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# Hepatocyte-like cells derived from human adipose tissue mesenchymal cells partially restore liver function in immunosuppressed mice with ccl4-induced fibrosis

**ARTICLE INFO**

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## ABSTRACT

**Introduction:** Liver diseases are a significant global health burden, causing roughly two million deaths annually. Liver fibrosis, characterized by excessive extracellular matrix accumulation, is a major contributor to morbidity and mortality. Liver transplantation remains the gold standard for severe fibrosis, but limitations exist. Cell therapy using mesenchymal stem cells offers a promising alternative. Hepatocyte-like cells derived from human adipose tissue mesenchymal stem cells are particularly attractive due to their potential for liver regeneration. This study aimed to compare the effectiveness of mesenchymal stem cells and hepatocyte-like cells in treating carbon tetrachloride induced liver fibrosis in immunosuppressed mice.

**Methods:** Twenty C57BL/6 mice were divided into four groups: (1) control, (2) fibrotic/untreated, (3 Mesenchymal stem cell-treated, (4) Hepatocyte-like cell-treated. fibrosis was induced in groups 2-4 using intraperitoneal carbon tetrachloride injection in immunosuppressed (cyclosporine A) mice. Mesenchymal stem cells and Hepatocyte-like cells were transplanted via tail vein injection in groups 3 and 4, respectively. Liver function tests were measured in all groups.

**Results:** Both Mesenchymal stem cells and Hepatocyte-like cells treatment improved liver function as evidenced by histopathology and biochemical analyses. In the fibrotic group, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin levels were significantly elevated, while Albumin levels decreased compared to the control group. Following treatment, these parameters significantly improved ($p < 0.05$) in both treatment groups.

**Conclusion:** Our findings suggest that both Hepatocyte-like cells and Mesenchymal stem Cells have therapeutic potential for moderating liver fibrosis regression. However, Mesenchymal stem cells therapy may be more cost-effective and time-efficient.

**Key words**: Liver fibrosis, Mesenchymal stem cell, Hepatocyte-like cell, Cell therapy.



## INTRODUCTION

Chronic liver diseases are a significant global health burden, causing roughly two million deaths annually. These diseases can be caused by various factors, including viral infections (hepatitis B and C), autoimmune disorders, and genetic conditions [4]. Liver fibrosis, a major contributor to morbidity and mortality, is characterized by the progressive accumulation of extracellular matrix in liver tissue, which disrupts its normal architecture and function [4, 6, 7]. Currently, liver transplantation is the only effective treatment for advanced fibrosis; however, limitations such as donor scarcity, high costs, and transplant rejection necessitate the exploration of alternative therapies [8-10].

Cell-based therapy offers a promising alternative for patients with end-stage liver disease [11]. While transplanting human hepatocytes has shown some success, challenges like limited cell availability, cryopreservation damage, and poor engraftment remain [12, 13]. Therefore, researchers are investigating the therapeutic potential of stem cells from various sources [14].

Mesenchymal stem cells (MSCs), particularly human mesenchymal stem cells (hMSCs), are a promising cell source for liver disease treatment due to their self-renewal ability and potential to differentiate into various cell types, including hepatocytes [15, 16, 20]. Studies have shown that hMSCs can be induced to differentiate into hepatocyte-like cells (HLCs) in vitro using specific growth factors [16].

Several studies suggest that HLCs derived from human adipose tissue Mesenchymal stem cells (HLCs-hADSCs) could be a particularly promising cell source for liver disease therapy [21]. This study aimed to investigate whether HLCs-hADSCs are more effective than hADSCs alone in promoting liver regeneration in a mouse model of immunosuppressed, carbon tetrachloride (CCl4)-induced fibrosis.

## MATERIALS AND METHODS

### Isolation and culture of human adipose tissue-derived stem cells (hADSCs)

Adipose tissue was obtained from discarded, physician-removed lipoma samples from the abdominal area during cosmetic surgery. hADSCs were isolated according to established protocols [22, 23]. Briefly, samples were washed with sterile PBS, digested with 0.075% collagenase type I in PBS for one hour at 37°C, and centrifuged to remove enzymes and lipids. The isolated cells were plated in 75 $cm^2$ flasks with DMEM containing 10% FBS and 1% penicillin/streptomycin (P/S). Incubation occurred at 37°C in a 5% CO2 atmosphere. After one day, the medium was changed to remove non-adherent cells. Culture medium was refreshed every three days until cells reached 80-90% confluency. Detachment for further analysis was performed using 0.025% Trypsin-EDTA.

### Stemness Confirmation of hADSCs

Flow cytometry analysis was used to assess the expression of cell surface markers (CD90, CD105, CD44, CD45, CD34) using FITC or PE-conjugated antibodies and a BD FACSCalibur™ platform. hADSCs were also evaluated for their differentiation potential towards osteogenic and adipogenic lineages using established protocols [22].

### Hepatic differentiation of hADSCs into hepatocyte-like cells (HLCs)

hADSCs at passage two to five were seeded in four-well plates and differentiated into HLCs using a two-step growth factor approach. First, cells were treated with high-glucose DMEM containing 10% FBS, 1% P/S, 20 ng/mL hepatocyte growth factor (HGF), and $10^{-7}$ mol/L dexamethasone for one week. Subsequently, the medium was supplemented with 10 ng/mL oncostatin M (OSM) for an additional 14 days. The culture medium was changed twice weekly. After three weeks, hepatic differentiation was confirmed using the following methods:

### Hepatic differentiation and function analysis

**qRT-PCR assay for ALB production:** Total RNA was isolated at various differentiation time points (days 1, 7, 14, and 21) using commercially available kits. Reverse transcription and qRT-PCR were performed to quantify ALB gene expression (normalized to β-actin) using specific primers (Table 1) and SYBR Green chemistry.

**Table 1.** Primers applied for qRT-PCR assays

| Gene | | Primer sequence |
|---|---|---|
| Human albumin | F | 5′-GAGACCAGAGGTTGATGTGATG-3′ |
| | R | 5′-AGGCAGGCAGCTTTATCAGCA-3′ |

**Glycogen staining:** Periodic acid-Schiff staining was used to detect glycogen deposition in HLCs.

**Urea production:** Culture medium was collected throughout differentiation for colorimetric urea quantification using a commercially available kit.

**Immunocytochemistry for ALB and AFP:** Cells were fixed, permeabilized, and blocked before incubation with primary antibodies against human ALB and alpha-fetoprotein (AFP). Alexa Fluor 594 donkey anti-mouse IgG was used as a secondary antibody. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole). Fluorescence microscopy was used for visualization.

**In vivo study design and analysis**

Liver fibrosis was induced in 6-8 weeks-old male C57BL/6 mice by intraperitoneal injection of 1.0 ml/kg CCl4 twice weekly for eight weeks (total of 16 injections). Control mice (group 1) received only PBS injections. Fibrotic mice (group 2) received 16 CCl4 injections over eight weeks and one PBS injection. Group 3 and 4 received 16 CCl4 injections and after four weeks, received tail vein injections of 1 x $10^6$ cells in 100 µl: hADSCs for group 3 and HLCs for group 4. Following transplantation, CCl4 injections continued for an additional 28 days (total of 16). Animals in groups 3 and 4 received daily intraperitoneal injections of cyclosporine A (20 mg/kg/day) starting five days before transplantation and continuing throughout the experiment. Five animals were included in each group.

At the study endpoint, all animals were sacrificed. Blood serum was collected for biochemical analysis, and liver samples were collected for histopathological evaluation. Liver tissues were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT). At least three sections from each animal were evaluated for fibrosis and inflammation. The percentage of fibrosis area was quantified using ImageJ software based on Masson's trichrome staining.

The maintenance and care of experimental animals complies with National Institutes of Health guidelines for the human use of laboratory animals, and has been confirmed by Tarbiat Modares University (Ethical Code: IR.TMU.REC.1396.675).

**Biochemical Analysis**

The following liver function markers were measured in serum using commercially available photometric assay kits: Albumin (ALB), total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST).

**Statistical Analysis**

Experiments were performed in triplicate. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc test. Results are expressed as mean ± SEM.

## RESULTS

**hADSC Characterization**

Flow cytometry analysis confirmed the expression of mesenchymal stem cell (MSC) markers CD44 (97.1%), CD90 (95.5%), and CD105 (96.9%) by hADSCs. hADSCs were negative for hematopoietic markers CD45 (0.76%) and CD34 (0.83%). Oil Red O staining confirmed adipogenic differentiation potential, while Alizarin Red staining demonstrated osteogenic differentiation capability (Figure 1).

**Hepatic differentiation and function of HLCs**

qRT-PCR analysis revealed a significant increase in ALB gene expression in HLCs compared to undifferentiated hADSCs, with expression levels normalized to β-actin. Periodic acid-Schiff staining confirmed glycogen storage in HLCs, while undifferentiated hADSCs showed no glycogen deposition. Immunostaining on day 21 of differentiation demonstrated positive staining for ALB and AFP proteins in HLCs compared to undifferentiated hADSCs. Urea production in the culture medium progressively increased throughout the 21-day differentiation process (Figure 2 & 3).

**In vivo study results**

**Histopathological analysis**

Hematoxylin and eosin (H&E) staining revealed normal liver architecture in control mice. Fibrotic mice showed disrupted architecture with signs of cell death (ballooning, karyolysis, karyorrhexis).hADSC-treated mice exhibited inflammation and partial tissue recovery. HLC-treated mice displayed the most significant improvement, with evidence of regeneration and minimal cell death (Figure 4).

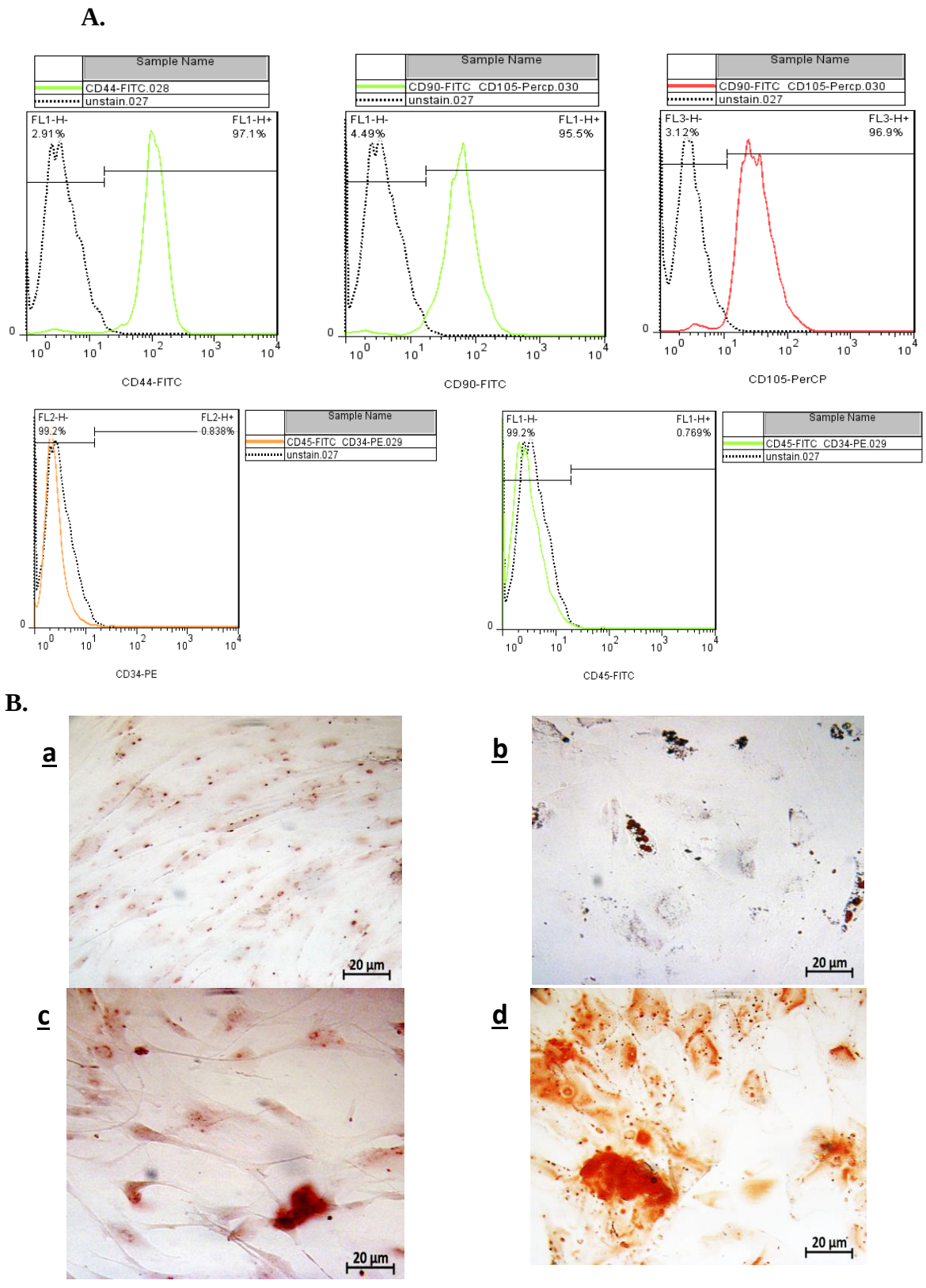


**Figure 1.** Characterization of hADSCs: (A) Flow cytometry analysis of hADSCs. All the cells expressed MSC-specific markers like CD90 (95.5%), CD105 (96.9%), and CD44 (97.1%), and were negative for CD45 (0.76%) and CD34 (0.83%). (B) Differentiation of hADSCs into adipocytes and osteoblasts. The hADSCs were treated with adipogenic and osteogenic medium. They were stained with Alizarin Red dye to identify mineralization of calcium and Oil Red dye to find fat droplets. The differentiated cells were positive for Oil Red staining (b) and Alizarin Red staining (d), while no staining was identified in undifferentiated cells with the same staining (a & c) (Abbreviations: hADSCs, human adipose-derived stem cells).

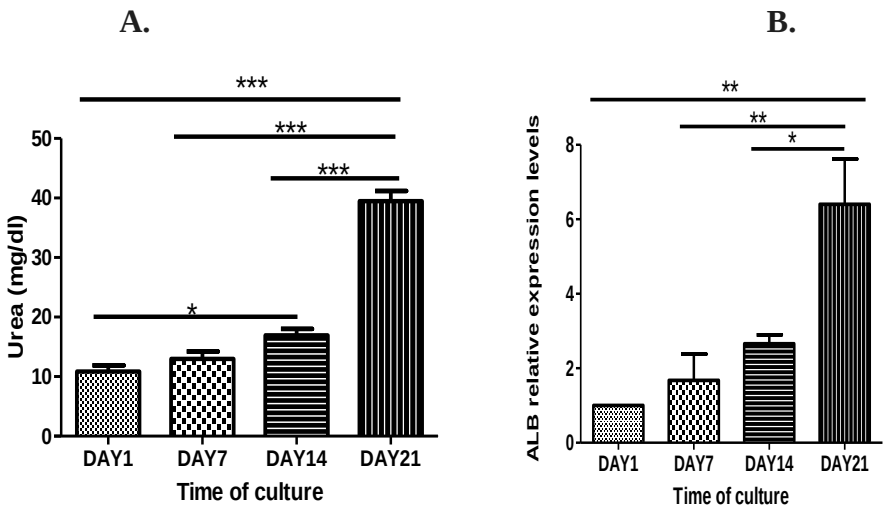


**Figure 2.** Differentiation of hADSCs into HLCs using growth factors. Detection of Urea in the cell culture medium at various intervals of the differentiation process. Urea concentration was significantly different on day 21 compared to other days of the differentiation process ($p<0.001$) (A). The ALB expression was determined by qRT-PCR assay. Data were normalized by β-actin and are shown relative to undifferentiated hADSCs. The ALB expression on day 21 was significantly different compared to day 1 and day 7($p<0.001$) and day 14($p<0.05$) of the differentiation process. The findings are provided as mean±SEM (B) (Abbreviations: hADSCs, human adipose-derived stem cells; ALB, albumin; qRT-PCR, quantitative real-time PCR). (*P- value < 0.05, **P- value < 0.01, ***P- value < 0.001).

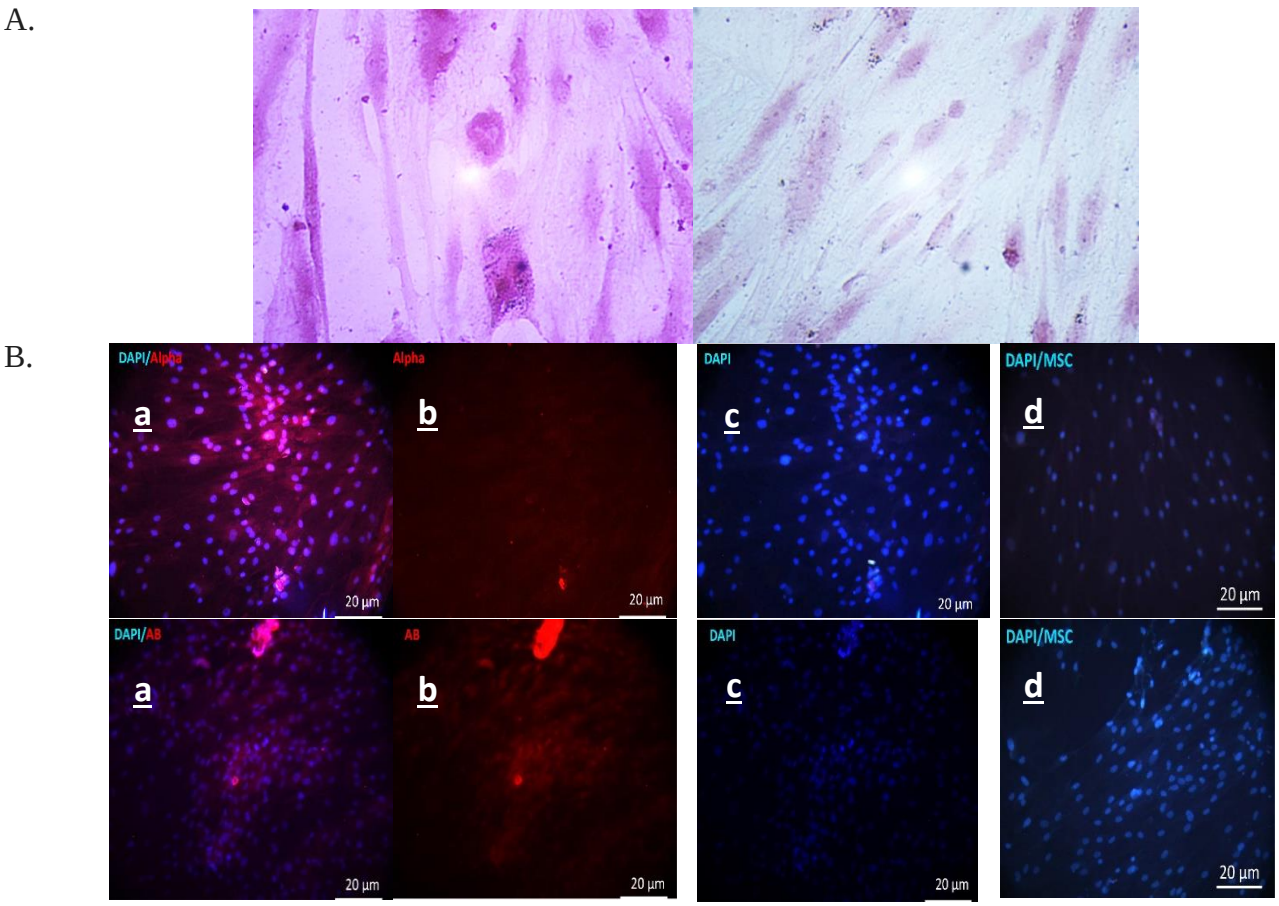


**Figure 3.** Differentiation of hADSCs into HLCs using growth factors. The PAS staining of hADSC-derived HLCs (a) and undifferentiated hADSCs (b) (A). The immunocytochemistry assay for AFP and ALB in HLCs, respectively (B) a:hADSC-derived hepatocyte-like cells, b: stained cytoplasm with Alexa Fluor 594 donkey anti-mouse IgG, c: stained nucleus with DAPI, d:undifferentiated hADSCs. (Abbreviations: hADSCs, human adipose-derived stem cells; PAS, periodic acid Schiff; ALB, albumin; AFP, alpha fetoprotein, DAPI, 4',6-diamidino-2-phenylindole).

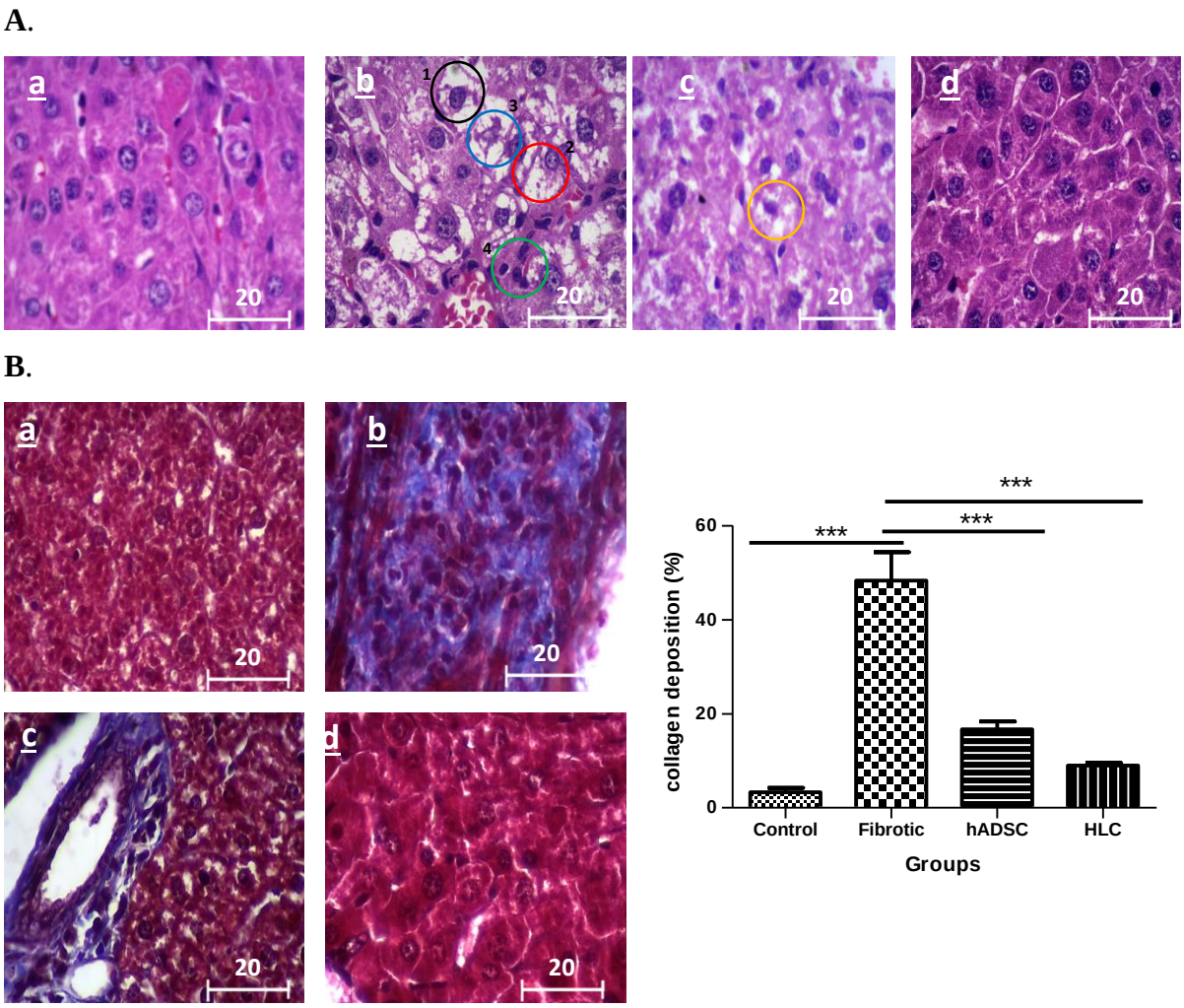


**Figure 4.** Representative liver histopathology of H&E-stained mice livers in different experimental groups (A).normal control mice liver showing classic hepatic lobules (a); non-treated fibrotic mice liver showing: circle 1. Ballooned cell, circle 2. Vacuolation, circle 3. Karyolysis, and circle 4. Karyorrhexis. (b); hADSC-treated liver showing vacuolation (c); and HLC-treated liver showing almost normal architecture (d). The representative liver histopathology of MT-stained mice livers and analysis of the percentage of collagen deposition in different experimental groups. Fibrotic group was significantly different from control, hADSC and HLC-treated ($p<0.001$). There is no significant difference between treatment groups. (B). (*P- value < 0.05, **P- value < 0.01, ***P- value < 0.001).

## MT staining

Masson's trichrome (MT) staining revealed significantly increased collagen deposition (48%) in the fibrotic group compared to the control group ($p<0.001$). Collagen deposition was significantly reduced in both hADSC-treated (16.5%) and HLC-treated (9%) groups compared to the fibrotic group ($p<0.001$), with no significant difference between these treatment groups with each other and with the control group (Figure 4).

## Biochemical analysis

Serum albumin (ALB) levels were significantly lower in the fibrotic group compared to the control group ($p<0.001$). Both hADSC and HLC treatment significantly increased ALB levels compared to the fibrotic group ($p<0.05$ between fibrotic and hADSC-treated, $p<0.01$ between fibrotic and HLC-treated). Also there is no significant difference between treatment groups with each other and with the control group. Total bilirubin, ALT, and AST levels were significantly elevated in the fibrotic group compared to control

(p<0.001) and significantly decreased in both treatment groups (p<0.001). Although there is no significant difference between HLC-treated and control group, hADSC-treated animals still exhibited slightly higher levels of these markers compared to the control group (p<0.01). Serum ALP levels were significantly higher in all study groups compared to controls (p<0.001) but showed a significant decrease in hADSC-treated (p<0.01) and HLC-treated (p<0.001) groups compared to the fibrotic group. Also there is a significant difference between treatment groups (p<0.001) (Figure 5).

So, according to histopathological and biochemical analysis both HLCs and hADSCs have therapeutic potential for moderating liver fibrosis regression and it seems that HLCs are more effective.

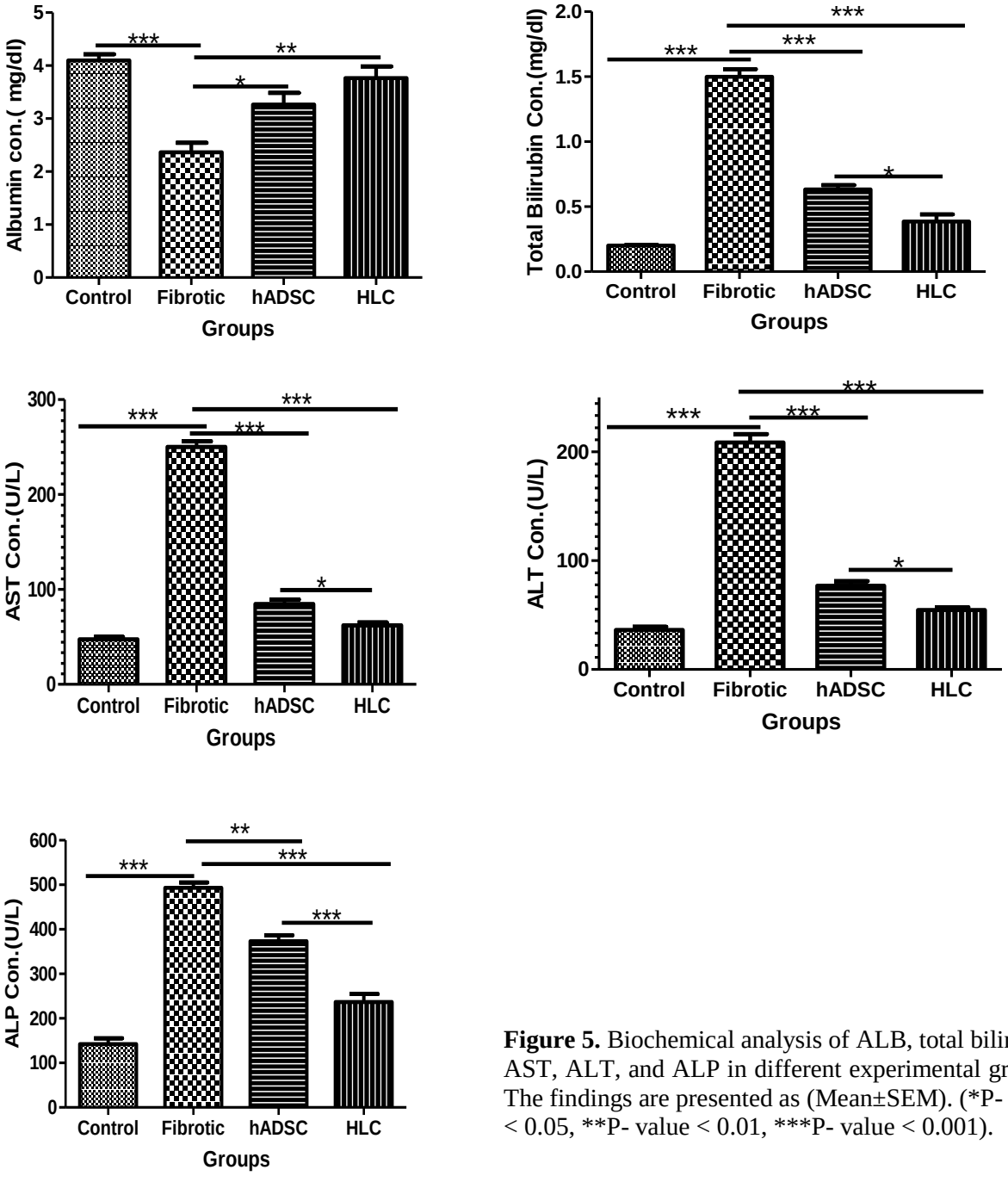


**Figure 5.** Biochemical analysis of ALB, total bilirubin, AST, ALT, and ALP in different experimental groups. The findings are presented as (Mean±SEM). (*P- value < 0.05, **P- value < 0.01, ***P- value < 0.001).

## DISCUSSION

This study investigated the therapeutic potential of human adipose tissue-derived hepatocyte-like cells (HLCs) for treating Liver fibrosis in a CCl4-induced mice model. Histopathological analysis revealed significant improvements in liver architecture and reduced collagen deposition in the HLC-treated group compared to the fibrotic group. These findings suggest that HLC transplantation can promote liver regeneration and fibrosis reduction.

Our results are consistent with previous studies demonstrating the efficacy of MSCs in alleviating Liver fibrosis [27, 28]. However, our study utilized HLCs derived from human adipose tissue MSCs, a readily accessible and minimally invasive source compared to bone marrow or Wharton's jelly MSCs used in other studies [27, 28].

Banas et al. reported improved liver function in mice transplanted with HLCs derived from human adipose tissue MSCs [29]. Our findings extend these observations by demonstrating HLC efficacy in reducing fibrosis, potentially due to a longer differentiation period allowing for more mature HLCs.

Ewida et al. also observed reduced fibrosis in a rat model treated with UC-MSC-derived HLCs [28]. While their study reported minimal periportal fibrosis, our results showed a more substantial reduction in overall collagen deposition. This difference may be attributed to variations in cell source, differentiation protocols, or the severity of fibrosis induction.

In conclusion, this study adds to the growing body of evidence supporting the therapeutic potential of HLCs for Liver fibrosis. Further research is needed to optimize HLC differentiation protocols and evaluate their efficacy in larger animal models.

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## CONFLICTS OF INTEREST

There is no conflict of interest.

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