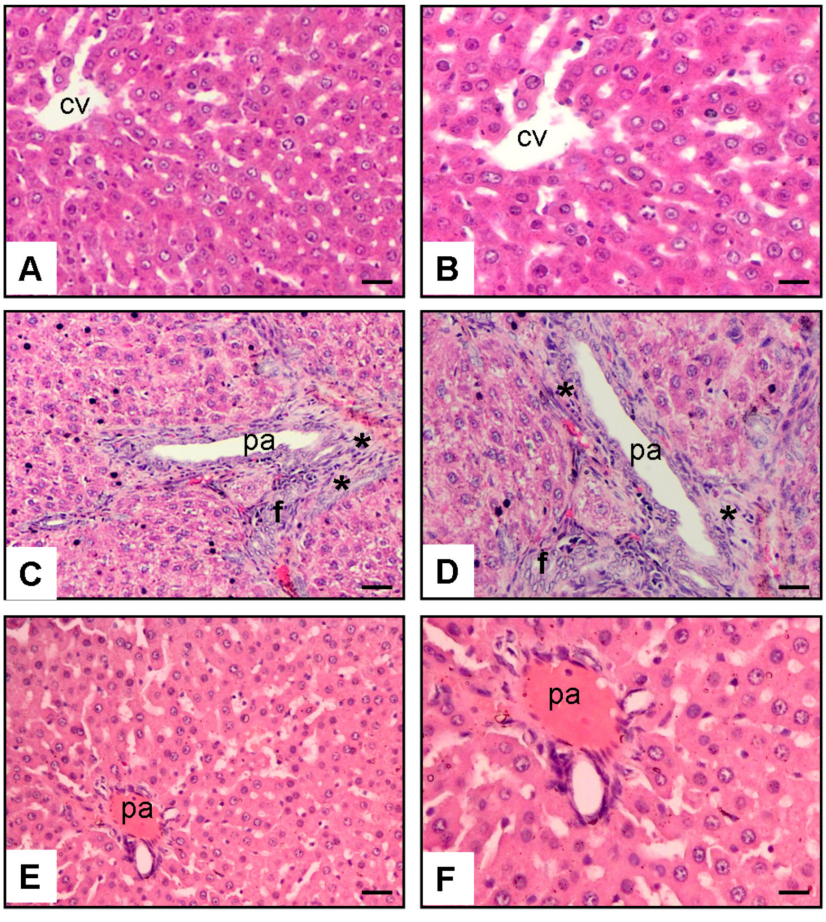

Figure 1. Liver histolopathology Hematoxylin and eosin stain (Magnification a,c,e (x10) and b,d,f (x20)), **A-B**: Normal Group Rats have normal liver. central vein (cv), **C-D**: MTX and saline Group Rats have Bridging necrosis, fibrosis (f) and celluar infiltration (*) (asterisk) in portal area (pa), **E-F**: MTX-+MSC Group Rats have decreased bridging necrosis fibrosis and celluar infiltration. (scale bar: 100 uM).

Discussion

Many causes of drug-induced liver injury are known[18,19]; the most well-known is MTX-induced liver injury due to its frequent use and also because of its lack of alternatives in some diseases[19]. Adipose-derived mesenchymal stem cells (ADMSCs) have demonstrated the ability to improve liver damage and inflammation. Notably, ADMSCs have been seen to effectively facilitate the recovery of liver injury in rats with MTX-induced hepatotoxicity. This discovery indicates a possible method by which ADMSC therapy may mitigate the inflammatory response in rats with hepatotoxicity.

Presently, the primary approach for managing hepatotoxicity involves using anti-inflammatory agents, particularly exogenous glucocorticoids. However, there has been a growing interest in the potential therapeutic application of bone marrow-derived stem cells in treating inflammatory conditions and organ injury[19,20]. It has been documented that MSCs possess various advantageous characteristics in the context of inflammatory illnesses. These include their ability to facilitate the migration of cells to specific locations, encourage the formation of new blood vessels, mitigate the inflammatory response by influencing immune cells, stimulate the regeneration of tissues, and diminish harm to organs[21,22]. Consequently, this work aimed to examine the potential of ADMSCs to mitigate the inflammatory response and organ damage generated by MTX.

In the present investigation, the administration of methotrexate resulted in a significant elevation in MDA levels. MDA, a metabolite that remains stable during the lipid peroxidation cascade mediated by free radicals, is commonly employed as a biomarker for assessing oxidative stress and lipid layer degradation[23]. As previously mentioned, the administration of methotrexate-induced lipid peroxidation is evidenced by a notable elevation in MDA levels. The process of lipid peroxidation, facilitated by oxygen-free radicals, is widely recognized as a significant mechanism responsible for the degradation and impairment of cellular membranes. It has been proposed that this process plays a role in the progression of methotrexate-induced tissue damage[23,24]. Moreover, several investigations[25,26] have been conducted about methotrexate-induced lipid peroxidations in the hepatic tissue of rats, wherein heightened levels of MDA were observed. The effects of methotrexate have been hypothesized by us and other researchers to potentially arise from its interaction with lipids in the cell membrane[27,28].

The administration of MTX resulted in a significant increase in plasma and tissue MDA levels compared to the control group. In a study conducted by Hadi et al[29], it was found that the administration of MTX to rats increased serum and tissue levels of MDA[29]. Moreover, numerous studies have demonstrated the antioxidant agents' capability to reduce MDA levels[30,31]. According to this study, MDA levels in tissue and plasma may be reduced by lactulose administration.

The investigation revealed that the administration of MTX therapy resulted in notable alterations in liver histology, characterized by hepatocyte necrosis, fibrosis, and heightened cellular infiltration. These findings are consistent with earlier studies[30-32]. Furthermore, we established a correlation between these histological alterations and the results obtained from serum and biochemical analyses, which provided insights into liver damage. Previous investigations[31,32] have demonstrated that therapy with MTX elevates serum ALT levels.

The use of MTX leads to a rise in blood TGF-β levels, hence emphasizing the involvement of TGF-β in developing MTX-induced hepatotoxicity. The anti-inflammatory action of MSCs may potentially entail additional mechanisms of MSC immunoregulation, such as TGF-β[33]. TGF-β plays a pivotal role as a regulatory factor in chronic liver disease, exerting its influence on the continuum of events spanning from the initial damage to the development of fibrosis and, ultimately, cancer. In this investigation, we have also demonstrated the ability of ADMSCs to reduce the concentration of liver TGF-β, a pivotal factor in the advancement of liver fibrosis.

The platelet-derived growth factor (PDGF) is recognized as the most influential factor in promoting the proliferation, differentiation, and migration of Hepatic stellate cells (HSCs), which play a significant role in the process of fibrogenesis following liver damage[34]. In addition, PDGF facilitates the synthesis and accumulation of collagen while inducing the differentiation of HSCs into myofibroblasts. The inhibition of PDGF signaling has been shown to effectively suppress the proliferation of HSCs and mitigate the progression of liver fibrogenesis[35]. This study examines the impact of ADMSCs on the levels of cytokines associated with liver fibrosis following liver damage produced by MTX. Our findings demonstrate a reduction in the expression of these cytokines in response to ADMSC treatment.

In hepatic tissues, CK-18 is a prominent cytoskeletal protein that primarily represents the intermediate filament family. The complete form of

the molecule is released from cells undergoing necrosis, whereas a caspase-cleaved fragment is produced because of the structural changes that occur during apoptosis. Utilizing CK-18 knockout mice has highlighted the significance of CK-18 in the liver. It has been observed that the absence of CK-18 in hepatocytes leads to the spontaneous formation of liver lesions that closely resemble the morphological characteristics of steatohepatitis-associated liver carcinogenesis. In addition, the absence of CK-18 has been linked to the growth of liver tumors in the liver[36]. In another study[37], it has been reported that CK-18 is a marker that can be used in liver damage monitoring in non-alcoholic fatty liver disease. In this study, toxic processes like the processes of fatty liver may be present. CK-18 levels decreased dramatically with the administration of ADMSC compared to the damaged liver. These results show that ADMSC stops the apoptosis process in the liver and its anti-inflammatory and antioxidant effects. Although this effect of ADMSC is still experimental in similar processes, it may be a ray of hope.

Conclusions

This study demonstrated the inhibitory and significant corrective effect of ADMSCs on methotrexate-induced liver injury with anti-oxidative anti-apoptotic effects biochemically and histopathological. Future studies will further elucidate the effects of ADMSCs in this type of injury.

Authors' Contributions

Omay Sorgun (Orcid: 0000-0002-0376-7173), Oytun Erbaş (Orcid: 0000-0002-2515-2946) contribute equally during study and making critical revisions related to the relevant intellectual content of the manuscript.

Conflict of Interest

The authors have no conflict of interest to declare.

Informed Consent

Not applicable.

Ethics Approval

The Institutional Animal Care and Ethical Committee of the Demiroğlu Science University, Istanbul approved the study protocol with the assigned ethical number (Ethical number: 14.01.2022/0723225721).

Availability of Data and Materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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