Original Article
ATSC transplantation contributes to liver regeneration following paracetamol-induced acute liver injury through differentiation into hepatic-like cells

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Abstract: Introduction: Drug-induced liver injury (DILI) is a leading cause of acute liver injury (ALI). Acetaminophen (also termed paracetamol), can often be found in drugs that may be abused (i.e., prescription for pain relief). Animal experiments have shown that mesenchymal stem cell transplantation can ameliorate or even reverse hepatic injury. Material and methods: ALI was induced in Wistar rats using paracetamol. ATSCs were transplanted via the intravenous, portal vein, or intrahepatic route directly onto the liver parenchyma. Histological evaluation was conducted to assess drug-induced injury following transplantation. Fluorescence in situ hybridization (FISH) was used to verify the location of stem cells on the liver parenchyma. The effect of those cells on liver regeneration was tested by immunohistochemistry for hepatic growth factor (HGF). In addition, reverse transcription-quantitative PCR (qRT-PCR) was used to assess hepatic growth factor (HGF), hepatic nuclear factor 4α (HNF4α), cytochrome P450 1A2 (CYP1A2) and α-fetoprotein (AFP) mRNA expression. Results: Immunohistochemical staining for HGF was stronger in the transplanted groups than that in the control group (P<0.001). HNF4α and HGF mRNA levels were increased on day 7 following transplantation (P<0.001 and P=0.009, respectively). CYP1A2 mRNA levels were also increased (P=0.013) in the intravenous groups, while AFP levels were higher in the intrahepatic groups (P=0.006). ATSC transplantation attenuates ALI injury and promotes liver regeneration. Furthermore, expression of specific hepatic enzymes points to ATSC hepatic differentiation. Conclusion: The study showed the positive effects of transplanted adipose tissue stem cells (ATSCs) on liver regeneration (LG) through hepatotrophic factors. Furthermore, increased expression of hepatic specific proteins was recorded in ATSC transplanted groups that indicate stem cells differentiation into hepatic cells.

Keywords: Adipose tissue stem cells, hepatic growth factor, hepatic nuclear factor 4, cytochrome P450 1A2, α-fetoprotein, drug-induced liver injury, acute liver injury, liver regeneration

Introduction

Acetaminophen abuse induced ALI that is a complex medical condition with high morbidity and mortality rates. Several treatment options have been developed with the aim of attenuating or completely reversing the complications of hepatic failure. Mesenchymal stem cell therapies with bone marrow and adipose tissue stem cells (ATSCs) have been tested on experimental models of drug-induced liver injury (DILI) in recent years [1-4]. Early findings have suggested that the incubation of stem cells with hepatic growth factors and subsequent transplantation after ALI can reduce hepatic necrosis and accelerate recovery [5-11]. The administration of undifferentiated stem cells following acute liver injury (ALI) contributes significantly to the healing mechanism by promoting the processes of hepatic mass recovery and stem cell differentiation into hepatic-like cells [10-12].

Recent studies have reported that stem cells promote liver regeneration, mainly through paracrine secretion of hepatic growth factors. In addition, mesenchymal stem cells undergo cellular proliferation and differentiation into hepatic-like cells. The latter cells possess histological and biochemical properties of mature hepatocytes. Among all stem cells, ATSCs are the most prominent candidate for liver regeneration due to their proliferative capacity and high rate of differentiation into hepatic-like cells. Although ATSCs are a promising therapeutic option for ALI, many aspects of these stem cells need further consideration. Wistar rats present a reproducible experimental model for assessing massive DILI that is associated with similar effects of DILI in humans. ATSCs may pose an alternative treatment to ALI for humans due to the severe shortage of liver donors.

The optimal administration route and number of ATSCs for transplantation remain to be investigated. The aims of the present study were as follows: i) to study the attenuation of ALI following ATSC transplantation; ii) to study the expression of critical hepatotropic factors affecting liver regeneration, such as hepatic growth factor (HGF) and hepatic nuclear factor 4α (HNF4α); iii) to study the expression of functional hepatic proteins that support ATSC hepatic differentiation; and iv) to determine the optimal administration route and number of ATSCs for transplantation.

Materials and methods

Animals

The present study used female Wistar rats to achieve the greatest hepatic damage after paracetamol abuse. Liver injury is accentuated under the effects of female hormones [15-19]. As a result, female Wistar rats were deemed the ideal specimen to measure the effects of ATSCs under the most severe drug-induced hepatic damage. The female Wistar rats were matched for age, 2-3 months, and their weights ranged between 200 and 240 g. The laboratory animals were divided into six groups as follows: i) control group (15 animals), normal animals were exposed in acetaminophen toxic dose (2,000 mg/kg) and without subsequent ATSCs transplantation post DILI; ii) sham group (15 animals), normal animals without exposure to acetaminophen toxic dose (2,000 mg/kg) and

without subsequent ATSCs transplantation post DILI); iii) group 1×10^6 IV (18 animals), normal animals were exposed in acetaminophen toxic dose (2,000 mg/kg) and were transplanted with 1×10^6 ATSCs intravenously post DILI; iv) group 2×10^6 IV (18 animals), normal animals were exposed in acetaminophen toxic dose (2,000 mg/kg) and were transplanted with 2×10^6 ATSCs intravenously post DILI; v) group 1×10^6 IH (18 animals), normal animals were exposed in acetaminophen toxic dose (2,000 mg/kg) and were transplanted with 1×10^6 ATSCs directly on hepatic parenchyma post DILI; and vi) group 2×10^6 IH (18 animals), normal animals were exposed in acetaminophen toxic dose (200 mg/kg) and were transplanted with 2×10^6 ATSCs directly on liver parenchyma post DILI. Control and sham groups contained 15 animals/group, while all other groups contained 18 animals/group (**Table 1**). A single animal represents an experimental unit. A total of 102 rats were included in our experimental protocol based on γ-power analysis with 3 predictors, a medium effect of 0.15 and a power of 0.91. Wistar rats of conventional microbiological status were purchased from the same breeder (National Centre of Scientific Research "Demokritos" Athens, Greece). All rats were group-housed in type IV cages with a 400 cm^2 floor area/rat. The animal house had a controlled environment of a 12:12 h light-dark cycle (light from 7:00 a.m. to 7:00 p.m.), a temperature of 21°C, a relative humidity of 55%, and ventilation of 15 air changes/h. Animals were fed commercial food (food for Ratti-Topi, Company Vergerio Mangimi s.r.l.; http://www.vergeriomangimi.it/catalogo/menu.html). All animals had *ad libitum* access to food and water. They were allowed to acclimate to the laboratory conditions for at least 1 week prior to the experiment. All studies carried out at the Experimental, Educational Research Center ELPEN conformed to the Presidential Decree 56/2013 for the Protection of Animals used for Scientific Purposes (EU Directive 63/2010).

Isolation and culture of adipose tissue-derived stem cells

A total of four male Wistar rats were obtained from the animal house of the "Demokritus" National Research Center under the official codes for breeding and provision of animals (EL

Table 1. Clinical characteristics of the different experimental groups

Characteristic	Experimental group					
	Group A (control group)	Group B (sham group)	Group C	Group D	Group E	Group F
No. of animals	15	15	18	18	18	18
Dose of paracetamol, mg/kg	2,000	-	2,000	2,000	2,000	2,000
Transplantation of ATSCs	-	-	1×10^6 IV	2×10^6 IV	1×10^6 IH	2×10^6 IH
4th day post transplantation euthanasia	5	5	6	6	6	6
7th day post transplantation euthanasia	5	5	6	6	6	6
15th day post transplantation euthanasia	5	5	6	6	6	6
Total proteins 4th day (g/dl)	-	-	5.87	5.83	5.52	5.7
Total proteins 7th day (g/dl)	-	-	6.08	6.27	5.26	5.76
Total proteins 15th day (g/dl)	-	-	6.8[a]	6.78[a]	6.96[a]	5.83
Creatinine 4th day (mg/dl)	-	-	0.4	0.5	0.32	0.75
Creatinine 7th day (mg/dl)	-	-	0.4	0.5	0.35	0.6
Creatinine 15th day (mg/dl)	-	-	0.53	0.55	0.5	0.5[a]
Total bilirubin 4th day (mg/dl)	-	-	0.056	0.182	0.04	0.108
Total bilirubin 7th day (mg/dl)	-	-	0.064	0.03	0.03	0.9
Total bilirubin 15th day (mg/dl)	-	-	0.029	0.02[a]	0.038	0.03[a]
GGT 4th day (IU/l)	-	-	3.3	5	3.6	7.3
GGT 7th day (IU/l)	-	-	2.85	1.8	2	6.3
GGT 15th day (IU/l)	-	-	2	0.6	4	1.5[a]
Albumin 4th day (g/dl)	-	-	3.78	3.7	3.84	3.58
Albumin 7th day (g/dl)	-	-	3.91	3.86	3.64	3.77
Albumin 15th day (g/dl)	-	-	4.38[a]	3.78	4	3.85

[a]P<0.05 for values on the 15th day vs values on the 4th day within each group. ATSCs, adipose tissue stem cells; IV, intravenous; IH, intrahepatic; GGT, γ-glutamyl transferase, ANOVA test. P<0.05 was considered to indicate a statistically significant difference.

25 BIO 019 and EL 25 BIO 020, respectively). These 3-month-old male Wistar rats had a weight range of 290-300 g. The housing conditions for the male rats were type IV cages with a 400 cm² floor area/rat, a temperature of 19-23°C, humidity of 40-60% and a 12-h light/dark cycle with *ad libitum* access to food and water. Male Wistar rats were anesthetized using sevoflurane, (SEVORANE VO.LIQ.G. A; 100% W/W; six flasks x250 ml) at the laboratory on the day prior to ATSC transplantation. The induction of anesthesia was performed for 8 min using sevoflurane at 100% W/W; an exact dose of 6% was used to achieve 100% anesthetic depth [20]. No maintenance dosing was necessary as the duration of the whole process was <10 min. Adipose tissue was collected from the subcutaneous layer of the abdominal wall of male Wistar rats with liposuction aspiration using a syringe and immediately kept at 40°C. The tissues were washed with PBS, minced using two scalpels and then digested in crude collagenase (1 mg/ml final concentration of collagenase; DMEM, Thermo Fisher Scientific, Inc.) for 30 min at 37°C. Subsequently, the digest was centrifuged (200× *g* for 5 min) at 37°C to discard the supernatant, and the pellet was resuspended in DMEM, 10% FBS (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin and then transferred to a culture flask. Following incubation overnight at 37°C, the medium was changed to remove the nonadherent cells, and the attached cells were further cultured in the same medium. The stem cells were resuspended from culture medium and counted (samples were taken and counted under a light microscope). In order to estimate the proliferative ability of the cells, novel DNA synthesis was measured with dual labeling with 5-bromo-2'-deoxyuridine (BrdU) and 4',6-diamino-2-phenylindole (DAPI) dihydrochloride (Sigma), as previously described (1). In brief, adipose tissue stem cells were plated sparsely on glass coverslips and allowed to attach for 48 hours prior to 50 µM BrdU labeling in DMEM containing 10% (v/v) FBS. After an additional 48-hour incubation cells were fixed with freshly prepared 4% (w/v) formaldehyde in phosphate buffered saline (PBS), blocked for 30 minutes with 0.5% (v/v) cold water fish gelatin in PBS,

and finally incubated overnight at 4°C with anti-BrdU FITC-conjugated antibody (Roche Diagnostics GmbH, Mannheim, Germany). Subsequently, cells were counterstained with 2.5 µg/ml DAPI in PBS for 20 min. DAPI- and BrdU-positive nuclei were observed under a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss, Germany). Furthermore, ATSCs cell surface markers were examined with ICH. The results showed that ATSCs were negative for panleukocyte marker CD45 and positive up to 97% for markers CD105, CD73, CD44 and CD29 [21]. The final volume of stem cells was then washed using cell culture medium and diluted again in PBS. The cells were preserved in Eppendorf tubes (1 ml total volume) on ice and then transplanted into female Wistar rats within 1 h. Transplanted ATSCs were located by tracing the Y chromosome with fluorescence *in situ* hybridization (FISH). All studies carried out at the "Demokritus" National Research Center conformed to the Presidential Decree 56/2013 for the Protection of Animals used for Scientific Purposes (EU Directive 63/2010).

Experimental models of ALI and treatment with ATSCs

All groups, except for sham group, were exposed to a single toxic dose of paracetamol (2,000 mg/kg) diluted in water for injection to a total volume of 4 ml. This dose was administered *per os* by oral gavage, which resulted in severe liver necrosis (>60% of the liver parenchyma) by the 48 hours later. The extent of hepatic necrosis was confirmed as follows: A few rats of the same gender, age and weight were exposed to different doses of paracetamol. The rats were sacrificed on day 2, and histological examination of their respective hepatic tissues confirmed the extent of necrosis.

The animals in groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH were transplanted with ATSCs 2 days after the toxic injury occurred. The administration route and quantity of stem cells differed among groups (**Table 1**). All quantities of stem cells were suspended in normal saline 0.9% to a total volume of 1 ml just before administration in each group 48 hours after paracetamol administration. Groups 1×10^6 IV and 2×10^6 IV received 1×10^6 and 2×10^6 ATSCs, respectively, via intravenous route through the portal vein. Groups 1×10^6 IH and 2×10^6 IH received 1×10^6 and 2×

10^6 ATSCs, respectively, as direct transplants into the liver parenchyma representing the intrahepatic route; this amount was equally subdivided and administered across all five hepatic lobes of each rat. The animals were sacrificed 4, 7 and 15 days after ATSC transplantation (**Table 1**).

Transplantation of ATSCs

Isolated ATSCs were transferred to female Wistar rats as isotransplants. The animals were anesthetized using sevoflurane at the laboratory. The doses of the anesthetic agent, sevoflurane (100% W/W), were lower than those recommended by the majority of protocols (8% for induction and 2% for maintenance). Deep anesthesia and pain control were achieved during the surgical procedure. Reflexes of rats were checked periodically to confirm the depth of anesthesia. An anterior abdominal midline incision was made, and the hepatic lobes were dissected thoroughly, followed by the identification and preparation of the portal vein adjacent to the hepatic hilum. Subsequently, ATSCs were administered either via the intravenous method (IV) or directly into the liver parenchyma via the intrahepatic method (IH). The stem cells were contained in 1 ml of normal saline solution. The 15-min operation was completed with hemostasis and closure of the abdominal wall. Rat awakening was followed by extubation.

Tissue harvesting and blood samples

All animals, except two, survived postoperatively. These two animals succumbed to severe acute lung injury, as concluded from histological findings. The animals were sacrificed 4, 7 and 15 days following ATSC administration. Five rats were sacrificed at each time point (day 4, day 7 and day 15) for the control group and sham group. Six rats were sacrificed at each time point (day 4, day 7 and day 15) for each transplanted group (1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH). According to the protocol, all animals were anesthetized using sevoflurane (100% W/W) at an exact dose of 6% for an 8-min duration. A high-dose high-volume pentobarbital administration (800 mg/kg) was implemented via the endocardial instead of the intraperitoneal route [20]. This route of administration results in even faster euthanasia than the intraperitoneal route [22]. Subsequently, the liver was dissected and removed,

and blood samples were collected. Hepatic tissue samples were equally placed in 10% formalin solution or in liquid nitrogen at -80°C. Blood samples were centrifuged at 1500× g for 10 minutes at room temperature, and the yellow supernatant (serum) was analyzed for total protein, creatinine, γ-glutamyl transferase (GGT), albumin (ALB) and total bilirubin (TB) levels using a clinical biochemical analyzer (JEOL, Ltd.).

Histological analysis

Hepatic tissues were fixed with 10% formalin for 72 h at room temperature, embedded in paraffin blocks, sectioned at 4 μm and stained with hematoxylin-eosin, i.e., hematoxylin staining for 5 min followed by eosin Y solution for 2 min at room temperature. Drug-induced hepatic injury was assessed using a semiquantitative four-grade scoring system for cellular damage. The formation of hepatocellular vacuoles (1st grade) was defined as mild injury, the presentation of inflammatory cells (2nd grade) as moderate injury, hepatocellular congestion (3rd grade) as severe injury and hepatocellular necrosis as the most severe injury (4th grade) [1].

ATSC detection with FISH

FISH analysis was performed on the 4th day post transplantation. Using 4 μm sections of formalin-fixed, paraffin-embedded rat liver tissue. The sections were deparaffinized with xylene and rehydrated in descending graded ethanol concentrations of 100, 95 and 80% and dH$_2$O. After they were completely air-dried, the slides were immersed in pretreatment solution [2X saline-sodium citrate (SSC), pH 7.0] for 2 min at 73°C. Pepsin solution (ZytoVision GmbH) was applied in a dropwise manner to the tissue sections, which were then incubated for 10 min at 37°C. The slides were rinsed in 1X PBS for 5 min at room temperature (RT) and fixed in 1% formaldehyde for 5 min at RT. Subsequently, the slides were rinsed with 1X PBS with a few drops of 1 M glycine (pH 8.5) for 5 min at RT and dehydrated in 70, 85 and 100% ethanol solution for 1 min each at RT.

Immunohistochemistry (IHC) of HGF

IHC was performed on 4-μm sections of formalin-fixed, paraffin-embedded rat liver tissue. A two-step technique using a peroxidase-conju-

gated polymer was implemented (Dako Envision kit; Agilent Technologies, Inc.). The slides were washed twice at 5 min each in TBS plus 0.025% Triton X-100 with gentle agitation, followed by blocking in 10% normal serum with 1% BSA diluted in TBS for 2 h at room temperature. Slides were drained for a few seconds without rinsing before tissue paper was used to wipe around the sections. Polyclonal anti-HGF antibody (1:50; cat. no. 83760; Abcam) was applied and diluted in TBS with 1% BSA at a concentration of 10 μg/ml. Slides were subsequently incubated in 0.3% H$_2$O$_2$ in TBS for 15 min at room temperature before goat anti-rabbit IgG H&L horseradish peroxidase-conjugated secondary antibody (1:50; cat. no. ab205718; Abcam) diluted in TBS with 1% BSA was applied to the slides and incubated for 1 h at room temperature. The slides were then developed with chromogen solution (Abcam) for 10 min at room temperature and rinsed under running tap water for 5 min. Finally, the slides were dehydrated using a descending ethanol gradient, cleared with running cold tap water and mounted. Antigen retrieval was carried out with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) while the heating temperature was 95°C with an incubation of 20 min. The alcohol concentrations used for rehydration were two changes of 100% ethanol for 3 min each, followed by 95 and 80% ethanol for 1 min each. Finally, the sections were rinsed out in PBS plus Tween 20 twice for 2 min each. The primary antibody dilution used was 1:50, and the incubation time was 1 h at room temperature. A semiquantitative measurement of the HGF-Ab anti-HGF complex was conducted using a four-grade scoring system: Grade 0, no reaction or focal weak reaction; Grade 1, intense focal or diffuse weak reaction; Grade 2, moderate diffuse reaction; and Grade 3, intense diffuse reaction [23]. An experienced pathologist made the measurements using a light microscope (magnifications, ×200 and ×400). A total of 10 high-power fields were examined for each block. Furthermore, a mean score of each paraffin block was conducted manually.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted using NucleoSpin® RNA Plus (Thermo Fisher Scientific, Inc.). The RNA concentration and quality were determi-

ned using a NanoDrop 2000 UV-Vis Spectro-photometer. Total RNA (250 ng) was converted to cDNA using a Superscript™ II RT-qPCR kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol using the following temperature protocol: initial heating at 65°C for 5 min followed by quick incubation on ice; cDNA extension at 42°C for 52 min; and final denatur-ation at 70°C for 15 min. The relative mRNA expression levels of genes associated with liver regeneration [HGF, α-fetoprotein (AFP), HNF4α and cytochrome P450 1A2 (CYP1A2)] were determined by RT-qPCR relative quantification on a LightCycler® 480 System (Roche Diagnostics GmbH) using a Maxima® SYBR Green/ROX kit (Thermo Fisher Scientific, Inc.). The conditions used in the LightCycler 480 were an initial cycle at 95°C for 10 min, followed by 90 cycles of 95°C for 15 sec, annealing at 61°C for 15 sec, 72°C for 30 sec and one cycle at 95°C for 1 min. The samples were run at least in duplicate, and for each sample, the mean Cp value was calculated using the $-2^{\Delta\Delta Cq}$ method [24]. GAPDH was selected as an appropriate endogenous control. The sequences of the gene and rat-specific primers used were as follows: HGF forward, 5'-CCCTATTTCCCGTTGTGAAGGA-3' and reverse, 5'-ACCATCCACCCTACTGTTGTTT-3'; HNF4α forward, 5'-AGGATGAAGAAGTTGC-CCCC-3' and reverse, 5'-GATGTGTCTGGTGGGT-CCTG-3'; CYP1A2 forward, 5'-CATCCTTTGTCC-CCTTCACCA-3' and reverse, 5'-GGTCTTTCCACT-GCTTCTCATC-3'; AFP forward, 5'-AGAAAACAG-GGCGATGTCCA-3' and reverse, 5'-TGCCTTGT-CATACTGAGCGG-3'. The primers for GAPDH were as follows: forward, 5'-CTCTCTGCTCCTC-CCTGTTC-3' and reverse, 5'-TACGGCCAAATCC-GTTCACA-3'.

Statistical analysis

Data are expressed as the mean ± SD, representing four experimental repeats. Analysis was conducted using the Kruskal-Wallis and Mann-Whitney tests (paired two-sided comparisons) with Bonferroni correction applied in both tests. Kolmogorov-Smirnov and normal probability plot tests were used to evaluate whether the data followed a normal distribution. P<0.05 was considered to indicate a statistically significant difference. Furthermore, ANOVA test was conducted for the numerical data of biochemical results and quantitative RT-PCR. P<0.05 was considered to indicate a statistically signifi-

cant difference. Statistical analysis was conducted using SPSS version 17 (SPSS, Inc.).

Results

Histological findings with decreased congestion and vacuolization and increased inflammation

Massive confluent necrosis was recorded after acetaminophen administration while coagulative necrosis that led to denaturation of cytoplasmic proteins and intense cytoplasmic eosinophilia was the main histopathologic finding. Furthermore, histopathologic findings of inflammation were acute hepatitis with hepatocytes ballooning and lymphocyte infiltration at multi lobular and periportal areas throughout the liver parenchyma. A representative image on 4th day posttransplantation in group 2×10^6 IH is displayed (**Figure 1A**), where extensive necrosis was observed between two portal tracts (magnification, ×200). Similar extension and grade of injuries were detected to control, 1×10^6 IV, 2×10^6 IV and 1×10^6 IH groups. Furthermore, are depicted inflammation in group 1×10^6 IV (**Figure 1B**), congestion in group 1×10^6 IV (**Figure 1C**), and vacuolization in group 1×10^6 IH (**Figure 1D**). Inflammation was significantly increased, and cellular vacuolization was significantly reduced between days 4 and 7 following injury (P<0.05 and P<0.005, respectively; **Figure 1E**). In addition, a significant increase in hepatic inflammation and a decrease in vacuolization were recorded between days 4 and 15 posttransplantation (P<0.005 and P<0.005, respectively; **Figure 1E**). Each type of drug-induced injury (necrosis, inflammation, congestion and vacuolization) was summarized collectively for all time points. Necrosis from groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH on day 4 was summarized as necrosis on day 4; inflammation from groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH on day 4 was summarized as inflammation on day 4; congestion from groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH was summarized as congestion on day 4; and vacuolization from groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH on day 4 was summarized as vacuolization on day 4, and the same process was applied for injuries on days 7 and 15 (**Figure 1E**). In addition, congestion is reduced over time but not to a statistically significant level while necrosis is slightly
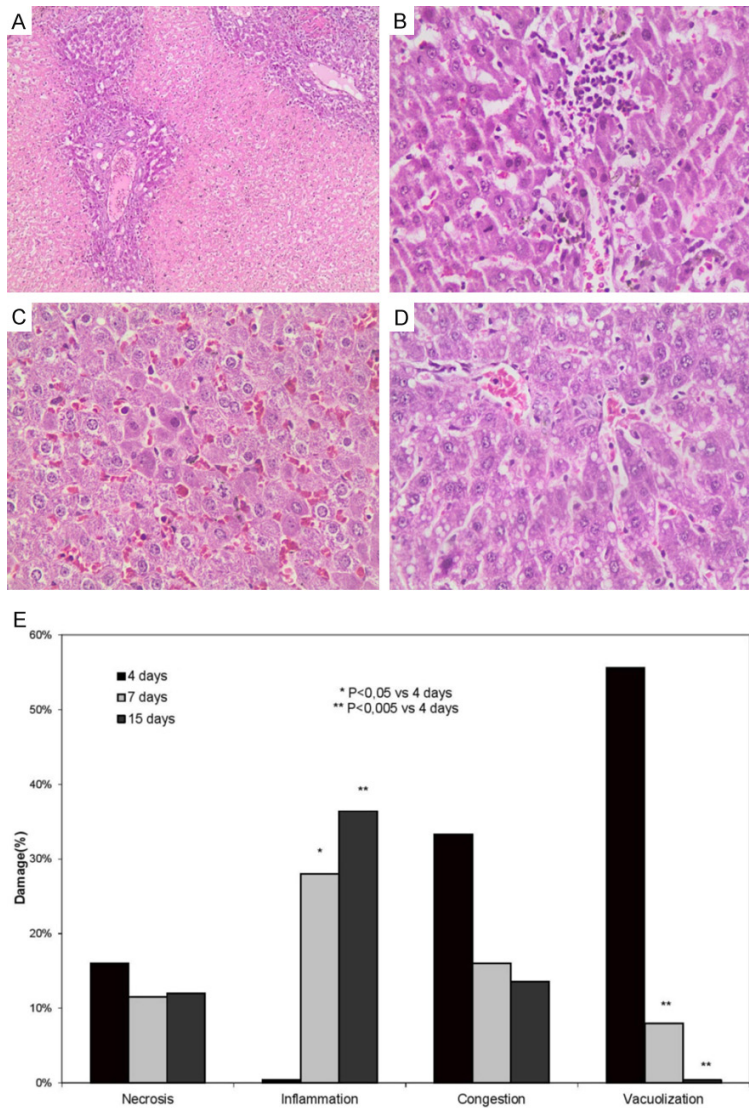
Figure 1. Histopathological findings of hepatic injury. Depicted the main 4 types of injury (necrosis, inflammation, congestion and vacuolization) across control, 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH groups. Significantly increased inflammation and decreased vacuolization across time. A. Drug-induced hepatic injury. Histopathological examination of liver tissue for histological injury where extensive necrosis was observed between two portal tracts, group 2×10^6 IH. Similar extension and grade of injuries were detected to control, 1×10^6 IV, 2×10^6 IV and 1×10^6 IH groups; Magnification, ×200. B. Inflammation in group 1×10^6 IV; Magnification, ×400. C. Congestion in group 2×10^6 IV; Magnification, ×400. D. Vacuolization in group 1×10^6 IH; Magnification, ×400. E. Overall intensity of hepatic injury across all transplanted groups on days 4, 7 and 15 independent of the quantity and the route of ATSC administration. Statistically significant inflammation on day 7 and 15 compared to day 4 (*P<0.05). Statistically significant reduction of vacuolization on day 4 and 15 compared to day 4 (**P<0.005). Statistical information: mean ± SD, the Kruskal-Wallis and Mann-Whitney tests with Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference.

reduced (**Figure 1E**). Overall, vacuolization was significantly reduced while inflammation was

significantly increased at transplanted groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH compared to control group (**Figure 1**). This latter inflammation partially explained by administration of ATSCs (**Figure 1**).

A statistically significant improvement in hepatic congestion was observed in group 1×10^6 IV compared with that in the control group (P<0.005) (**Figure 2**). Similar results were observed when groups 2×10^6 IV, 1×10^6 IH and 2×10^6 IH were compared with the control group (P<0.005; **Figure 2**). In addition, the inflammation intensity was lower following intrahepatic (IH) transplantation compared with the IV method. Specifically, groups 1×10^6 IH and 2×10^6 IH were less affected by inflammatory processes following ALI (P<0.005 in both groups; **Figure 2**) compared with those in group 2×10^6 IV. Attenuation of hepatic inflammation was recorded in group 2×10^6 IH when compared with that of group 1×10^6 IV (P<0.05), and no significant differences in inflammation were observed between groups 1×10^6 IV and 1×10^6 IH (**Figure 2**). Furthermore, group 1×10^6 IV showed statistically significant decreased necrosis compared to control group (P<0.05). The other groups 2×10^6 IV, 1×10^6 IH and 2×10^6 IH showed reduced necrosis compared to control group but not significant. In conclusion congestion is significantly attenuated in each transplanted group compared to control group while vacuolization is significantly reduced for IH arm and not for IV arm (**Figure 2**). In addition, necrosis is reduced between transplanted
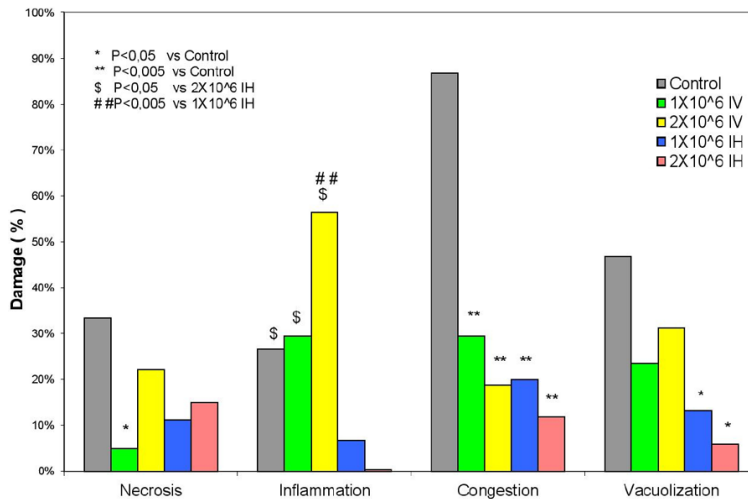
Figure 2. Intensity of hepatic injury (necrosis, inflammation, congestion and vacuolization) across transplanted groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH, as well as the control group independent of time. Significantly increased inflammation in IV arm, decreased congestion across all transplanted groups and decreased vacuolization in IH arm of the study. Furthermore, 1×10^6 IV group of the study showed significantly reduced necrosis compared to control group while the other groups (2×10^6 IV, 1×10^6 IH and 2×10^6 IH) showed reduced necrosis but not statistically significant. Each type of injury is depicted as the summary of injury from all time points (days 4, 7 and 15) in relation to each group (control group, 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6). A statistically significant difference between control group and group 1×10^6 IV (*P<0.05) for necrosis injury, a statistically significant difference between group 2×10^6 IH with control group, 1×10^6 IV and 2×10^6 IV (**P<0.05) for inflammation injury, a statistically significant difference between group 1×10^6 IH and group 2×10^6 IV (##P<0.005) for inflammation injury, a statistically significant difference between control group and groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH (**P<0.005 in all cases) for congestion injury and a statistically significant difference between control group and group 1×10^6 IH and group 2×10^6 IH (*P<0.05 in both cases) for vacuolization injury. *P<0.05 vs control group, $P<0.05 vs group 2×10^6 IH, ##P<0.005 vs group 1×10^6 IH, **P<0.005 vs control group. Statistical information: mean ± SD, the Kruskal-Wallis and Mann-Whitney tests with Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference.

groups and control group but only 1×10^6 IV showed significant decrease. Furthermore, IV route of administration of ATSCs and control group correlated with significantly higher inflammatory response compared to IH route (**Figure 2**).

Biochemical findings of increased total proteins-albumin and decrease creatinine, bilirubin and γ-glutamyl transferase over time

Total protein levels were improved significantly following ALI in the transplanted groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH between the 4th and 15th days posttransplantation (P=0.003 for 1×10^6 IV; P=0.042 for 2×10^6 IV; and P<0.001 for 1×10^6 IH and only slightly for 2×10^6 IH group; **Table 1**). Creatinine, γ-glu-

tamyl transferase (GGT) and total bilirubin (TB) levels were significantly decreased in group 2×10^6 IH between days 4 and 15 posttransplantation. Specifically, creatinine levels dropped from 0.75 to 0.5 mg/dl (P=0.045), GGT from 7.3 to 1.5 IU/l (P=0.002) and total bilirubin from 0.108 to 0.03 mg/dl (P=0.017) (**Table 1**). In addition, an significant attenuation of GGT and total bilirubin was observed in group 2×10^6 IV over time, GGT levels decreased from 5 to 0.6 IU/l (P=0.009) and bilirubin from 0.182 to 0.02 mg/dl P=0.029, which resembled the pattern in group 2×10^6 IH (**Table 1**). Albumin levels increased significantly in group 1×10^6 IV between days 4 and 15 (3.78 vs 4.38 mg/dl; P= 0.002) while groups 2×10^6 IV, 1×10^6 IH and 2×10^6 IH showed increased albumin levels but not statistically significant (**Table 1**). Creatinine levels increased for groups 1× 10^6 IV, 2×10^6 IV and 1× 10^6 IH but not to statistically significant level (**Table 1**). Total bilirubin decreased for groups 1×10^6 IV and 1×10^6 IH but not to a statistically significant level (**Table 1**). Finally, γ-glutamyl transference levels decreased for group 1×10^6 IV between 4th and 15th day while group 1×10^6 IH showed decreased levels of γ-glutamyl transference on 7th day and increased levels on 15th day (**Table 1**). Overall, total proteins significantly increased in groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and the albumin was significantly increased in 1×10^6 IV group (**Table 1**). Furthermore, bilirubin and γ-glutamyl transferase significantly reduced in groups 2×10^6 IV and 2×10^6 IH while creatinine significantly reduced only for 2×10^6 IH group (**Table 1**).

BrdU incorporation and ATSC identification in the liver with FISH

BrdU staining for ATSCs showed that incorporation was up to 95.2% (**Figure 3A**). The suc-
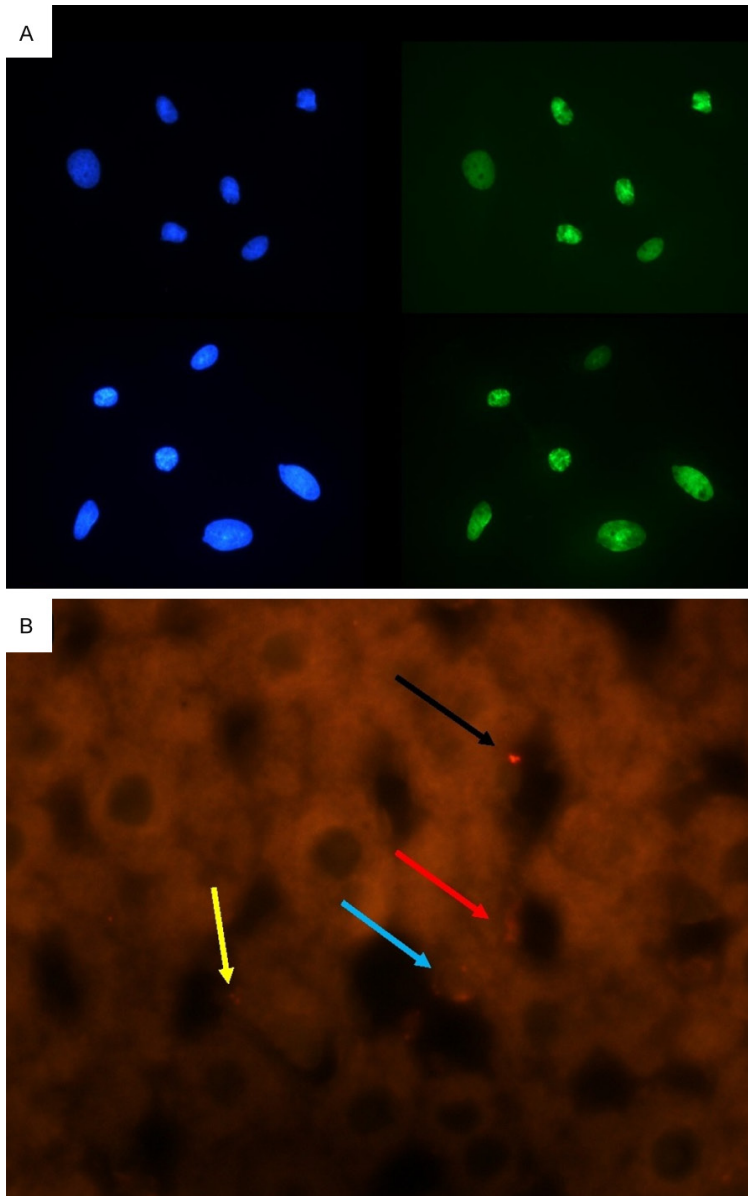
mation from fibroblast-like (corrugated shape) to hepatic-like (oval shape) cells.

HGF IHC revealed increased expression of HGF across all transplanted groups compared to control and sham group for all time points as well as independent of time and quantity of ATSCs

Increased levels of HGF were recorded following ATSC transplantation independent of time (HGF levels of control group from days 4, 7 and 15 were summarized as HGF levels of control group; HGF levels of sham group from days 4, 7 and 15 were summarized as HGF levels of sham group; HGF levels of groups 1×10^6 IV + 1×10^6 IH from days 4, 7 and 15 were summarized as HGF levels of groups 1×10^6 IV + 1×10^6 IH; and HGF levels of groups 2×10^6 IV + 2×10^6 IH on days 4, 7 and 15 were summarized as HGF levels of groups 2×10^6 IV + 2×10^6 IH) (**Figures 4A-C**, **5A** and **5B**). Statistically significant differences (P<0.001) were observed between the control group and transplanted groups 1×10^6 IV + 1×10^6 IH and groups 2×10^6 IV + 2×10^6 IH, (1.07 vs 2.32 and 1.07 vs 2.45 respectively) independent of the time or route of administration (**Figure 4C**). Statistically higher HGF values were recorded for groups 1×10^6 IV + 1×10^6 IH and groups 2×10^6 IV + 2×10^6 IH compared to values in the sham group (P<0.001 in both

Figure 3. Detecting ATSCs with BrdU staining and FISH method. A. BrdU incorporation at ATSCs up to 95, 2% is depicted (magnification, ×400). B. Detecting transplanted adipose stem cells from male Wistar rats in the female rat liver (magnification, ×400). ATSC identification on the liver parenchyma using fluorescence *in situ* hybridization. The bright red signal between polygonal hepatocytes is identified as the Y chromosome of the ATSC. The FISH signal is attached to cells with oval and corrugated cell shapes. Black and yellow arrows indicate oval ATSCs, while red and blue arrows indicate ATSCs. ATSCs, adipose tissue stem cells.

cessful transplantation of ATSCs from male Wistar rats among mature hepatocytes in female Wistar rats was confirmed using FISH (**Figure 3B**). The ATSCs varied in shape and size from small corrugated cells to larger oval cells. This observation suggested that ATSCs had undergone a gradual morphological transfor-

cases; **Figure 4C**) (2.32 vs 0.8 and 2.45 vs 0.8 respectively). As a result, groups transplanted with of lower quantities of ATSCs (1×10^6 IV + 1×10^6 IH) and those transplanted with higher quantities of ATSCs (2×10^6 IV + 2×10^6 IH) showed significantly increased levels of HGF compared to control and sham groups (**Figure**
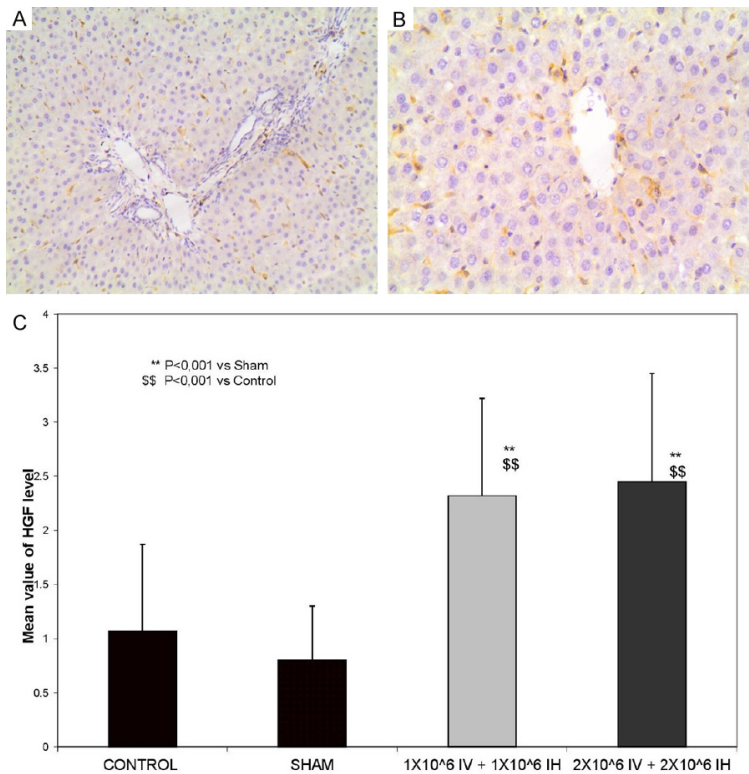
Figure 4. HGF expression analysis using IHC. Detection of HGF IHC in IV arm of the study. Significantly increased HGF detection in IV and IH arms of the study compared to control and sham group. A. Pattern of immunohistochemical staining for HGF in liver tissue from group 1×10^6 IV, showing an intense immunopositive reaction around a portal tract (yellow-brown immunostaining; magnification, ×200). B. HGF IHC close to the central lobar vein. Pattern of immunohistochemical staining for HGF in liver tissue from group 2×10^6 IV, showing an intense immunopositive reaction around a central vein (yellow-brown immunostaining; magnification, ×400). C. Significantly higher differences were observed between the sham group and groups 1×10^6 IV + 1×10^6 IH (P<0.001) and between the sham group and groups 2×10^6 IV + 2×10^6 IH (P<0.001) for the IHC analysis of HGF. In addition, groups 1×10^6 IV + 1×10^6 IH and 2×10^6 IV + 2×10^6 IH showed significantly higher differences compared to the control group (P<0.001 in both comparisons, respectively). **P<0.001 vs sham group and $$P<0.001 vs control group. HGF, hepatic growth factor; IHC, immunohistochemistry; IV, intravenous; IH, intrahepatic. Statistical information: mean ± SD, the Kruskal-Wallis and Mann-Whitney tests with Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference.

4). The four groups that received ATSCs retained significantly higher levels of HGF compared with levels in the control group [P<0.001 for groups 1×10^6 IV + 2×10^6 IV (2.39) vs control group (1.07) and P<0.001 for groups 1×10^6 IH + 2×10^6 IH (2.37) vs control group (1.07)] independently of the time (HGF levels of control group from days 4, 7 and 15 were summarized as HGF levels of control group; HGF levels of sham group from days 4, 7 and 15 were summarized as HGF levels of sham group; HGF lev-

els of groups 1×10^6 IV + 2×10^6 IV from days 4, 7 and 15 were summarized as HGF levels of groups 1×10^6 IV + 2×10^6 IV; and HGF levels of groups 1×10^6 IH + 2×10^6 IH on days 4, 7 and 15 were summarized as HGF levels of groups 1×10^6 IH + 2×10^6 IH) independent of time and the quantity of transplanted stem cells (**Figure 5B**). The same significant difference was observed between sham group (0.8) and groups 1×10^6 IV + 2×10^6 IV (2.39) and groups 1×10^6 IH + 2×10^6 IH (2.37) (P<0.001 in both cases; **Figure 5B**).

No significant differences were observed in the HGF levels between the transplanted groups in relation to the route of administration [1×10^6 IV + 2×10^6 IV groups (2.39) vs 1×10^6 IH + 2×10^6 IH groups (2.37)] and quantity of transplanted stem cells, (P=0.841 and P=0.498, respectively; **Figure 5B**). Overall, IV groups (1×10^6 IV + 2×10^6 IV) and IH groups (1×10^6 IH + 2×10^6 IH) showed significant increase of HGF levels compared to control and sham groups but no significant difference between them (IV groups vs IH groups) (**Figure 5**).

The effects of the different doses of stem cells (1×10^6 and 2×10^6) were studied independently of time. The absolute values were higher in group 1×10^6 IV and group 1×10^6 IH (2.25 and 2.39, respectively) than those in the control group (1.07) (P<0.001 for group 1×10^6 IV and P=0.001 for group 1×10^6 IH) (**Figure 6A**). The values in groups 2×10^6 IV and 2×10^6 IH (2.56 and 2.35, respectively) were also significantly higher than those in control group (P=0.001 for group 2×10^6 IV and P<0.001 for group 2×10^6 IH) (**Figure 6A**). No significant difference was ob-
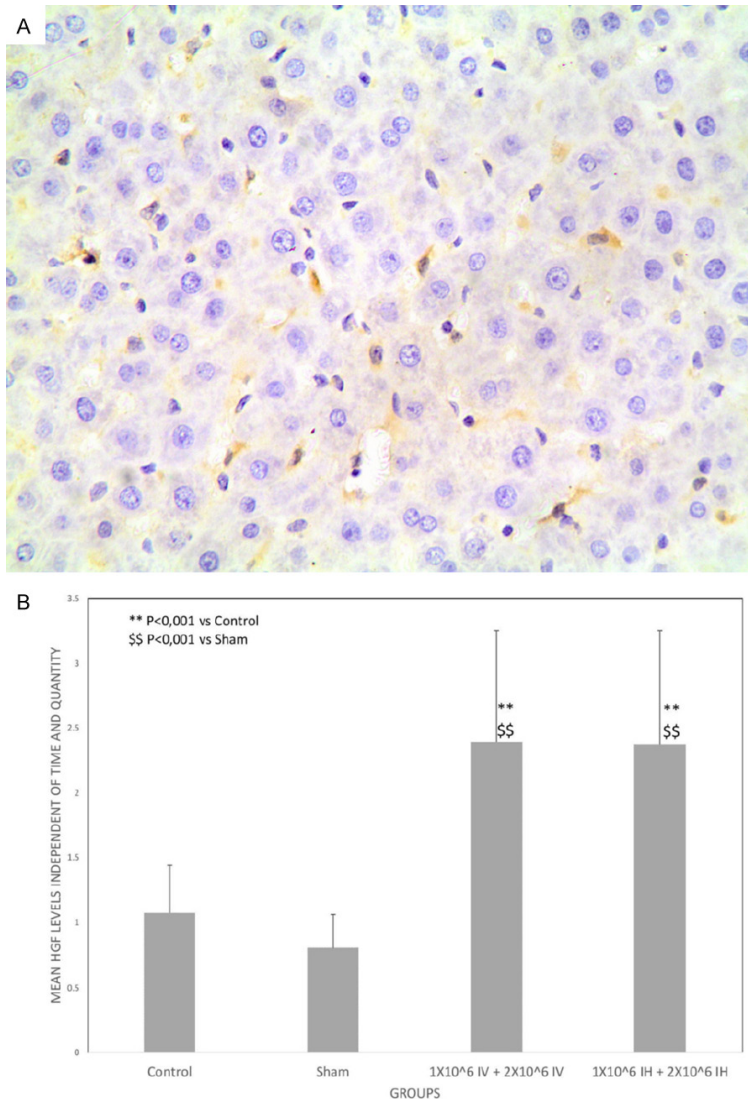
Figure 5. Detection of HGF expression using IHC. Depiction of HGF IHC in IH arm of the study. Significantly increased HGF detection across all transplanted groups independent of time and quantity of ATSCs administration in relation to control and sham group. A. Pattern of immunohistochemical staining for HGF in liver tissue from group 1×10^6 IH, showing an immunopositive reaction around a central vein (yellow-brown immunostaining; magnification, ×400). B. Comparisons between the groups independent of time and quantity of ATSCs showed a significantly higher difference between the control group and groups 1×10^6 IV + 2×10^6 IV (P<0.001), as well as a significantly higher difference between the control group and groups 1×10^6 IH + 2×10^6 IH (P<0.05). Furthermore, significantly higher differences were recorded between the sham group and groups 1×10^6 IV + 2×10^6 IV (P<0.001) as well as between the sham group and groups 1×10^6 IH + 2×10^6 IH (P<0.001). **P<0.001 vs control group and $$P<0.001 vs sham group. HGF, hepatic growth factor; IHC, immunohistochemistry; IV, intravenous; IH, intrahepatic. Statistical information: mean ± SD, the Kruskal-Wallis and Mann-Whitney tests with Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference.

served between groups 1×10^6 IV and 1×10^6 IH or between groups 2×10^6 IV and 2×10^6

IH (P=0.784 and P=0.515, respectively; **Figure 6A**).

On day 4, the transplanted groups showed higher HGF levels than the control group for both the IV and IH arms of the study (**Figure 6B**). The absolute values recorded in the IV groups (groups 1×10^6 IV + 2×10^6 IV) (2.42) and IH groups (groups 1×10^6 IH + 2×10^6 IH) (2.25) were increased compared to that in the control group (1.00) (**Figure 6B**). These differences were statistically significant in the IV and IH transplantation groups compared to group (P<0.001 for groups 1×10^6 IV + 2×10^6 IV and P<0.05 for groups 1×10^6 IH + 2×10^6 IH; **Figure 6B**). Furthermore, significantly higher levels were recorded between the sham group (1.00) and groups 1×10^6 IV + 2×10^6 IV (2.42) (P<0.001) as well as between sham group (1.00) and groups 1×10^6 IH + 2×10^6 IH (2.25) (P<0.05; **Figure 6B**). Overall, significantly higher absolute levels of HGF recorded between transplanted groups and control group as well as between transplanted groups and sham group. Those HGF levels were higher both on 4th day and independent of time (**Figure 6**).

On day 15, significantly higher differences were observed in both arms of the study [P= 0.007 for the IV groups (groups 1×10^6 IV + 2×10^6 IV) (2.46) and P=0.019 for the IH groups (groups 1×10^6 IH + 2×10^6 IH) (2.25)]; compared to control group (0.8) (**Figure 7A**). Furthermore, significant difference were observed between sham group (1.00) and groups 1×10^6 IV, 2×10^6 IV (2.46) (P<0.05) as well as between sham group (1.00) and groups 1×
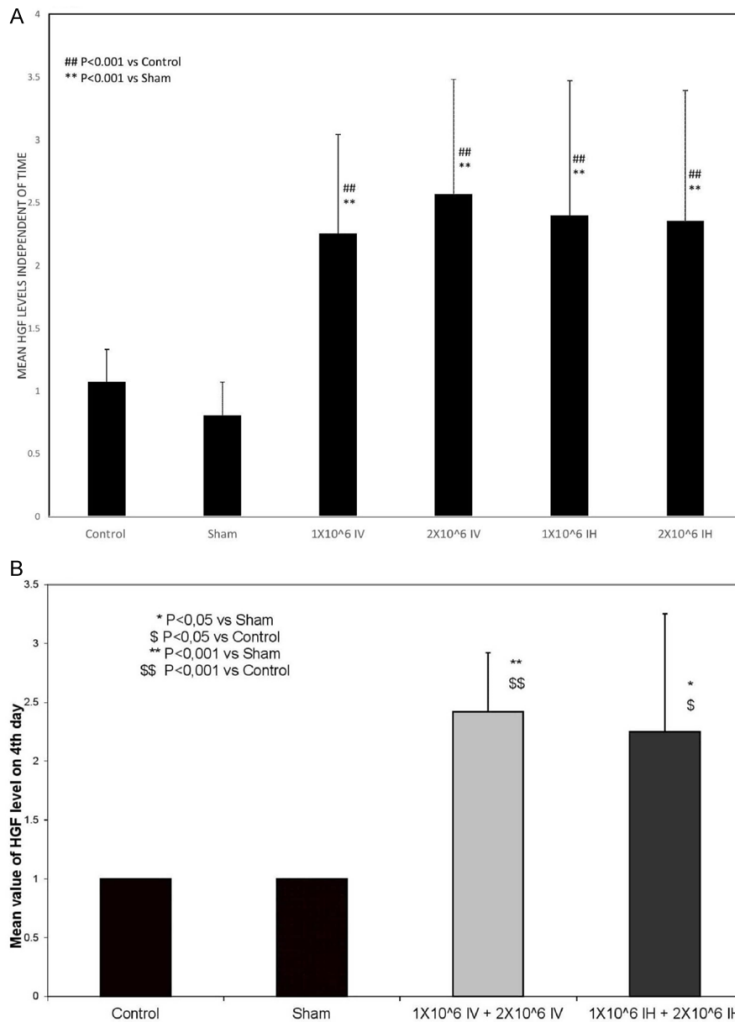
Figure 6. Detection of HGF levels using immunohistochemistry. Increased levels of HGF both for IV and IH arm independent of time and on 4th post transplantation day. A. HGF levels were higher in groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH compared with the control group (P<0.001), independent of time. Statistically significant differences were recorded between the sham group and groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH (P<0.001). ##P<0.001 vs control group and **P<0.001 vs sham group. B. HGF levels measured on day 4 posttransplantation. Statistically higher differences were recorded between the control group and groups 1×10^6 IV + 2×10^6 IV (P<0.001) and between control group and groups 1×10^6 IH + 2×10^6 IH (P<0.05). Furthermore, statistically significant differences were observed between the sham group and groups 1×10^6 IV + 2×10^6 IV (P<0.001) and between sham group B and groups 1×10^6 IH + 2×10^6 IH (P<0.05). No statistically significant difference was recorded among the transplanted groups. *P<0.05 vs sham group, $P<0.05 vs control group, **P<0.001 vs sham group and $$P<0.001 vs control group. HGF, hepatic growth factor. Statistical information: mean ± SD, the Kruskal-Wallis and Mann-Whitney tests with Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference.

10^6 IH + 2×10^6 IH (2.25) (P<0.05). On day 7, no statistically significant difference was observed between control group (1.40) and

groups 1×10^6 IV + 2×10^6 IV (2.31) (P=0.075), while statistically higher values were recorded between control group (1.40) and groups 1×10^6 IH + 2×10^6 IH (2.57) (P<0.05) (**Figure 7B**). On day 7th groups 1×10^6 IV + 2×10^6 IV (2.31) and 1×10^6 IH + 2×10^6 IH (2.57) showed statistically significant higher values compared to sham group (0.40) (P<0.001). Furthermore, a statistically significant difference between control group (1.40) and sham group (0.40) (P< 0.05) is recorded. This discrepancy on day 7 is attributed to the temporarily increased production of HGF in the control group (**Figure 7B**). However, this production in control group was reduced on the 15th day to a greater extent compared with the expression of HGF in the IV arm (groups 1×10^6 IV + 2×10^6 IV) and IH arm (groups 1×10^6 IH + 2×10^6 IH) (P< 0.05; **Figure 7A**). The IH arm showed slightly reduced HGF expression on the 15th day compared with the 7th day for groups 1×10^6 IH + 2×10^6 IH (2.25 vs 2.57), while the IV arm showed slightly increased HGF expression on day 15 compared to day 7 for groups 1× 10^6 IV + 2×10^6 IV (2.46 vs 2.31) (HGF levels on day 15; **Figure 7A**, and HGF levels on day 7; **Figure 7B**).

Regarding the routes of administration, significantly higher HGF expression was observed between groups 1×10^6 IV + 2×10^6 IV and the sham group on day 7 (P<0.05; **Figure 7B**). A statistically significant difference was recorded on day 4 for group 1×10^6 IV and group 2×10^6 IV (P<0.005 in each group) compared to control group (**Figure 7C**). The difference was not sta-

there was a significant difference between the control group and IH groups (groups 1×10^6 IH + 2×10^6 IH) (P<0.05). In addition, there were statistically significant difference (P<0.05) between the control and sham groups, while between the IV groups (groups 1×10^6 IV + 2×10^6 IV) and the sham group, the difference was significant (P<0.001). *P<0.05 vs sham, $P<0.05 vs control and **P<0.001 vs sham. C. Significantly higher HGF levels were recorded between the control group and groups (1×10^6 IV, 2×10^6 IV and 1×10^6 IH) on 4th day (P<0.005). A significant increase between control group and group 2×10^6 IH on day 15 posttransplantation (P<0.005). Additionally, a statistically higher value was recorded between control group and group 1×10^6 IV on day 15 (P<0.05). No other statistically significant differences were observed between the other groups. #P<0.05 vs control group and $P<0.005 vs control (group A). HGF, hepatic growth factor; IV, intravenous; IH, intrahepatic. Statistical information: mean ± SD, the Kruskal-Wallis and Mann-Whitney tests with Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference.

Figure 7. HGF factor levels using immunohistochemistry on days 7 and 15 post ATSC administration. Significantly increased levels of HGF on IV and IH arm of study compared to Control and Sham groups. A. Comparisons on the 15th day showed statistically significant differences between the control group and IV groups (groups 1×10^6 IV + 2×10^6 IV) (P=0.007) as well as between the control group and IH groups (groups 1×10^6 IH + 2×10^6 IH) (P=0.019). *P<0.05 vs sham and $P<0.05 vs control. B. No statistically significant differences were found between the control group and IV groups (groups 1×10^6 IV + 2×10^6 IV) on the 7th day (P=0.075), although

tistically significant on day 7 (P=0.268 for group 1×10^6 IV and P=0.052 for group 2×10^6 IV) for each group individually compared with the control group (**Figure 7C**). In addition, a significant difference was observed for group 1×10^6 IV (P=0.010) and not for group 2×10^6 IV (P=0.052) on day 15 compared with control group. The IH arm, consisting of groups 1×10^6 IH and 2×10^6 IH, presented conflicting results. Group 1×10^6 IH showed a statistically significant difference when compared with control group (P=0.004) on day 4, but group 2×10^6 IH did not (P=0.177; **Figure 7C**). Neither of the IH groups 1×10^6 IH and 2×10^6 IH showed a
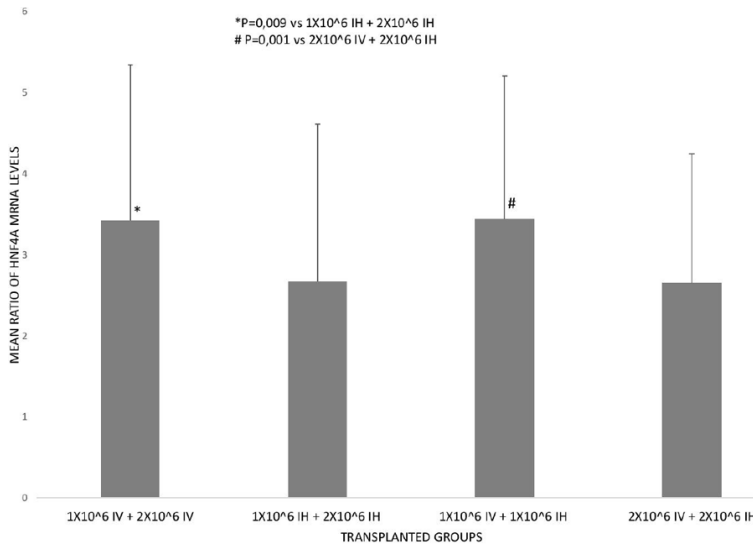
Figure 8. HNF4α expression levels across all transplanted groups (groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH) independent of time, route of administration and quantity of ATSCs. A statistically significant difference was recorded between the IV route (groups 1×10^6 IV + 2×10^6 IV) and the IH route (groups 1×10^6 IH + 2×10^6 IH) of ATSC administration (P=0.009). In addition, a statistically significant difference was recorded between the quantity of ATSCs for groups 1×10^6 IV + 1×10^6 IH and ATSCs for groups 2×10^6 IV + 2×10^6 IH (P=0.001). *P=0.009 vs groups 1×10^6 IH + 2×10^6 IH, #P=0.001 vs groups 2×10^6 IV + 2×10^6 IH. HNF4α, hepatic nuclear factor 4α; IV, intravenous; IH, intrahepatic; ATSCs, adipose tissue stem cells. Statistical information: mean ± SD and ANOVA test. P<0.05 was considered to indicate a statistically significant difference.

statistically significant difference when compared with the control group individually on day 7 (P=0.052 for group 1×10^6 IH and P=0.127 for group 2×10^6 IH; **Figure 7C**). Finally, group 2×10^6 IH exhibited significantly elevated HGF levels when compared with the control group on day 15 (P=0.004), while the HGF levels in group 1×10^6 IH were comparable to those of control group within the same timeframe (P=0.247; **Figure 7C**). Overall, transplanted groups 1×10^6 IV, 2×10^6 IV and 1×10^6 IH showed significantly increased HGF levels on 4th day while group 1×10^6 IV and 2×10^6 IH showed significant increase of HGF levels on 15th day post-transplantation toward control group. Furthermore, all transplanted groups showed no statistically significant increase of HGF levels on 7th day compared to control group (**Figure 7**). Furthermore, the same significant increase recorded between IH groups (1×10^6 IH and 2×10^6 IH) and sham group for the 7th and 15th day while the increase of HGF levels was significant for the 7th and 15th day betw-

een IV groups (1×10^6 IV and 2×10^6 IV) and sham group (**Figure 7**).

HNF4α mRNA levels increased for IV groups and for lower quantities of transplanted ATSCs while higher HNF4α expression recorded 4th and 7th day

HNF4α expression levels were examined independently of time, quantity, and route of administration. The expression of HNF4α was significantly higher in the IV groups (groups 1×10^6 IV and 2×10^6 IV) (3.42) compared to that in the IH groups (groups 1×10^6 IH + 2×10^6 IH) (2.67) (P=0.009). Furthermore, 1×10^6 transplanted ATSCs (groups 1×10^6 IV + 1×10^6 IH) (3.64) were associated with higher HNF4α expression when compared to 2×10^6 transplanted ATSCs (groups 2×10^6 IV + 2×10^6 IH) (2.65) (P=0.001; **Figure 8**). Overall, IV route of administration and lower quantities of transplanted ATSCs are associated with higher expression of HNF4α levels (**Figure 8**).

Analysis of each transplanted group showed a time-dependent accentuation of HNF4α levels (**Figure 9A**). IV group 1×10^6 IV, which received 1×10^6 ATSCs, showed a 3-fold increase in HNF4α from day 4 to day 7 compared to the cut-off value of 1, that is the reference for control group. Furthermore, HNF4α levels for group 1×10^6 IV were normalized on day 15. Significant differences were observed for group 1×10^6 IV between days 4 and 7 (P=0.002) and between days 7 and 15 (P=0.002). IV group 2×10^6 IV, which received 2×10^6 ATSCs, exhibited the same pattern of HNF4α expression as group 1×10^6 IV, with elevated levels on days 4 and 7 (4- and 10-fold, respectively; P=0.065 between days 4 and 7 and P=0.002 between days 7 and 15; **Figure 9A**). In the IH arm of the study, group 1×10^6 IH had a similar course as the IV arm in terms of HNF4α transcription levels, with a 2-fold increase com-
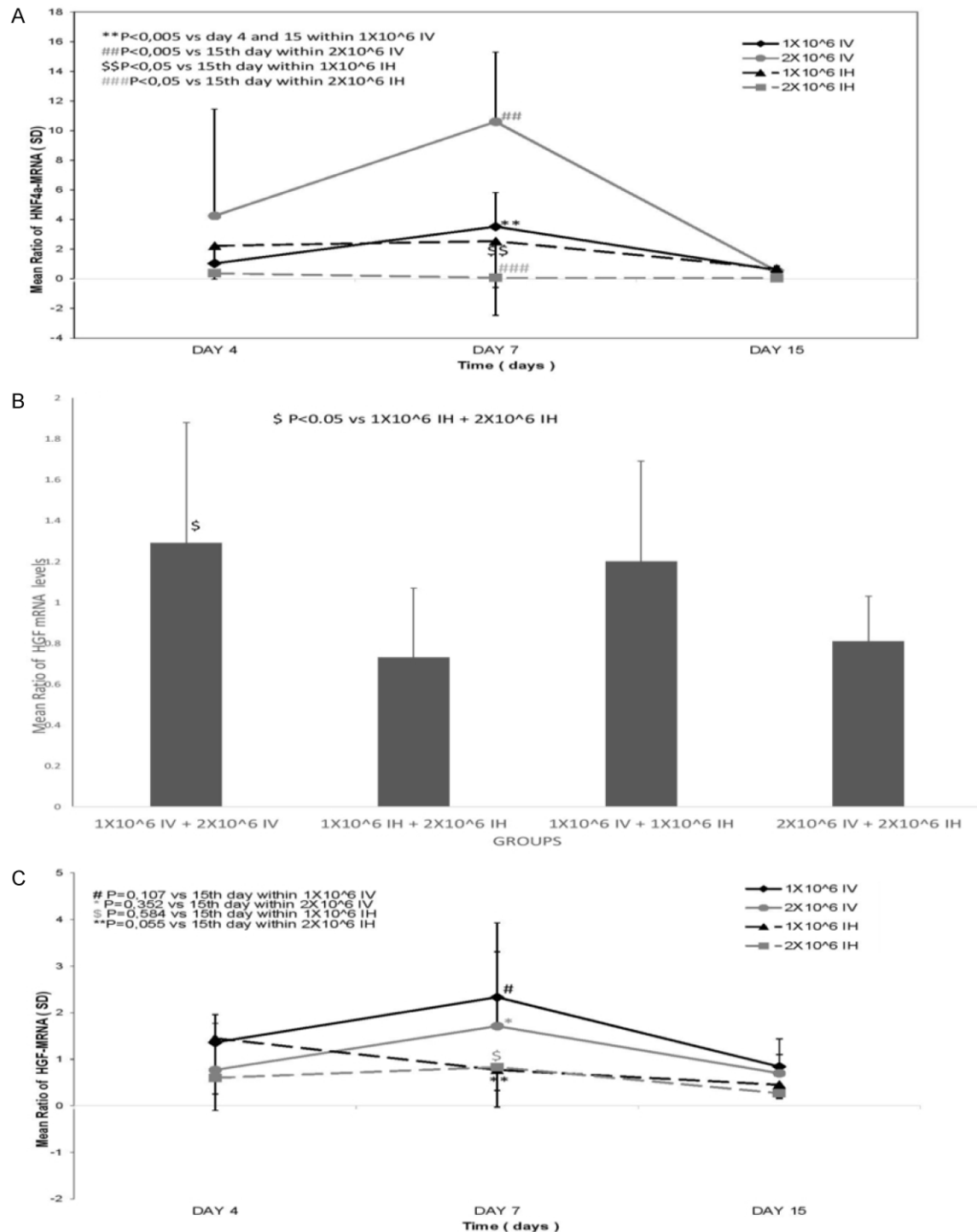
Figure 9. Hepatic-specific gene levels were increased among the transplanted groups using quantitative reverse transcription-quantitative PCR. HNF4α and HFG levels were higher on 7th day post transplantation and normalized on 15th day. A. Mean HNF4α mRNA levels of the transplanted groups. The expression levels of HNF4α within groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH differed significantly between days 7 and 15 (P<0.005 for 1×10^6 IV, P<0.005 for 2×10^6 IV, P<0.05 for 1×10^6 IH and P<0.05 for 2×10^6 IH within each group). Furthermore, a statistically higher value was recorded between days 4 and 7 within group 1×10^6 IV (P<0.005). Those *P*-values represent the fluctuation of HNF4α levels within each group that were statistically significant. **P<0.005 vs days 4 and 15 within group 1×10^6 IV, ##P<0.005 vs day 15 within group 2×10^6 IV, $$P<0.05 vs day 15 within group 1×10^6 IH and ###P<0.05 vs day 15 within group 2×10^6 IH. B. HGF mRNA levels are increased in groups 1×10^6 IV + 2×10^6 IV compared to levels in groups 1×10^6 IH + 2×10^6 IH. No other significant difference was

recorded. $^\$$P<0.05 vs groups 1×10^6 IH + 2×10^6 IH. C. Mean HGF mRNA levels of the transplanted groups. Groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH on days 4, 7 and 15. No statistically significant differences were observed. The expression levels of HGF within groups 1×10^6 IV, 2×10^6 IV 1×10^6 IH, and 2×10^6 IH differ between days 7 and 15 ($^#$P=0.107 for 1×10^6 IV, *P=0.352 for 2×10^6 IV, $^\$$P=0.584 for 1×10^6 IH and **P=0.055 for 2×10^6 IH group). mRNA levels were defined as significant for cut-off values >1, while values <1 were considered equal to that of the control group. HNF4α, hepatic nuclear factor 4α; HGF, hepatic growth factor; IV, intravenous; IH, intrahepatic. Statistical information: mean ± SD and ANOVA test. P<0.05 was considered to indicate a statistically significant difference.

pared to the cut-off value of 1, that is the reference for control group, from days 4 to 7 and normalization of HNF4α levels for group 1×10^6 IH on day 15 (**Figure 9A**). Comparison among the three time points revealed significant differences for group 1×10^6 IV (P=0.002 between days 4 and 7 as well as between days 7 and 15). Finally, IH group 2×10^6 IH, which received 2×10^6 ATSCs, showed no increase in HNF4α above normal levels, the cut-off value of 1 that is the reference for control group, on days 4, 7 and 15 (**Figure 9A**). Peak HNF4α transcription occurred on day 7 following stem cell administration, with levels significantly higher compared with those with a cut-off value of 1. HNF4α transcription levels were defined as significant for a cut-off value >1, while values <1 were considered as equal to that of control group. In conclusion, groups 1×10^6 IV, 2×10^6 IV and 1×10^6 IH showed increased levels of HNF4α on 4th and 7th day post transplantation that were normalized on 15th day while group 2×10^6 IH showed no such increase across all time points (**Figure 9A**).

HGF mRNA levels are increased in IV groups on 7th day and slightly for 1×10^6 IH on 4th day

Overall, the IV arm of the study exhibited significantly higher expression of HGF than the IH arm (P=0.024), independent of time and the quantity of transplanted ATSCs. The quantity of stem cells did not affect HGF transcription (P=0.145 for 1×10^6 vs 2×10^6; **Figure 9B**). HGF mRNA levels increased for groups 1×10^6 IV (1.36 on 4th day to 2.33 on 7th day) and 2×10^6 IV (0.77 on 4th day to 1.71 on 7th day) from day 4 to day 7 and then decreased on day 15 (0.84 for 1×10^6 IV group and 0.70 for 2×10^6 IV), but no significant difference was observed within each group (**Figure 9C**). Group 1×10^6 IH showed increased levels on day 4 (1.45) that gradually decreased on days 7 (0.77) and 15 (0.45), but no significant difference was recorded while group 2×10^6 IH showed low level of HGF expression across all time points (**Figure 9C**). Further assessment

revealed that the highest HGF levels were recorded in the IV groups on day 7 (2.33 in group 1×10^6 IV and 1.77 in group 2×10^6 IV), while those in the IH arm were lower on day 7 (0.77 in group 1×10^6 IH and 0.83 in group 1×10^6 IH). HGF transcription was even lower in the IH arm on day 15 following transplantation (0.45 for 1×10^6 IH and 0.27 for 2×10^6 IH) (**Figure 9C**). Overall, HGF levels were increased on IV arm of the study on 7th day post-transplantation but not significantly. Furthermore, HGF levels increased on slightly for 1×10^6 IH group on 4th day and not for 2×10^6 IH across all time points (**Figure 9B** and **9C**).

CYP1A2 and AFP increased mRNA levels on 7th postoperative day on IV arm and mixed results on IH arm across all time points

CYP1A2 mRNA levels were within the normal range (close to 1) on days 4 and 15 but elevated on day 7 following transplantation (**Figure 10A**). The highest value was recorded in group 1×10^6 IV (3.8) (P=0.013; **Figure 10A**). This pattern in CYP1A2 values was in accordance with the time-dependent fluctuations in the HNF4α levels, with increased values on day 7 and decreased values on days 4 and 15. Specifically, groups 1×10^6 IV (3.8) and 2×10^6 IV (2.4) showed an increase in CYP1A2 mRNA levels (>1 that is the reference for control group) compared with groups 1×10^6 IH and 2×10^6 IH on day 7. A statistically significant difference was recorded between groups 1×10^6 IV and 2×10^6 IH on the 7th day (P<0.05; **Figure 10A**). Furthermore, group 1×10^6 IH showed an increase in CYP1A2 mRNA levels (1.3) on day 4 above the cut-off value of 1 that is the reference for control group (red line; **Figure 10A**). On the other hand, group 2×10^6 IH exhibited CYP1A2 expression levels of <1 independently of time. Furthermore, AFP expression followed a similar trend as that of CYP1A2 following ATSC administration in groups 1×10^6 IV (3.4 on 7th day), 2×10^6 IV (3.4 on 7th day) and 1×10^6 IH (1.8 on 7th day) (**Figure 10B**). AFP levels were continuously sig-
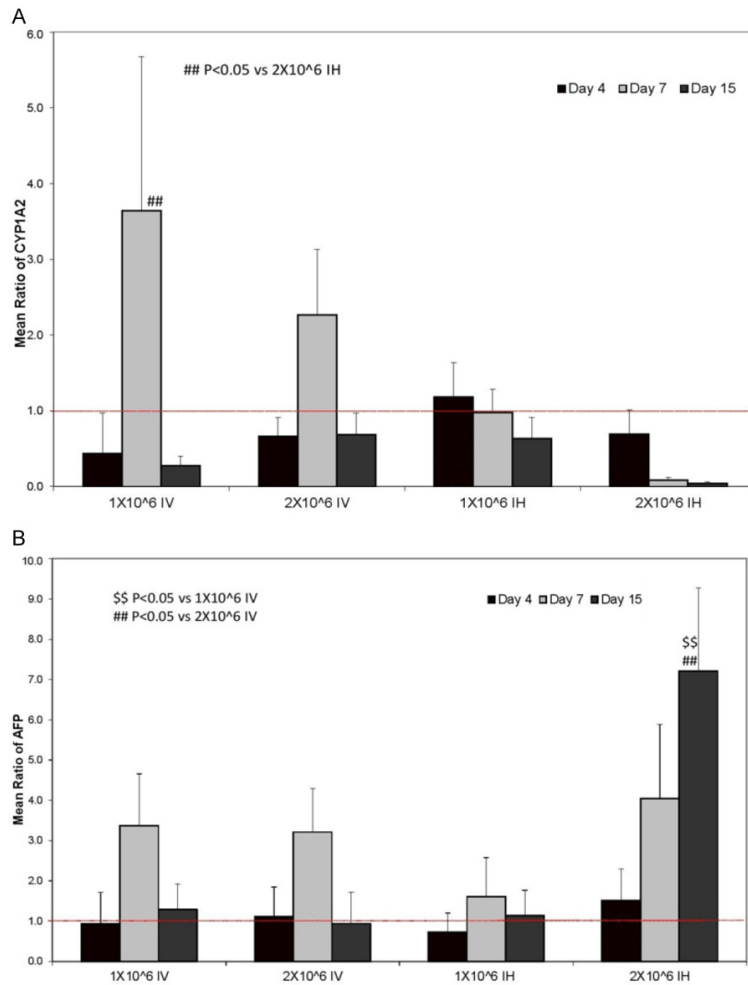
A

B

Figure 10. Measurement of hepatic-specific enzymes CYP1A2 and AFP with significant increase of the mRNA expression rate in transplanted vs control group. A. Mean mRNA expression of the transplanted groups compared to that of the control group for CYP1A2. Transplanted groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH on days 4, 7 and 15. CYP1A2 levels were designated as significant for values >1, while values <1 were considered equal to that of the control group. The red line represents the cut-off value of 1, where values above this level are significant and values below this level are insignificant. CYP1A2 levels between groups 1×10^6 IV and 2×10^6 IH showed significant differences on day 7 (P<0.05). ##P<0.05 vs group 2×10^6 IH. B. Mean mRNA expression of the transplanted groups compared to that of the control group for AFP. Transplanted groups 1×10^6 IV, 2×10^6 IV, 1×10^6 IH and 2×10^6 IH on days 4, 7 and 15. AFP levels were designated as significant for values >1, while values <1 were considered equal to that of the control group. The red line represents the cut-off value of 1, where values above this level are significant and values below this level are insignificant. AFP levels were significantly higher between groups 1×10^6 IV and 2×10^6 IH (P<0.05 on day 15) as well as between groups 2×10^6 IV and 2×10^6 IH (P<0.05 on day 15). $$P<0.05 vs group 1×10^6 IV, ##P<0.05 vs group 2×10^6 IV. CYP1A2, cytochrome P450 1A2; AFP, α-fetoprotein. Statistical information: mean ± SD and ANOVA test. P<0.05 was considered to indicate a statistically significant difference.

timeframe (P=0.006 and P= 0.008, respectively, on day 15; **Figure 10B**). Conclusively, IV arm groups 1×10^6 IV and 2×10^6 IV showed an increased expression of CYP1A2 and AFP on 7th day post transplantation which was significant for group 1×10^6 IV compared to 2×10^6 IH group for CYP1A2 on 7th day (**Figure 10**). Furthermore, IH groups 1×10^6 IH and 2×10^6 IH showed mix results with increased expression of CYP1A2 and AFP for 1×10^6 IH group (on 4th and 7th day respectively) and significant increase for AFP expression on group 2× 10^6 IH on 15th day (**Figure 10**).

Discussion

ATSCs have been studied previously due to their ability to proliferate and differentiate into a hepatic lineage at higher rates than bone marrow stem cells [2-5]. These stem cells produce a wide range of trophic factors that mitigate the severity of hepatic injury and contribute significantly to liver regeneration through the recovery of cellular mass [6, 13, 14, 25, 26]. HGF is a prominent trophic factor produced by ATSCs [27-31]. This mediator can affect the extent of DILI through the activation of detoxifying enzymes from the cytochrome P450 family and can attenuate hepatic fibrosis [32, 33]. In addition, HGF is essential during the proliferating phase of liver regeneration [34-38].

The present study explored the impact of ATSCs on DILI and liver regeneration. Female Wistar rats provided the optimum specimen to measure the effects of ATSCs under the most severe drug-induced hepatic damage [15-19]. Histological examination confirmed that hepat-

nificantly increased in group 2×10^6 IH (1.6 on 4th day, 4.1 on 7th day and 7.2 on day 15) following transplantation compared to those in groups 1×10^6 IV and 1×10^6 IH in the same

ic injury was less severe and had occurred to a lesser extent in the transplanted groups than that in the control group. Furthermore, stem cells were identified on the liver parenchyma using the FISH technique on day 4 posttransplantation. These results suggest that undifferentiated ATSCs are likely responsible for the observed hepatic recovery. This conclusion is in accordance with recent experimental protocols that support the improvement of hepatic injury following mesenchymal stem cell administration [14, 25, 26, 31, 33].

The results of the present study demonstrated that ATSCs are a critical source of HGF. Increased levels of this mediator were recorded across all treated groups compared with levels in the control. The IV route was proven to be the most efficient out of the two administration routes in achieving higher amounts of HGF at an earlier time point (day 4), independent of the quantity of transplanted ATSCs, while the IH arm had this effect only at low ATSC doses (1×10^6); the unexpected increase in HGF levels in group 2×10^6 IH on day 15 was probably the result of increased numbers of undifferentiated ATSCs on the hepatic tissue. No difference was observed between the treated and control groups on day 7, most likely due to increased HGF production in control group on day 7.

HNF4α was found to be strongly correlated with hepatic differentiation, with its expression levels significantly increased on day 7 posttransplantation, in groups 1×10^6 IV, 2×10^6 IV and 1×10^6 IH, as expected. ATSCs are exposed to high levels of HNF4α, which promotes stem cell transformation into hepatic-like cells [34, 39, 40]. A proportional increase in HNF4α levels was observed in response to high doses of stem cells transplanted via the IV route. In contrast, only lower doses of ATSCs were found to affect HNF4α production in the IH route groups. The above finding represents why the 1×10^6 IV-administered ATSCs yielded better overall results than 2×10^6 IH-administered ATSCs.

Differentiation might explain the reduced levels of HGF mRNA between days 7 and 15 in groups 1×10^6 IV, 2×10^6 IV and 1×10^6 IH [41, 42]. This observation was further supported by the HGF IHC findings, which identified lower levels of this mediator in groups 1×10^6 IV, 2×10^6 IV and 1×10^6 IH on day 15 when compared to levels at earlier time points. Group 2×10^6 IH

exhibited a nonsignificant increase in HNF4α, and it appeared that transplanted ATSCs preserved their stemness while HGF mRNA levels were quite low. However, IHC identified elevated HGF levels in group 2×10^6 IH on day 15, which was due to the increased number of transplanted ATSCs (2×10^6) in that group. The stem cells likely remained undifferentiated and preserved their capacity as a steady source of HGF.

The mRNA expression levels of CYP1A2 were found to be significantly increased in parallel with the HNF4α values across the IV groups 1×10^6 IV and 2×10^6 IV compared to control group. A similar pattern was observed in the 1×10^6 IH group, but lower CYP1A2 mRNA values were recorded. It appears that higher levels of HNF4α induce the overexpression of liver-specific proteins (CYP1A2 and AFP) in groups 1×10^6 IV, 2×10^6 IV and 1×10^6 IH. Consequently, a lack of HNF4α expression, as found in the 2×10^6 IH group, was correlated with sparse CYP1A2 values across all time points. These findings strongly support the central role of HNF4α in the hepatic differentiation of ATSCs [43-46].

Peak α-fetoprotein levels were recorded on day 7 following transplantation, which subsequently declined during the course of the hepatic differentiation of ATSCs, which was in accordance with recent studies [47]. Groups 1×10^6 IV, 2×10^6 IV and 1×10^6 IH all presented this pattern of AFP expression, which suggested potential ATSC differentiation in these groups [47]. Low HNF4α and CYP1A2 expression levels in group 2×10^6 IH were combined with continuously elevated AFP levels, further indicating the lack of differentiation in this group.

It is essential to focus on the limitations of the present study. An increased number of specimens could further support the findings of this study. Furthermore, certain biomarkers of hepatic differentiation of ATSCs, such as albumin and urea metabolism enzymes, were not found to be significantly increased during the first 15 days following transplantation, but a longer period of observation could reveal more definitive results. Finally, other biomarkers, such as cytokeratin (CK) 18, CK19 and E-cadherin, were not explored in the present study.

In conclusion, ATSC transplantation following acetaminophen-induced ALI may serve as a

viable treatment option because it can attenuate hepatic injury and strongly support liver regeneration through the production of hepatotropic factors, such as HGF and HNF4α. These factors accentuate ATSC differentiation into hepatic-like cells. In the present study, the transplanted stem cells were found to differentiate into hepatic-like cells, as they expressed liver-specific proteins such as CYP1A2 and AFP. The results of this study point toward an alternative and effective treatment for severe DILI in humans.

Acknowledgements

Disclosure of conflict of interest

None.

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