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Experimental Research Article

A new accessible source of endometrial mesenchymal stem cells: a promising strategy for liver regeneration and repair in a rat model of chemotherapy-induced injury

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ABSTRACT

Introduction: Alkylating agents, commonly used in chemotherapy, can cause significant liver damage by inducing cell death pathways. Human menstrual blood-derived mesenchymal stem cells (HuMenSCs), with their remarkable proliferative capacity and easy accessibility, offer a potential treatment for this injury.

Methods: We investigated the impact of transplanting HuMenSCs in rats using a liver damage model caused by Busulfan injection (36 mg/kg, i.p.). Engraftment was confirmed using fluorescence microscopy and flow cytometry. Hepatic morphology was assessed via histopathological examination. Apoptosis was quantified by TUNEL assay and real-time PCR analysis of apoptotic (Bax) and anti-apoptotic (Bcl2) gene expression.

Results: HuMenSCs effectively engrafted into injured liver tissue. Histopathological examination revealed hepatic morphology significantly improved in the group treated with HuMenSCs, with reduced inflammation, congestion, and sinusoidal dilation. Additionally, both the TUNEL assay and real-time PCR analysis demonstrated a significant decrease in apoptosis and a shift toward the downregulation of pro-apoptotic gene expression (Bax) in the group that received HuMenSCs treatment when compared with the sham ($p < 0.0001$) and negative control groups ($p < 0.001$).

Conclusion: The results of our research suggest that HuMenSCs transplantation effectively mitigates Busulfan-induced liver injury by inhibiting apoptosis and promoting liver regeneration. These findings demonstrate the promise of HuMenSCs as a novel therapeutic approach for treating liver damage caused by chemotherapy.

Keywords:
Apoptosis,
Endometrial mesenchymal stem cells
Liver regeneration
Hepatotoxicity
Menstrual blood

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Introduction

A crucial organ in charge of metabolism, detoxification, and the production of necessary proteins is the liver (Cao et al., 2023; Kakizaki et al., 2021). Unfortunately, many therapeutic drugs, particularly chemotherapeutic agents, can cause liver damage, known as hepatotoxicity (Senior 2010). Consequently, this adverse effect may precipitate additional complications such as fibrosis, necrosis, vascular injury, cholestasis, and steatosis, which significantly compromise treatment outcomes and patient well-being (Ishak and Zimmerman 1995). Most hepatotoxicity incidents resulting from drug side effects are attributed to immunological mechanisms and perturbation of the patient's metabolic response. Notably, chemotherapeutic agents often induce idiosyncratic reactions, regardless of the dosage. Nonetheless, dose reduction strategies are contemplated to mitigate the risk of liver toxicity (Ciurea and Andersson 2009; King and Perry 2001).

Alkylating agents can damage the liver by depleting glutathione levels and causing oxidative stress (Bahirwani and Reddy 2014). Busulfan, an alkylating agent commonly used in cancer treatment, poses a particular challenge due to its hepatotoxic potential. Busulfan disrupts DNA replication by forming cross-links within DNA strands, specifically at the 5′-GA-3′ sequence (Thatishetty et al., 2013). This disruption leads to cell death through apoptosis (Thatishetty et al., 2013). Previous studies have shown that busulfan-induced liver injury can manifest as hepatitis, cholestasis, and veno-occlusive disease (VOD) (Grigorian and O'Brien 2014).

High doses of busulfan, exceeding 16 mg/kg, have been linked to hepatic venous occlusion disease in approximately 20% of patients (Grochow et al., 1989). VOD is characterized by congestion and hepatocyte apoptosis resulting from the obstruction of small hepatic veins by fibrin deposits beneath the endothelial lining (Rollins 1986). This blockage leads to a buildup of blood within the liver, ultimately causing damage to the liver cells (Rollins 1986).

Mesenchymal stem cells (MSCs), found in multiple organs, including the liver (Cabezas et al., 2014; Sun 2014), have gained attention as a possible treatment approach for liver regeneration (De Cesaris et al., 2017; Meng et al., 2007). MSCs can alter immunological responses, self-renew, and specialize into several cell types (De Cesaris et al., 2017; Jung et al., 2013). They also offer ethical advantages over embryonic stem cells, making their clinical application more feasible (Meng et al., 2007). Research has demonstrated that MSCs, which may be acquired from several sources, such as tonsils, adipose tissue, bone marrow, and placenta, can promote liver repair by reducing apoptosis, fibrosis, and inflammation (Ayatollahi et al., 2014; Fang et al., 2023; Kozub et al., 2017; Markovic et al., 2017; Milosavljevic et al., 2017; Ryu et al., 2014; Salomone et al., 2013; Xagorari et al., 2013; Yan et al., 2009; Zhou et al., 2009). Furthermore, MSCs secrete signaling molecules that stimulate liver cell growth and regeneration (Zhou et al., 2009).

The endometrium, the dynamic uterus lining, changes monthly in cyclical patterns throughout a woman's reproductive years. MSCs are found in this tissue and are essential for its shedding, differentiation, and regeneration (Gargett et al., 2009). MSCs are found in the endometrium's basal and functional layers (Hong 2024). During menstruation, the shedding of the endometrial lining releases these cells into the menstrual blood (Rodrigues et al., 2016). This offers a conveniently available supply of MSCs from menstrual blood (Bozorgmehr et al., 2020).

According to studies, MSCs obtained from menstrual blood (HuMenSCs) are able to differentiate into cells resembling liver cells (hepatocytes) when cultivated with hepatic growth factors (FGF-4, OSM, and HGF) in a specific medium (Mou et al., 2013). These differentiated cells exhibit key functions of mature hepatocytes, including urea production, glycogen storage, and the ability to take up indocyanine green (ICG) dye (Mou et al., 2013). The ability of HuMenSCs to treat a range of disorders and models has been investigated in earlier research, including POF (Premature Ovarian Failure) (Noory et al., 2019), stroke (Borlongan et al., 2010; Rodrigues et al., 2012), type 1 diabetes (Santamaria et al., 2011; Wu et al., 2014), myocardial infarction (Zhang et al., 2013), Duchenne muscular dystrophy (Cui et al., 2007), and acute lung injury (Xiang et al., 2017).

Although HuMenSCs show promise as a treatment in several disease models, their protective properties against Busulfan-induced hepatic impairment have not been fully elucidated. This research seeks to examine the potential side effects of busulfan administration on rat liver tissue and investigate the protective effects that HuMenSCs provide against busulfan-induced hepatic injury.

Materials and Methods

Obtaining and Culturing HuMenSCs

Blood samples were taken from five healthy women volunteers between the ages of 25 and 30 using a sterile menstrual cup on the third day of their menstrual period to isolate and cultivate HuMenSCs consistent with our previous study (Noory et al., 2019). The Ethics Committee of TUMS (Tehran University of Medical Sciences) authorized the study's guidelines, and each participant gave their informed permission (IR.TUMS.MEDICINE.REC.1395.1069).

The collected menstrual blood samples were transferred to sterile tubes containing antibiotics and PBS (phosphate-buffered saline). After dilution with PBS, the cellular fraction was separated from the supernatant by centrifuging the blood for 15 minutes at 400g. The diluted blood was centrifuged after being spread out over Ficoll (GE Healthcare, Amersham, UK) to isolate mononuclear cells, including MSCs, from other blood components. After carefully aspirating the buffy coat layer, PBS was used for washing. The isolated mononuclear cells were cultured in a humidified environment with 5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium (DMEM/F12, Bioidea, Iran) containing 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). Non-adherent cells were eliminated by changing the culture medium every two to three days.

The HuMenSCs were detached using trypsin-EDTA (Gibco, USA) once they had achieved 90% confluence. Before further characterization, HuMenSCs from all five donors were pooled in equal numbers. This pooling strategy was employed to minimize potential donor-specific variability, ensure sufficient cell yield for our experiments, and create a more representative HuMenSC population (Kannan et al., 2024; Kuçi et al., 2016; Widholz et al., 2019). After that, they were subcultured in flasks of 25 cm^2 and examined using phase-contrast microscopy (Olympus CKX41, Japan) to assess morphological characteristics. For subsequent experiments, injection-ready cells were taken from passage three. The MSC phenotype of the pooled cells was confirmed by flow cytometry analysis, where HuMenSCs were negative for CD45 and CD34 (hematopoietic and immune markers) and expressed positive markers such as CD73 and CD90.

Experimental Design and Animal Groups

Forty adult Wistar albino rats, aged 6–8 weeks and weighing 200–250 grams, were used in this investigation. They were acquired from the Pharmacy Faculty of TUMS. For the course of the investigation, the rats were kept in regular settings concerning humidity (55–65%), lighting (12-hour light/dark cycle), and temperature (23 ± 2 °C). The TUMS Ethical Committee approved every method used in this investigation.

To induce chemotherapy-induced liver injury, adult rats were treated with Busulfan (36 mg/kg) through intraperitoneal injection (Figure 1). Seven days post-Busulfan administration, the animals were randomly allocated to four distinct groups, each consisting of eight rats. The groups included: a negative control group that received just Busulfan treatment, a normal control group that was left untreated, a treatment group that received an intravenous injection of 1×10^6 HuMenSCs in 1 ml PBS seven days after Busulfan administration, and a sham group that was intravenously injected via the tail vein with 1 ml PBS seven days post-Busulfan administration. Additionally, the homing experiment was conducted on eight animals. After four weeks from cell transplantation, we euthanized the rats and removed the livers for further assessment (Figure 1).

Cell Tracking Analysis

To monitor the migration of transplanted cells, the rat model with chemotherapy-induced liver injury received intravenous injections of 1×10^6 HuMenSCs tagged with CM-Dil (red fluorescence, C7000, Molecular Probes™, USA) in 1 ml of PBS. After one month post-cell transplantation, five-micrometer sections were prepared from livers fixed in 10% neutral buffered formalin and embedded in paraffin. The sections were stained with Hoechst dye (33258, 25MG, Sigma, USA) to identify the nuclei for 30 minutes at room temperature in a dark environment following deparaffinization and permeabilization with 0.2% Triton X-100 (Merck, Germany) and rinsing with PBS. Imaging was performed using an Olympus BX51 microscope with an E.30 digital camera (Olympus, Japan).

Furthermore, Flow cytometry analysis was conducted four weeks post-transplantation to verify the presence of HuMenSCs and determine the proportion of retained HuMenSCs. Liver samples were obtained, mechanically minced, digested with collagenase IV (Invitrogen, USA), and then subjected to centrifugation and lysis.

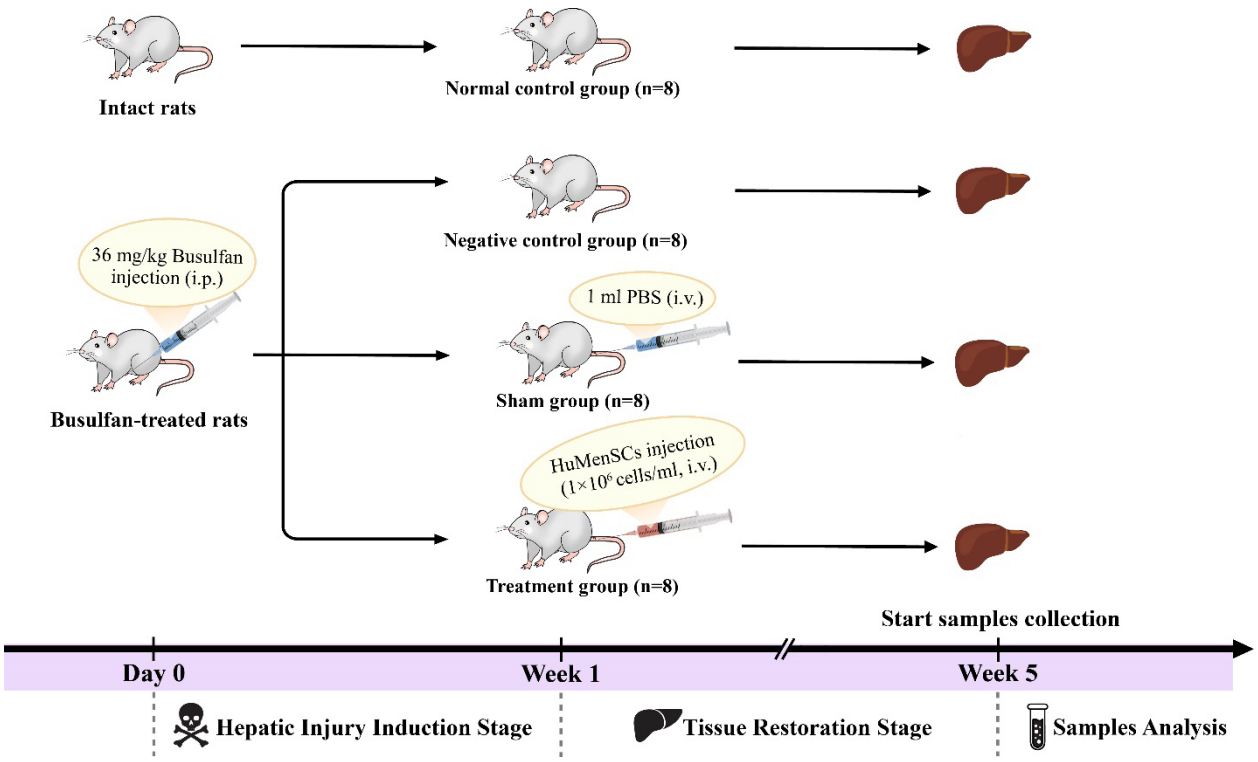

FIGURE 1. Graphical timeline illustrating the experimental design for all studied groups. Normal (No Treatment), Negative (Busulfan Day 0 i.p.), SHAM (Busulfan Day 0 i.p. + PBS Day 7 i.v.), and Treatment (Busulfan Day 0 i.p. + HuMenSCs Day 7 i.v.). See Methods for full details. Livers were collected from all groups on Week 5 (four weeks after HuMenSCs and PBS administration in the treatment and sham groups).

The resulting cell suspensions were treated at room temperature for an hour with FITC-conjugated rabbit monoclonal antibodies against CD44 at a 1:100 dilution (eBioscience, USA). PE-labeled mouse IgGs served as the negative controls (BD Biosciences). Data analysis was carried out using Flowjo software to interpret our results accurately.

Histopathological Examination

The liver samples were collected from all groups one month after injection to assess histopathological changes. After fixation (at least 24 hours in 4% paraformaldehyde) and paraffin embedding, the livers were sectioned (5 µm) and stained with hematoxylin-eosin. was then used to stain the slices. A Japanese optical microscope (Olympus CX31) was used to view the slides.

TUNEL Assay for Apoptotic Cell Detection

The TUNEL test (terminal deoxynucleotidyl transferase dUTP nick end labeling) was used to evaluate apoptosis using a commercially available kit (Fluorescein Roche, 11684795910). Two µm-thick liver slices fixed in paraffin were deparaffinized and given three PBS washes for five minutes each. Sections were twice treated for 10 minutes in 10% aqueous hydrogen peroxide to inhibit endogenous peroxidase activity. To improve DNA accessibility, slices were then subjected to a 30-minute proteinase K treatment at 37°C. Cell membranes were permeabilized by incubating sections with 3% Triton X-100 at ambient temperature for ten minutes (Noory et al., 2019).

Each sample received 25 µl of the TdT enzyme reaction mixture for the TUNEL assay. The slides were incubated under humid conditions for two hours at 37°C in the dark. The nuclei were counterstained with propidium iodide (PI) (10 µg/ml; Invitrogen) for a few seconds following washing three times with PBS. A drop of 50% glycerol was applied to a glass slide, and the stained cells were covered with a coverslip, ensuring no air bubbles were trapped. A fluorescent microscope (Olympus, Japan) was used to acquire the images. Apoptosis was quantified by manually counting fluorescein-stained (TUNEL-positive) cells in five randomly selected fields per sample; results are presented as the percentage of

TABLE 1: Forward and reverse sequencing primers and anticipated product length (Noory et al., 2019)

Gene name	Product size (bp)	annealing temp	Product size (bp)
Bax	'For: 5'- GAGTGGGATACTGGAGATGAAG -3 'Rev: 5'- TGGTAGCGACGAGAGAAGTCC -3	57.4	233
Bcl2	'For: 5'- GCAAACTGGTGCTCAAGG -3 'Rev: 5'- CAGCCACAAAGATGGTCA -3	56.63	183
GAPDH	'For: 5'- AAGTTCAACGGCACAGTCAAGG -3 'Rev: 5'- CATACTCAGCACCAGCATCACC -3	61.58	121

Bax, Bcl2 associated X protein; Bcl2, B cell lymphoma 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

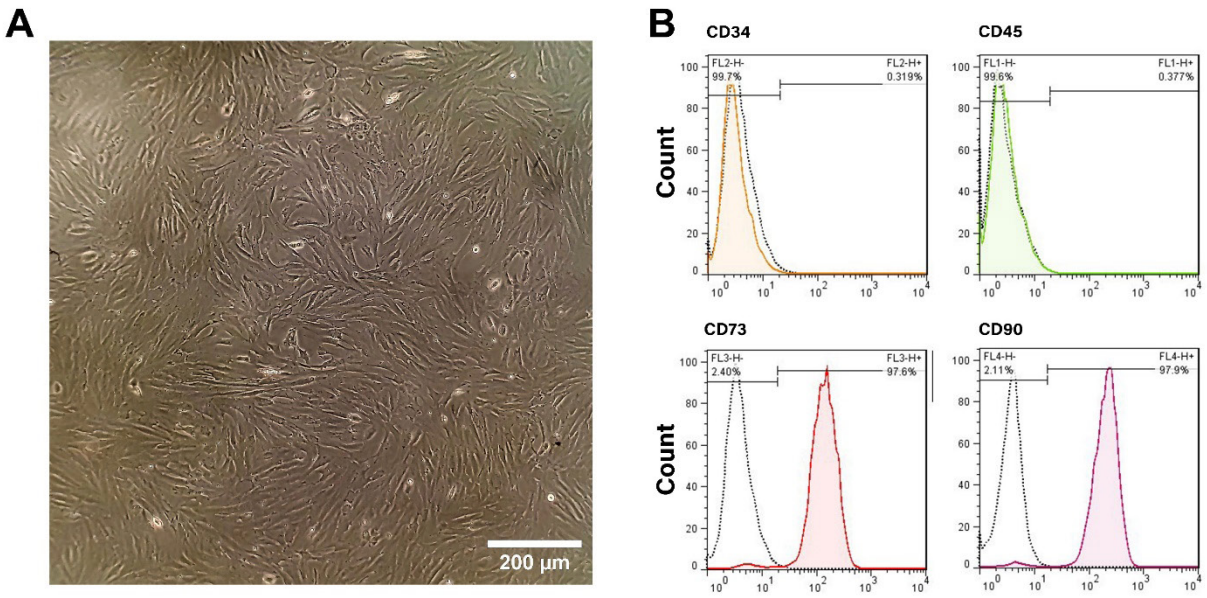

FIGURE 2. Characterization of HuMenSCs. (A) HuMenSCs displayed a characteristic fibroblast-like morphology and formed distinct mesenchymal colonies within ten days of culture. (B) Flow cytometry analysis demonstrated that HuMenSCs had elevated levels of characteristic MSC markers, including CD73 and CD90, whereas showing low hematopoietic cell markers, including CD34 and CD45 expression.

apoptotic cells per field.

qPCR Analysis of Bax and Bcl-2 Levels

Quantitative PCR (using SYBR Green on an ABI StepOne system) assessed Bax and Bcl-2 mRNA levels in liver samples, building upon our previous method (Noory et al., 2019). RNA, extracted using QIAzol (Qiagen, Germany) and treated with DNase I (Fermentas, USA), was reverse transcribed (Fermentas, USA) into cDNA. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to a reference gene, and compared to healthy controls. Statistical analysis employed one-way ANOVA with Tukey's post-hoc test ($p \leq 0.05$). Primer sequences are in Table 1.

Analytical statistics

One-way ANOVA with Tukey's post-hoc test was used to compare group means (mean ± SD). Statistical significance was defined as $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

Results

HuMenSCs Characterization

Isolated and cultured HuMenSCs demonstrated a consistent proliferative capacity in the culture medium and exhibited a characteristic fibroblast-like and spindle-shaped appearance. Within ten days of culture, these cells formed a homogeneous monolayer of distinct mesenchymal colonies (Figure 2A).

To further characterize the surface marker profile of

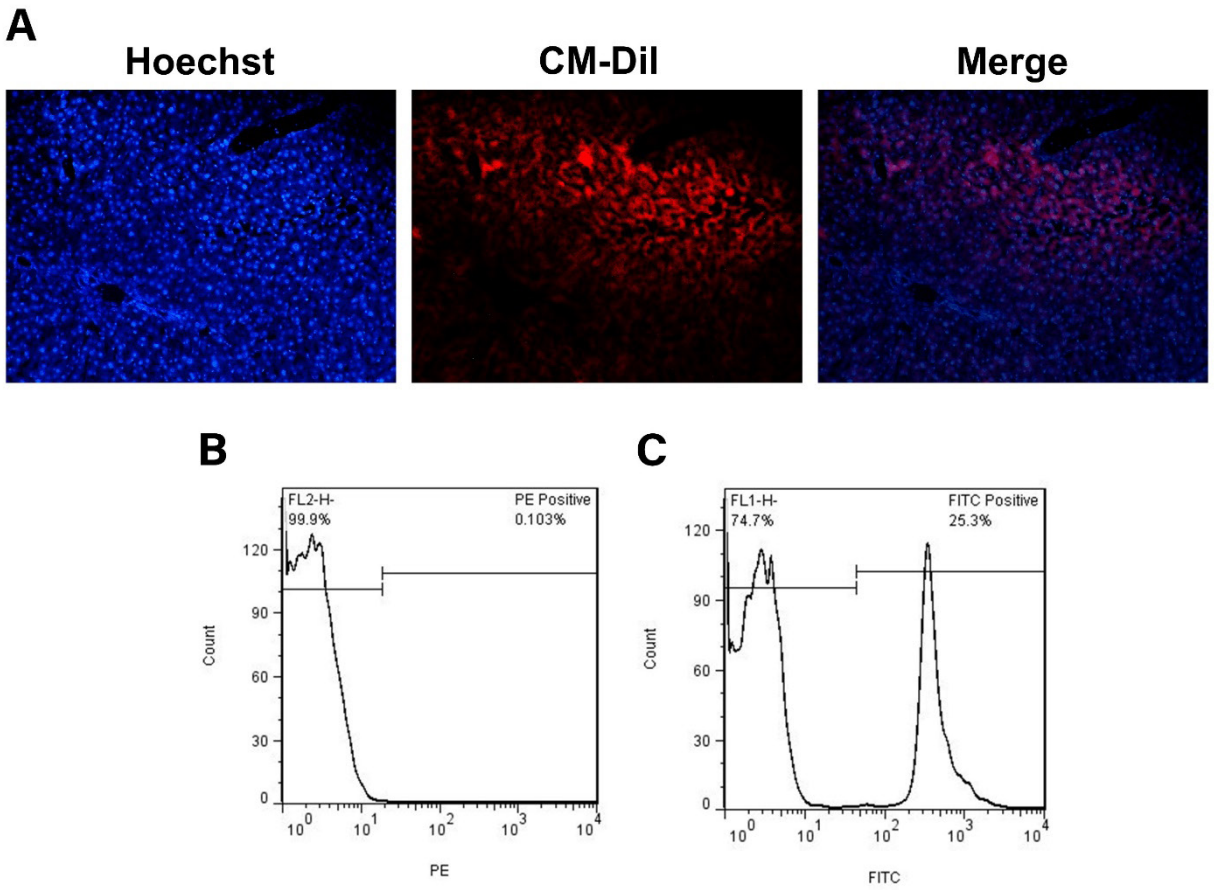

FIGURE 3. Tracking of HuMenSCs in Busulfan-Injured Livers. (A) Fluorescence microscopy images (200x magnification) show CM-DiI-labeled HuMenSCs (red) localized in the damaged areas of the liver four weeks after transplantation. Cell nuclei are stained with Hoechst dye (blue) for visualization. (B & C) Flow cytometric data confirm the successful engraftment of HuMenSCs within the liver tissue. The histograms demonstrate the presence of CD44-positive HuMenSCs (C) compared to an isotype control (B).

HuMenSCs, flow cytometry analysis was performed on cells at passage three (Figure 2B). HuMenSCs exhibited decreased hematopoietic cell marker expression, specifically CD34 (0.319%) and CD45 (0.377%). Conversely, these cells displayed elevated MSC marker expression, including CD73 (97.6%) and CD90 (97.9%).

Tracking and Quantification of Transplanted HuMenSCs

The presence of transplanted HuMenSCs in the injured liver was assessed one month after busulfan-induced hepatic injury. CM-DiI-labeled HuMenSCs were successfully traced in the injured liver tissue, as evidenced by red fluorescence signals detected using fluorescence microscopy (Figure 3A).

To further confirm the engraftment of HuMenSCs, flow cytometry analysis was conducted on the injured liver tissue using the CD44 marker, a common marker for mesenchymal stem cells. The results revealed that 25.3% of cells within the liver tissue were CD44-positive (Figure 3C), indicating the successful transplantation of HuMenSCs into the liver following intravenous injection.

Liver Tissue Histopathological Analysis

Histological analysis of hematoxylin and eosin-stained liver sections showed distinct morphological changes across the four experimental groups (Figure 4). Figure 3A depicts a normal control group liver section, displaying normal hepatocyte morphology, sinusoidal spaces, and central vein architecture. In contrast, Figures 4B and 4C (negative control and sham groups, respectively) demonstrate significant alterations. These sections exhibit moderate to severe inflammatory alterations, reduced portal space, congested and dilated sinusoidal spaces, and lymphocyte infiltration between hepatocytes. Furthermore, scattered areas of hepatocyte necrosis were observed. Figure 4D, representing the treatment

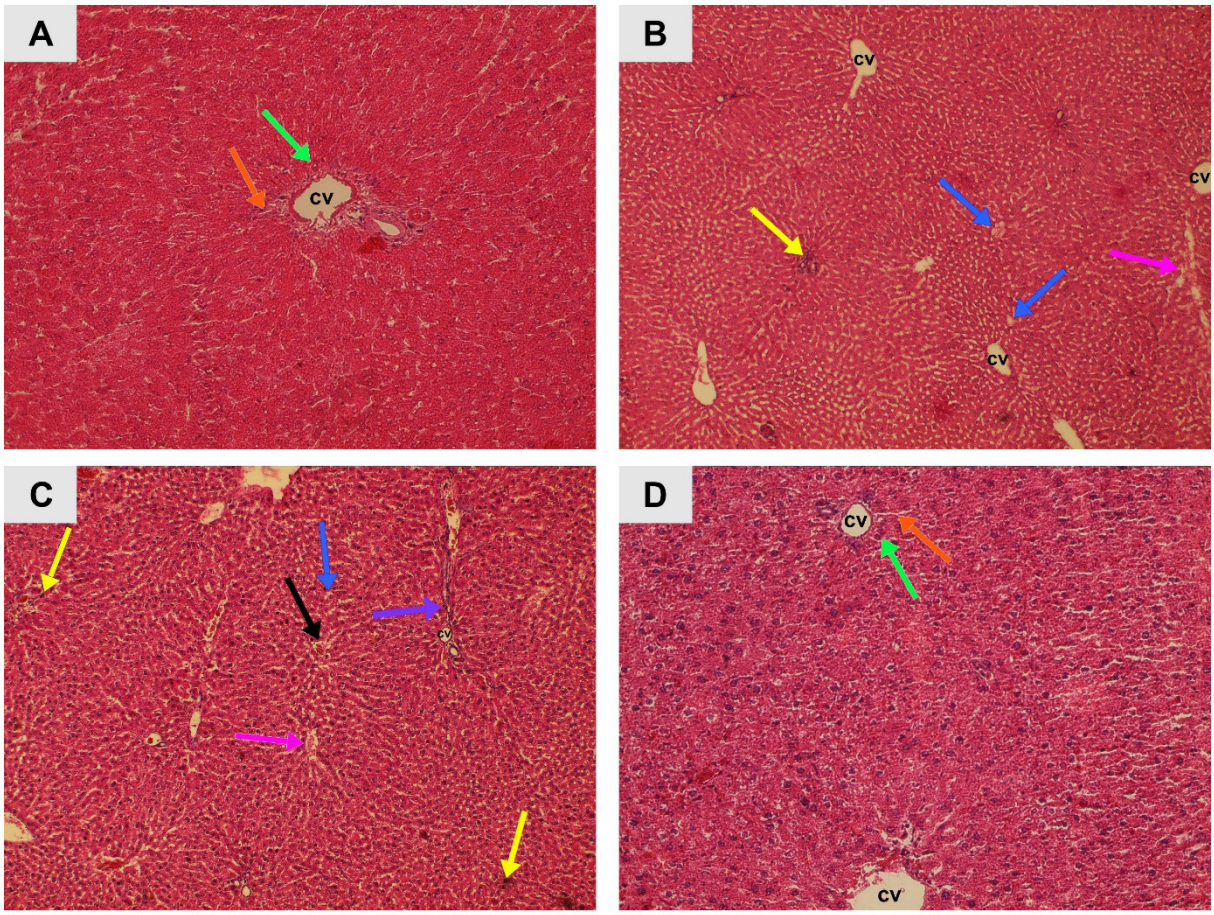

FIGURE 4. Histopathological Analysis of Liver Tissue in Four Experimental Groups. (A) Normal Control Group: A representative liver section from the normal control group displays normal hepatic architecture, characterized by well-defined hepatocytes, normal sinusoidal spaces, and a central vein (CV). (B) Negative Control Group: A liver section exhibiting significant pathological changes, including necrotic hepatocytes, lymphocyte infiltration, a reduced portal space with inflammatory cell infiltration (yellow arrow), dilated and congested sinusoidal spaces (pink arrow), and cytoplasmic vacuolations (blue arrow). (C) Sham Group: A liver section displaying similar pathological features as the negative control group, including necrotic hepatocytes, lymphocyte infiltration (yellow arrow), reduced portal space with inflammatory cell infiltration (purple arrow), dilated sinusoidal spaces (pink arrow), congestion (black arrow), and cytoplasmic vacuolations (blue arrow). (D) Treatment Group: A liver section showing improved liver morphology with a normal central vein (CV), near-normal hepatocytes (green arrow), and patent sinusoidal spaces (orange arrow). (Optical microscope, 200x magnification).

group, demonstrates marked improvements in liver morphology. The central vein and sinusoidal spaces appear normal with reduced congestion and dilation. Hepatocyte morphology is nearly restored to normal, with a decreased number of lymphocytes and a reduction in inflammation.

Assessment of Apoptosis using TUNEL Assay

The apoptotic impacts of alkylating agents on liver tissue and HuMenSCs' ability to prevent busulfan-induced liver damage were assessed by TUNEL staining four weeks post-transplantation (Figure 5). In each section, the quantity of TUNEL-positive cells was counted (Figure 6A). Compared to the normal group, TUNEL-stained cells significantly increased in both the negative control ($p < 0.001$) and the sham groups ($p < 0.05$). The percentage of TUNEL-positive cells in the treatment group was significantly lower ($30.03 \pm 0.70\%$) in comparison with the negative control group ($52.65 \pm 7.33\%$; $p < 0.01$). However, the decreased percentage did not reveal any statistical significance compared to the sham group ($33.84 \pm 2.34\%$).

Bax and Bcl-2 gene expression by qPCR

To further investigate the apoptotic response in the liver, anti- and pro-apoptotic gene expression (Bcl-2 and Bax) were evaluated using real-time PCR analysis in all four groups (Figure 6B&C).

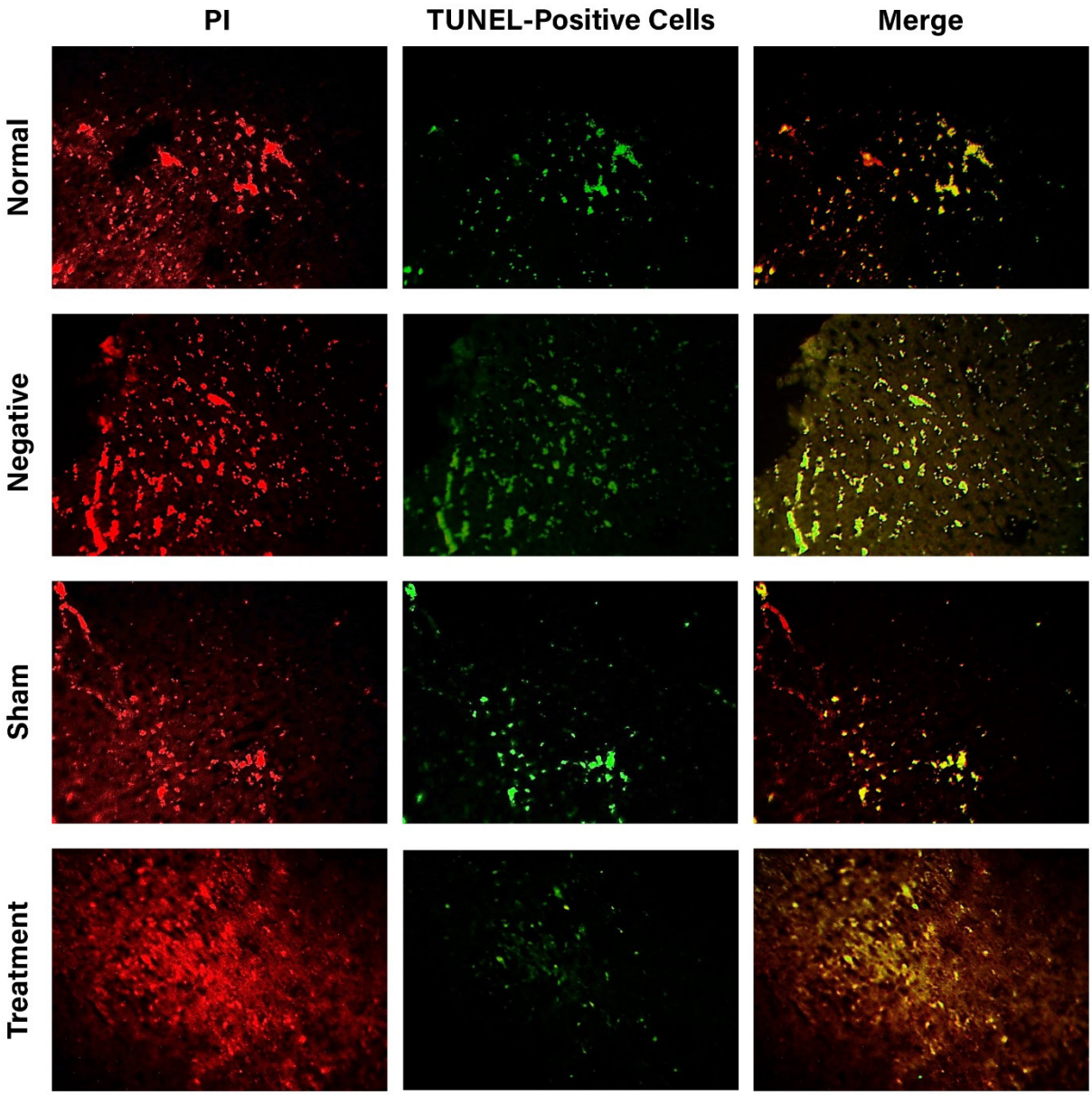

FIGURE 5. TUNEL Staining of Liver Tissue in Four Groups. TUNEL-positive cells, indicative of apoptosis, are labeled in green, while propidium iodide (PI) was used to counterstain nuclei in red. One month after the transplantation of HuMenSCs, the treatment group exhibited a reduction in TUNEL-positive cells compared to the sham control and negative control groups (200x magnification).

One week and one month after busulfan-induced liver injury, the Bcl2 and Bax expression levels in both the negative control and sham groups were significantly different (respectively, $p < 0.001$ and $p < 0.0001$) compared to the normal control group, indicating increased apoptosis in these groups. The expression of these genes was similar in the negative control and sham groups, indicating that the sham treatment without HuMenSCs transplantation did not improve ovarian function.

The treatment group showed significantly lower Bax expression (0.944 ± 0.1003) compared to both the sham (8.595 ± 0.9766; $p < 0.0001$) and negative control groups (6.929 ± 1.6652; $p < 0.001$). Additionally, while the expression of Bcl2 was upregulated in the treatment group (0.511 ± 0.0027) in comparison with the negative control (0.274 ± 0.0523) and sham groups (0.245 ± 0.026), the increase did not reach statistical significance. The findings suggest that the decrease in cell death in the treatment group after cell therapy is primarily due to the downregulation of pro-apoptotic gene expression.

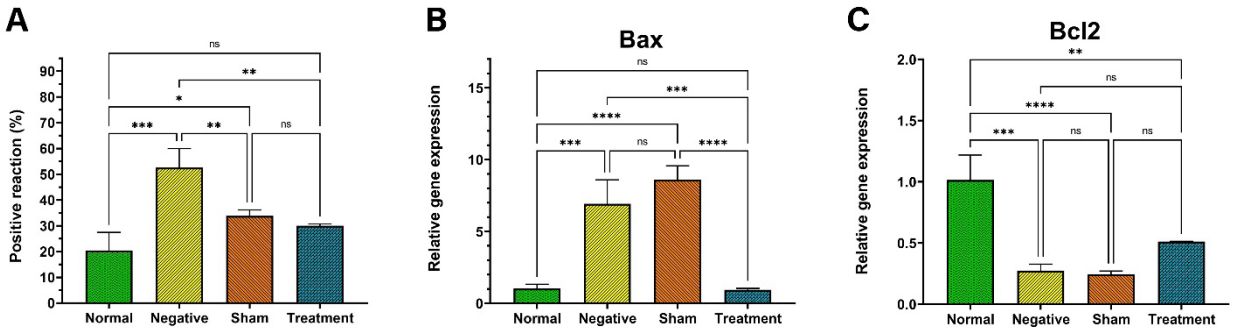

FIGURE 6. Assessment of Apoptosis in Liver Tissue. (A) TUNEL-positive cell quantification across the four groups demonstrates a significant reduction in the treatment group in contrast to the negative control group ($p < 0.01$). (B & C) Real-time PCR investigation of the Bcl2 and Bax reveals significant intergroup differences. Treatment significantly reduced Bax expression compared to the sham ($p < 0.0001$) and negative control ($p < 0.001$) groups. (Data presented as mean ± SD)

Discussion

This research explored the possible therapeutic benefits of HuMenSCs in mitigating chemotherapy-induced liver injury. Our findings demonstrate the successful engraftment of HuMenSCs into damaged liver tissue, suggesting a potential protective and regenerative effect. The observed anti-apoptotic effects of HuMenSCs may contribute to the preservation, restoration, and regeneration of hepatocytes, ultimately contributing to the recovery of liver function.

One major obstacle in clinical practice is DILI (drug-induced liver injury), often arising as a consequence of medication side effects (Meyer et al., 2024). The variable and often subtle clinical manifestations of DILI can delay diagnosis and potentially lead to the onset of severe liver failure. While the prevalence of DILI is estimated at 1-2 cases per 100,000 individuals, this rate is reportedly on the rise (Fontana et al., 2010). Chemotherapy, a mainstay in cancer treatment, significantly improves patient survival. However, alkylating agents, particularly at high doses utilized in HSCT (hematopoietic stem cell transplantation), can inflict severe damage to normal tissues, including the liver (Ciurea and Andersson 2009). Our previous research highlighted the detrimental effects of alkylating agents on ovarian tissue, posing a significant risk to fertility in women of reproductive age (Noory et al., 2019).

As a highly vascular organ crucial for metabolic function, the liver is particularly susceptible to the toxic effects of chemotherapy agents due to its extensive exposure (Lewis 2000). Hepatotoxicity manifests in various forms, including hepatocellular necrosis, steatosis, cholestasis, peliosis hepatitis, ductal injury, fibrosis, and veno-occlusion, with hepatocellular injury being the most prevalent. Hepatocellular damage often involves intracellular dysfunction, compromised membrane integrity, and injury mediated by immune system byproducts (Floyd et al., 2006). Furthermore, repeated exposure to chemotherapeutic agents can lead to a more severe recurrence of liver injury (Benichou et al., 1993).

Histopathological examination of liver tissue one week and one month after busulfan administration revealed significant damage, characterized by dilated and congested sinusoidal spaces, lymphocyte infiltration, reduced portal space, and hepatocyte necrosis. These findings suggest a robust inflammatory response within the liver, likely triggered via pro-inflammatory cytokine secretion. Previous research by Nafees et al. demonstrated elevated IL-6 and TNF-α levels in the liver following cyclophosphamide administration. Their study indicated that rutin, a flavonoid with anti-inflammatory properties, effectively reduced IL-6 and TNF-α levels (Nafees et al., 2015). Further research has highlighted the advantages of antioxidant and anti-inflammatory agents in mitigating inflammatory responses, cell necrosis, and oxidative damage in alkylating agent-induced liver injury (Fahmy et al., 2016; Habibi et al., 2015; Jiang et al., 2017; Mahmoud et al., 2017; Olayinka et al., 2015; Shokrzadeh et al., 2014). The findings imply that targeting inflammation might be a useful treatment approach for managing liver damage caused by chemotherapy.

MSCs have shown promise as treatment agents for various immune and non-immune conditions. The capacity of MSCs to specialize in diverse cell types and

self-renewal is remarkable. These cells are readily accessible from multiple sources, including adipose tissue, cord blood, amniotic fluid, and bone marrow (Domnina et al., 2016; Heidari et al., 2020). A key advantage of MSCs is their immunomodulatory potential, rendering them ethically favorable for therapeutic applications (Ryu et al., 2014). Salomone et al. showed that adipose tissue-derived MSCs transplanted into injured livers effectively suppress stress and inflammatory signaling via decreasing TNF-α and IL-1β expression. This, in turn, facilitates hepatocyte regeneration (Salomone et al., 2013).

MSCs with a strong propensity for multi-lineage differentiation and proliferation are abundant in human menstrual blood. (Gargett et al., 2016; Ghamari et al., 2021). These cells possess low immunogenicity and can be successfully passaged multiple times without compromising their genetic stability (Chen et al., 2017b). Given their accessibility through a non-invasive approach, we examined the therapeutic potential of HuMenSCs in mitigating alkylating agent-induced hepatotoxicity.

Our study demonstrates the successful homing of HuMenSCs to the injured liver tissue. This targeted migration is likely facilitated through an intricate interaction of signaling molecules, including matrix metalloproteinases (MMPs), adhesion molecules, and chemokines, which guide MSCs toward sites of inflammation and damage (Li et al., 2014; Wei et al., 2013). We confirmed the successful implantation and viability of HuMenSCs in the liver four weeks post-injection using fluorescent imaging of CM-DiI-labeled cells and flow cytometry analysis of CD44-positive cells, a key marker for MSCs. These findings suggest that HuMenSCs possess a remarkable ability to migrate to damaged tissues, proliferate, and exert therapeutic effects through signaling pathways. SDF-1 (stromal cell-derived factor-1) modulates MSC migration, engraftment, and biological response via its receptor CXCR4 (Ryu et al., 2010). The elevated SDF-1 expression in reaction to liver damage, coupled with the expression of CXCR4 on HuMenSCs (Mou et al., 2013), suggests that busulfan-induced hepatotoxicity may contribute to enhancing SDF-1 expression, thereby promoting cell migration.

Once implanted, mesenchymal stem cells (MSCs) interact with the microenvironment of damaged tissues, responding to stimuli like hypoxia, toll-like receptors (TLRs), and inflammatory cytokines (Wei et al., 2013). In response, MSCs produce numerous angiogenic, pro-survival, and regenerative growth factors, including CCL-2, VEGF, bFGF, HGF, IGF-1, and IL-6 (Gong et al., 2021; Wei et al., 2013). Histopathological analysis a month following a HuMenSCs intravenous injection revealed significant improvements in liver injury in the treatment group, including reduced inflammation, congestion, and sinusoidal dilation relative to the negative control group. The persistence of hepatotoxicity signs in the sham group highlights the absence of spontaneous healing in liver tissue without HuMenSCs transplantation and their associated protective effects.

Apoptosis, or programmed cell death, serves as a vital quality control mechanism, eliminating cells with mitotic abnormalities or insufficient survival factors (Wyllie 1993). Busulfan, an alkylating agent with multiple targets, induces apoptosis by cross-linking of DNA and protein-DNA, damaging both tumor cells and healthy tissues (Ciurea and Andersson 2009). Using the TUNEL assay, our findings confirmed the apoptotic effects of busulfan, revealing significantly more apoptotic cells in the negative control and sham groups relative to the normal group. Conversely, the treatment group demonstrated a marked reduction in TUNEL-positive cells, suggesting that HuMenSCs play a role in inhibiting apoptosis and promoting liver repair. Previous research has highlighted the cytoprotective effects of MSCs, including their anti-apoptotic and anti-inflammatory actions. For instance, research using hUCMSCs (mesenchymal stem cells derived from the human umbilical cord) demonstrated a reduction in apoptotic cells and normalized levels of serum aminotransferases (AST and ALT) in a liver damage mouse model (Dong et al., 2020; Yan et al., 2009). Xagorari et al. conducted a study on the therapeutic applications of mesenchymal stem cells-conditioned medium, which contains microparticles and factors, in CCl4-induced acute liver injury (Xagorari et al., 2013). Their findings suggest that these components can contribute to liver tissue repair. Another study by Gazdic et al. revealed that MSCs derived from murine bone marrow effectively treat acute liver injury models. They observed that MSCs effectively inhibit the expression of pro-apoptotic molecules, including FasL, TRAIL, CD107, Noxa, IFN-γ, Bax, and Bad, ultimately mitigating hepatotoxicity and reducing apoptosis (Gazdic et al., 2018).

Apoptosis, or programmed cell death, can be triggered by two distinct pathways: the intracellular death signaling system, involving mitochondrial events, and the extracellular death signaling pathway, which is initiated by death ligands interacting with cell surface receptors (Hussein et al., 2003). MSCs-derived exosomes have shown promise in mitigating drug-induced hepatotoxicity by promoting anti-apoptotic, regenerative, and proliferative effects (Tan et al., 2014). These exosomes exert their therapeutic action through signaling pathways that enhance the expression of Bcl-xL and STAT3, ultimately alleviating mitochondrial dysfunction (Tan et al., 2014). Our study further investigated the anti-apoptotic effects of HuMenSCs by examining Bcl-2 and Bax levels, key regulators of the mitochondrial death pathway. The balance between Bcl-2 and Bax expression profiles in response to programmed cell death stimuli determines cell fate, with a shift towards Bax promoting apoptosis and a change towards Bcl-2 promoting cell survival. Bax, when present as a homodimer, creates channels in the outer membrane of the mitochondria, permitting cytochrome c to seep into the cytosol and trigger caspase 3-mediated apoptosis. Conversely, Bcl-2, a protein typically found on the outer mitochondrial membrane, inhibits cytochrome c release and apoptosis by forming heterodimers with Bax (Qiao et al., 2016).

The observed increase in Bcl-2 levels and a simultaneous decrease in Bax levels following HuMenSCs transplantation suggest a mechanism of action that inhibits hepatocyte apoptosis. These intracellular regulators of the apoptotic pathway likely prevent Bax homodimer formation, which is essential for activating the caspase-3-mediated apoptotic cascade. This finding aligns with the results of Chen et al., who revealed that exosomes derived from menstrual stem cells effectively reduced caspase-3 activity of the acute hepatic failure model in murine (Chen et al., 2017a). These studies collectively support the notion that HuMenSCs demonstrate their therapeutic actions via signal transduction pathways promoting cell survival and inhibiting apoptosis.

The present study offers compelling evidence for the effectiveness of HuMenSCs in liver injury treatment. Our findings imply that these cells successfully migrate in the affected hepatic region, promoting regeneration and reducing cell death. The accessibility and non-invasive nature of menstrual stem cell sourcing make them a promising option for clinical applications, particularly in addressing chemotherapy-induced liver damage.

Nonetheless, it is critical to understand the particular limitations of this research. Firstly, while we observed improved liver function and reduced apoptosis, additional studies are required to conclusively verify the differentiation of HuMenSCs into functional hepatocytes. Secondly, our study focused on a single type of liver injury (chemotherapy-induced), further studies are necessary to analyze the effectiveness of HuMenSCs in other forms of liver damage. In addition, extended follow-up studies are needed to conduct a comprehensive long-term evaluation of the efficacy and safety profile of HuMenSCs transplantation for liver injury.

Notwithstanding these limitations, our data strongly suggest the significant potential of MSCs therapy for treating hepatic damage. Future research should focus on addressing these limitations through larger-scale human clinical trials with longer-term follow-up, investigation of different liver injury models, and exploration of optimized cell delivery methods and combination therapies. By doing so, we can advance the development of MSC therapy into an effective and safe treatment option for liver-damaged patients.

Conclusion

Our research demonstrates the therapeutic potential of HuMenSCs in treating hepatic injury. These cells effectively migrated to damaged liver tissue, promoting healing through two key mechanisms: reducing cell death (apoptosis) and stimulating the regeneration of liver cells (hepatocytes). Notably, significant recovery was observed within a month of transplantation. This study highlights the accessibility and non-invasive nature of human menstrual stem cells, making them a promising candidate for clinical applications, particularly in addressing chemotherapy-related liver damage. However, further research, particularly larger-scale human clinical trials with long-term follow-up, is crucial to fully evaluate the clinical safety, efficacy, and long-term effects of this novel therapeutic approach.

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Conflict of interest

The authors have no competing interests to declare.

Ethics approval

The study complied with the ethical standards set by the Ethics Committee of the Medical Faculty of Tehran University, ensuring the rights of the animals used. Human menstrual blood stem cells (HuMenSCs) were collected according to the ethical guidelines of the Tehran University of Medical Science, Iran (Ethics approval number IR.TUMS.MEDICINE.REC.1395.1069).

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