Multi-Targeted Therapy of Hepatic Fibrosis by Adipose Tissue Derived Mesenchymal Stem Cells

ElShebiny S A1*, Salem N A1, Ahmed H H2, Aglan H A2

1 Narcotics, Ergogenics and Poisons Department, Medical Research Division, National Research Centre, Giza, Egypt, Affiliations ID: 60014618.
2 Hormones Department, Medical Research Division, National Research Centre, Giza, Egypt, Affiliations ID: 60014618.

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ABSTRACT
Adipose tissue holds a promise for regenerative medicinal approaches. This study investigated the competency of stem cells derived from the adipose tissue in thioacetamide (TA)-induced liver fibrotic disease model in rats. Adipose tissue derived-mesenchymal stem cells (AT-MSCs) were isolated from female Wistar rats, characterized, and cultured in DMEM media (undifferentiated) or HGF enriched media (differentiated). Both AT-MSCs were labelled with PKH26. Forty male Wistar rats were used and divided into four groups; group (1) served as negative control group, groups (2), (3) and (4) received TA (i.p., 100 mg/kg, three times weekly for 6 weeks), thereafter group (2) served as positive control group, group (3) received a single injection of undifferentiated AT-MSCs (3x10^6 cells), and group (4) received a single injection of differentiated AT-MSCs (3x10^6 cells). After two months of AT-MSCs single injection, biochemical parameters and proteins expression were measured. The engraftment of PKH26-labeled differentiated AT-MSCs in injured liver was confirmed. TA administration produced fibrotic changes characterized by increased serum enzymes activity and reduced albumin and fibrinogen. Additionally, hepatic TGF-β and HGF contents were elevated. Treatment with AT-MSCs restored liver functions associated with decreased TGF-β and elevated HGF. The expression of HNF4a, CYP7a1, and albumin was upregulated after transplantation, while CK-7, AFP, and β-5-Tub were down-regulated. The aforementioned effects were more prominent after hepatocytes transplantation. The present work enlightens the aptitude of adipose tissue in producing functional in vitro differentiated hepatocytes that promote regeneration of fibrotic liver. The transplantation of hepatocytes proved superior proficiency rather than naïve stem cells.

Keywords: Adipose tissue; Hepatocytes; Liver fibrosis; Mesenchymal stem cells; Thioacetamide; Transplantation.

INTRODUCTION
Adipose tissue-derived mesenchymal stem cells (AT-MSCs) are an attractive approach for regenerative medicine. Adipose tissue represents a non-invasive and low-cost source of stem cells. AT-MSCs can differentiate along multiple cell lineage pathways in a reproducible manner. They can be safely and effectively transplanted into an either autologous or allogeneic host. Hepatocyte growth factor (HGF) acts as a mitogen for mature hepatocytes and it is important for liver development and regeneration. The supplementation of culture medium with HGF and fibroblast growth factor-4 (FGF-4) encourages the MSCs to differentiate into hepatocyte lineage. Liver diseases eventually end into fibrosis and loss of function thus death. At the end stage, liver transplantation remains to be the only choice to maintain life of patients. However, lack of donors, surgical and immunological complications limit its wide applicability and render the generation of hepatocytes from MSCs a considerable promise for future clinical applications. Transplantation of hepatocytes is more applicable than liver transplantation and non-problematic. Though use of undifferentiated stem cells in different diseases is extensively studied, the use of differentiated ones and optimization of their culture methods and protocols for isolating, maintaining, and transplanting functional hepatocytes is still required. The current study investigated the in vivo functioning potential of AT-MSCs in induced fibrotic liver rat model after their in vitro differentiation into hepatocytes.

MATERIAL AND METHODS
In vitro expansion of adipose tissue-derived MSCs
Adipose tissue (AT) was obtained from the abdominal fats of female adult Wistar rats, cut into small pieces, digested, filtered, and cultured according to the previously described conditions. Shorty, the small pieces of AT were digested using 0.075% collagenase II (Serva Electrophoresis GmbH, Mannheim) for 60 min at 37°C with shaking. Then, digested tissue was filtered and centrifuged, and erythrocytes were removed by treatment with erythrocyte lysis buffer. The cells were transferred to tissue culture flasks with Dulbecco modified eagle medium (DMEM, Gibco/BRL, USA) supplemented with 10% fetal bovine serum (FBS, Gibco/BRL) and after an attachment period of 24 hours, non-adherent cells were

*Author for Correspondence
removed by phosphate buffer solution (PBS; Gibco/BRL) wash. Attached cells were cultured in DMEM media supplemented with 10% FBS, 1% penicillin-streptomycin (Gibco/BRL) and 1.25 mg/L amphotericin B (Gibco/BRL) and expanded in vitro. At 80–90% confluence, cultures were washed twice with PBS and the cells were trypsinized with 0.25% trypsin in lmM EDTA (GIBCO/BRL) for 5 min at 37 °C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 75 cm² culture flask. The resulting cultures were referred to as first-passage cultures.

Flow cytometric characterization of AT-MSCs
Phenotype of the isolated AT-MSCs was confirmed by means of flow cytometry using CD44, CD45 and CD90 cell surface markers. The PE-conjugated CD44 antibody was procured from Miltenyi Biotec, Germany, the FITC-conjugated CD45 antibody was supplied from Immunotech SAS, France, while the PE-conjugated CD90 antibody was purchased from R&D Systems, UK. Briefly, the cells were incubated with the antibody against each of the surface markers for 10 min at 4 °C for CD44, 20 min at 25 °C for CD45 and 30 min at 4 °C for CD90 followed by flow cytometry analysis (Beckman Coulter Elite XL, USA).

Hepatogenic differentiation of AT-MSCs
AT-MSCs was seeded onto collagen-coated polystyrene plates at a density of 2 × 10⁴ cells/cm², and cultured with DMEM medium containing 10% FBS, 100 mg/l insulin, 0.067 mg/l selenium, and 55 mg/l transferrin (Sigma, USA). For differentiation into hepatocytes, 20 ng/ml of HGF (Genzyme, USA) and 10 nM dexamethasone (Sigma) were added to culture media. Seven to nine days later, hematopoietic stem cells and non-adherent cells were removed with every medium change. AT-MSCs cultured for 2 weeks in the presence of HGF were detached by trypsin treatment. Hepatic differentiation of AT-MSCs was confirmed by morphological examination and reverse-transcription polymerase chain reaction (RT-PCR) detection of albumin (ALB) and alpha-fetoprotein (AFP) genes expression.

RT-PCR detection of functional hepatic genes expression
Total RNA was extracted from cells using RNeasy Purification Reagent (Qiagen, CA). A sample of RNA (1 μg) was reverse transcribed with M-MLV (Moloney – Murine Leukemia virus) reverse transcriptase (RT) for 30 minutes at 42°C in the presence of oligo-dT primer. Polymerase chain reaction was performed using primers for ALB: F: 5’-TGGAAGCCGGTGATGGGTGA-3’, R: 5’-GAGGGAAATCGTGCGTGA-3’. AFP: F: 5’-AAATCTGTITCCTCATGGCTAC-3’, R: 5’-CGGACCGTTCTCCTCCTCCT-3’ and β-actin: F: 5’-GAGGAAAATCCTGCGTGA-3’, R: 5’-AGGAGCCAGGCGAGTAACTT-3’. PCR was adjusted at 34 cycles, with each cycle consisting of denaturation at 95°C for 20 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 15 seconds, with an additional 5-minute incubation at 72°C after completion of the last cycle. The PCR product was separated by electrophoresis through a 1% agarose gel, stained, and photographed under ultraviolet light.

Labeling of AT-MSCs with PKH-26
The undifferentiated and differentiated MSCs were labeled with PKH-26 fluorescent linker dye according to the manufacturer’s instructions (Sigma Aldrich, USA) prior to transplantation in experimental animals. After 2 months, liver tissues were examined using fluorescence microscope to detect and track the cells. PKH26-derived fluorescence was observed using a Typhoon 8600 variable mode imager (Amersham Biosciences, Piscataway, NJ, USA).

Experimental design
Forty adult male Wistar rats weighing 180±10 g were provided from the Animal House Colony of the National Research Centre (NRC) and housed in a temperature controlled environment (25 ± 2 °C) with a fixed light/dark cycle for one week as an adaptation period with free access to water and food. The guidelines for animal experiments approved by the Ethical Committee of Medical Research at National Research Centre, Giza, Egypt were followed. After the acclimatization period, the animals were classified into 4 groups (n=10 /group). The first group served as normal control group. Other groups were injected intraperitoneally with thioacetamide (TA; Sigma, USA) in a dose of 100 mg/kg three times weekly for 6 weeks to induce liver fibrosis. Thereafter, one set received 0.5ml of culture media and served as positive control group, other set was infused intravenously.
with 0.5 ml of culture media containing 3x10^6 undifferentiated AT-MSCs (TA+AT-MSCs) and the last set received differentiated AT-MSCs (3x10^6, i.v.) (TA+AT-MSCs+HGF). After completion of the experimental period (2 months), all animals were fasted for 12 h and blood samples were collected from retro-orbital venous plexus under anaesthesia. Plasma and sera were separated and stored at -20°C until analyzed. After blood collection, the whole liver of each rat was rapidly and carefully dissected. Then each liver was divided into two portions. The first portion was homogenized immediately to give 10% (w/v) homogenate in ice-cold medium containing 50 µm Tris-HCl and 300 µm sucrose. The resultant homogenate was centrifuged (1800 xg, 10 min, 4°C) and the supernatant was used for biochemical assays. The second portion was immediately frozen in liquid nitrogen and stored at -80°C prior to extraction for molecular study.
Table 1: Effect of treatment with undifferentiated and differentiated AT-MSCs on serum ALT and ALB as well as plasma FBG levels in liver fibrosis induced rats.

<table>
<thead>
<tr>
<th>ParametersGroups</th>
<th>ALT (U/L)</th>
<th>ALB (g/dL)</th>
<th>FBG (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>26.6 ± 0.2</td>
<td>3.9 ± 0.1</td>
<td>74500 ± 2353</td>
</tr>
<tr>
<td>Positive control (TA)</td>
<td>60.5 ± 0.7</td>
<td>2.9 ± 0.2</td>
<td>45588 ± 658</td>
</tr>
<tr>
<td>TA + AT-MSCs</td>
<td>39.7 ± 0.4</td>
<td>3.0 ± 0.1</td>
<td>59786 ± 1164</td>
</tr>
<tr>
<td>TA + AT-MSCs + HGF</td>
<td>39.3 ± 1.1</td>
<td>3.5 ± 0.2</td>
<td>63460 ± 1470</td>
</tr>
</tbody>
</table>

Rats were administered thioacetamide (TA, 100 mg/kg, i.p, three times weekly for 6 weeks) then only the third group received AT-MSCs treatment for two months. At the end of treatments, sera and plasma were collected. Data are represented as Mean ± S.E.M (n=8). a: significant from negative control, b: Significant from TA-positive control group, c: significant from TA+AT-MSCs at P<0.05. Statistical analysis was carried out by one-way ANOVA followed by LSD comparison test. ALT=alanine transaminase, ALB=albumin, FBG=Fibrinogen

Table 2: Effect of treatment with undifferentiated and differentiated AT-MSCs on hepatic TGF-β1, EGF and HGF levels in liver fibrosis induced rats.

<table>
<thead>
<tr>
<th>Parameter Groups</th>
<th>TGF-β1 (ng/mg protein)</th>
<th>EGF (ng/mg protein)</th>
<th>HGF (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>7.50 ± 0.60</td>
<td>13.00 ± 0.74</td>
<td>12.4 ± 0.4</td>
</tr>
<tr>
<td>Positive control (TA)</td>
<td>35.90 ± 3.20</td>
<td>15.16 ± 1.37</td>
<td>15.5 ± 1.0</td>
</tr>
<tr>
<td>TA + AT-MSCs</td>
<td>28.80 ± 1.70</td>
<td>15.50 ± 0.48</td>
<td>16.9 ± 1.2</td>
</tr>
<tr>
<td>TA + AT-MSCs + HGF</td>
<td>25.60 ± 2.10</td>
<td>15.80 ± 0.62</td>
<td>17.8 ± 0.8</td>
</tr>
</tbody>
</table>

Rats were administered thioacetamide (TA, 100 mg/kg, i.p, three times weekly for 6 weeks) then only the third group received AT-MSCs treatment for two months. At the end of treatments, sera and plasma were collected. Data are represented as Mean ± S.E.M (n=8). a: significant from negative control, b: Significant from TA-positive control group, c: significant from TA+AT-MSCs at P>0.05. Statistical analysis was carried out by one-way ANOVA followed by LSD comparison test. EGF: Epidermal growth factor, HGF: Hepatocyte growth factor, TGF-β1: Transforming growth factor-β1

Biochemical analyses

Serum alanine transaminase (ALT) activity was determined colorimetrically using the kit purchased from Quimica Clinica Aplicada S.A., Spain according to the method described by12. Serum albumin level was estimated colorimetrically using the kit purchased from Stanbio Laboratory Co., USA according to the method of16. Plasma fibrinogen (FBG) level was determined by enzyme-linked immunosorbert assay (ELISA) using the kit purchased from Assaypro, USA according to the method described by12. Hepatic transforming growth factor-β1 (TGF-β1) and hepatocyte growth factor (HGF) levels were determined by ELISA using the kit purchased from Glory Science Co., USA according to the manufacturer’s instructions. While, hepatic epidermal growth factor (EGF) was assayed by ELISA using the kit purchased from WKEA Med Supplies Corp., USA according to the manufacturer’s instructions. Moreover, hepatic tumor necrosis factor α (TNF-α) and interleukin-8 (IL-8) levels were determined by ELISA using the kit purchased from Assaypro, USA according to the methods described by15,16.

Semi-quantitative real time PCR (sqRT-PCR) analysis

The sqRT-PCR was used to detect the expression of the liver associated genes; cytokeratin 7 (CK7), alpha fetoprotein, β-5-tubulin (β-5-Tub), forkhead boxa 2 (FOXa2), hepatic nuclear factor-4α (HNF-4α), albumin and cytochrome P450,7A1 (CYP7A1) that were normalized on the bases of β-actin expression. Total RNA was isolated from liver tissues by the standard TRIzol® reagent extraction method (Invitrogen, USA). Then, the complete Poly(A)® RNA was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An iQ5-BIO-RAD Cycler (Cepheid, USA) was used to determine the rat cDNA copy number. PCR reactions were set up in 25 µl reaction mixtures containing 12.5 µl 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd., Germany), 0.5 µl of 0.2 µM forward primer, 0.5 µl of 0.2 µM reverse primer (the sequences used are listed in the supplementary data), 6.5 µl distilled water, and 5 µl of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided into 3 steps: (a) denaturation at 95.0°C for 15 secs; (b) annealing at 55.0°C for 30 secs and (c) extension at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C for 30 secs.

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95.0°C for melting curve analysis which was performed to check the quality of the used primers.

**Statistical analyses**

All results are expressed as Mean±S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) test. Difference was considered significant when P value was < 0.05.

**RESULTS**

**Characterization of AT-MSCs**

The isolated AT-MSCs were characterized using flow cytometry and were found to be positive for CD44 (80.2%), CD90 (82.7%) and negative for CD45 (0.03%) (Fig. 1).

**Morphological features of AT-MSCs after differentiation into hepatocyte-like cells**

Differentiation of AT-MSCs into hepatocytes under the effect of liver specific factors showed that AT-MSCs appeared with typical hexagonal shape of hepatocytes as compared with the undifferentiated AT-MSCs which lost the hexagonal shape of hepatocytes (Fig. 2).

**RT-PCR analysis of AT-MSCs after differentiation into hepatocyte-like cells**

The specific liver proteins, ALB and AFP were positively expressed confirming differentiation of AT-MSCs into hepatocyte-like cells (Fig. 3).

**Engraftment of undifferentiated and differentiated AT-MSCs**

Fig. (4) shows the engraftment of PKH-26 labelled AT-MSCs into the TA-injured liver tissue with strong red autofluorescence for the differentiated AT-MSCs than undifferentiated one.

**Biochemical markers**

Administration of TA increased serum ALT (+127%) activity associated with a decrease in serum ALB (-25%) and plasma FBG (-39%) levels in comparison with the negative control group (P< 0.05). On the other hand, undifferentiated AT-MSCs decreased ALT activity and increased plasma FBG level as compared to positive control group. Treatment with differentiated AT-MSCs retained the normal activities of serum ALT (-35%) in comparison with the untreated TA-administered group. Moreover, serum ALB and plasma FBG levels were normalized (Table 1). Thioacetamide administration elicits increase (p< 0.05) in hepatic TGF-β1 level (more than 450 folds) in comparison with the negative control group. However, treatment of TA administered rats with undifferentiated and differentiated AT-MSCs partially reduced the level of hepatic TGF-β1 (-19.7% and -28.7%) versus the untreated TA administered group (p<0.05) that was still significant from control level (>200 folds). Furthermore, in comparison with the untreated TA administered group, treatment with differentiated AT-MSCs enhanced hepatic EGF and HGF levels but the undifferentiated cells did not affect their levels (p<0.05) (Table 2). Moreover, hepatic TNF-α and IL-8 levels were elevated in TA group (+125% and 65%, respectively, p< 0.05) in comparison with the negative control animals. Treatment with undifferentiated and differentiated AT-MSCs restored the normal level of hepatic TNF-α. Differentiated AT-MSCs showed normal hepatic IL-8 level, while the undifferentiated ones did not affect its level as compared to the untreated TA group (Table 3). Rats were administered thioacetamide (TA, 100 mg/kg, i.p. three times weekly for 6 weeks) then only the third group received AT-MSCs treatment for two months. At the end of treatments, sera and plasma were collected. Data are represented as Mean ± S.E.M (n=8). Significant from negative control or TA-positive control groups, respectively at P<0.05. Statistical analysis was carried out by one-way ANOVA followed by LSD comparison test.

**IL-8: Interleukin-8, TNF-α: Tumor necrosis factor-α.**

**Expression of hepatic specific genes**

Figure 5: Hepatic CK7, AFP, β-5-Tub, FOXa2, HNF-4a, ALB and CYP7A1-mRNA expression levels in liver fibrosis induced rats treated with undifferentiated and differentiated AT-MSCs. Liver samples were taken two months after AT-MSCs transplantation into TA-treated group. Specified genes were determined by sqRT-PCR. Data are mean S.E.M (n=10), a: Significant change at P>0.05 in comparison with control group and b: Significant change at P>0.05 in comparison with the TA group and c: significant change at P>0.05 in comparison with TA+AT-MSCs group. Statistical analysis was carried out by one-way ANOVA followed by LSD comparison test.
The mRNA expression of liver ALB, HNF-4, FOXa2, and CYP7a1 genes was prohibited in fibrotic liver of TA-treated rats compared to negative control rats (by 53.5, 63, 64, 60% respectively). Hepatocyte transplantation resulted in a markedly improved expression of ALB (57%), HNF-4 (61%), FOXa2 (72%), and CYP7a1 (35.6%) genes in comparison to TA-injected rats (Fig. 5). On the other hand, TA-group showed increase in CK7 (279%), β-5-tub (330%) and AFP (245%) genes expression in comparison with the negative control group. Treatment with differentiated AT-MSCs caused down expression of AFP (18%), CK7 (27.6%) and β-5-tub (30%) genes compared to untreated group (Fig. 5).

**DISCUSSION**

Chronic liver injuries including toxin-induced, alcoholic disorder or viral hepatitis lead to hepatic fibrosis which is one of the major death causes17. Liver loses its regenerative capacity18. Unfortunately, the only effective treatment, liver transplantation, is limited by lack of donors and immune rejection. Alternatively, cell therapy strategies are being developed19. The present study showed the competency of AT-MSCs to differentiate into effective hepatocytes that succeeded in restoring the normal liver functions in 60 days after transplantation as assessed biochemically and genetically.

Liver fibrotic changes which were known to be induced by the administration of TA20,21 are represented in the current study by the elevation in the serum level of ALT, TGF-β1, EGF, HGF, TNF-α and IL-8 as well as the depletion of serum ALB and plasma FBG levels. These changes could be attributed to the ability of TA to increase the generation of reactive oxygen species, which induce hepatic damage and fibrosis, stimulate the innate immune system, and exacerbate liver inflammation5. Additionally,22 reported that TA administration induces a sequence of events with a defined up-regulation of gene expression of inflammatory chemokines and cytokines. AT-MSCs were proven to differentiate in vitro into hepatocyte-like cells and regenerate the liver tissue of CCl4-treated animals when transplanted in undifferentiated form6,23,24. Since hepatocyte-differentiated AT-MSCs produce better engraftment than undifferentiated ones25, the present study used enriched culture medium with HGF. Hepatocytes were successfully differentiated and engrafted into liver tissue of TA-injured rats as obvious in PKH26 stained specimens. Transplantation of either undifferentiated or differentiated AT-MSCs succeeded in alleviating the fibrotic changes efficiently. As our results revealed that treatment with differentiated AT-MSCs lessened ALT, TGF-β1, TNF-α, while elevated ALB and FBG levels. Moreover, transplanted hepatocytes restored the normal functions of liver tissue as indicated from the liver-injury markers; ALT, ALB, and FBG and induced proliferation as shown from the elevated growth factors HGF and EGF. These findings are consistent with the previous reports, where MSCs derived from bone marrow26 or AT27 enhanced the liver functioning. Furthermore, the mRNA expression of liver ALB gene was restored after the treatment representing a marker for the normal hepatic function. Together, these results demonstrate the functionality and the superiority of the engrafted hepatocytes. Bioactive cytokines, such as HGF, vascular endothelial growth factor, and FGF-4 are recognized for their ability to promote regeneration of tissues. Since hepatic HGF level was elevated in the current study, the observed enhancement of liver functions after differentiated AT-MSCs transplantation can be justified27. Indeed, HGF was reported to accelerate recovery of liver function and promote regeneration of damaged liver tissue through an anti-apoptotic effect by increasing hepatocyte proliferation, where it mediates the mesenchymal-epithelial transition factor (c-met) signaling pathway28. In addition, it stimulates the expression and activity of proteases that are involved in the breakdown of ECM proteins29,30. Moreover31 stated that MSCs modulate the immune system and attenuate tissue damage caused by excessive inflammation. One of the central mediators in the maintenance of liver architecture and function is HNF4α, which regulates not only the hepatocyte proliferation and differentiation but also the hepatic epithelial morphology32. Our observation revealed that the expression of HNF4α and CYP7a1 genes was down-regulated in liver tissue of TA-administered rats. In accordance, liver carcinogenesis, cirrhotic tissue was associated with decreased HNF4α expression32. However, transplantation of hepatocytes raised the expression level of HNF4α and CYP7a1 genes. The up-regulation of HNF4α is ideal for treatment of hepatic fibrosis, where HNF4α enhances the expression of CYP7A that contains binding site for HNF4α in its promoter region33. Furthermore, the presence of TGF-β impairs the efficiency of HNF4α34 explaining the TA-induced liver fibrosis and corroborating the role of AT-MSCs in regenerating liver function through balancing these critical hepatic factors. The present study revealed that the expression of FOXa2 gene was down-regulated by TA and up-regulated after treatment with AT-derived hepatic differentiated cells. FOXa2 is a liver transcription factor that regulates the development of liver organogenesis, as well as the expression of liver-specific genes, such as ALB35. In addition, FOXa2 expression is required for liver metabolic reactions37, which makes it a detrimental gene in treatment of liver-related diseases.

In the current study, the expression levels of CK7, β-5-Tub and AFP were elevated in TA-injured liver tissues. AFP is the characteristic hepatocyte marker for liver lineage process38. The observed increase in the mRNA expression of liver AFP gene can be ascribed to hepatocytes proliferation following the hepatic TA-induced injury39. This gene was not down-regulated in differentiated AT-MSCs -treated rats indicating the process of proliferation. CK7 gene can be an enhancer for AFP gene in cases of liver fibrosis. CK7 represent a regenerative component of hepatocytes contributing to liver regeneration40. It is found in tissue only in fibrotic states that is a valuable tool to detect liver disease progression41,42 reported that CK7 is expressed by differentiated hepatocytes from MSCs. The observed down-regulation of the mRNA expression level of these early markers could be related to the time at which...
the experiment of the current work ended as the scarification done after 2 months from MSCs transplantation. So, the differentiated hepatocytes like cells from MSCs could be in mature stage as evidenced in the current work by the expression of ALB and CYP7a1 genes. The observed increase in liver β-5-tub mRNA expression due to TA administration might be attributed to the increase in the proliferative activity of mature hepatocytes as a response to toxin-induced liver injury. β-5-Tub overexpression inhibits microtubule assembly, leaving cells with a very sparse and fragmented microtubule network. On the other hand, it has been reported that disruption of these microtubular networks is associated with activation of TGF-β-like signals. Thus, the up-regulation of the mRNA expression level of β-5-tub gene observed in the current investigation could be related to the increase in TGF-β level in liver fibrosis. Meanwhile, the down-regulation of the mRNA expression level of β-5-tub gene could be attributed to the ability of MSCs to diminish TGF-β as shown in the current work. TGF-β1 released from hepatic stellate cell (HSC) activates Smad3 in cases of liver injury and fibrogenesis, which stimulates Cyp7A1 gene expression and bile acid synthesis. TNF-α which is released from Kupffer cells activates mitogen-activated protein kinases/c-Jun N-terminal kinases signaling to attenuate TGF-β1 stimulation of Cyp7A1 gene expression in rats. The altered cell signaling play critical roles in liver injury, fibrogenesis, and regeneration. Moreover, TGF-β1 positive regulation of Cyp7a1 expression activates farnesoid X receptor (FXR) to stimulate liver regeneration. FXR inhibits Cyp7A1 expression in the liver. It was suggested that bile acids activate intestinal FXR to induce fibroblast growth factor 15, which is transported to the liver to inhibit Cyp7A1 expression. In summary, the data of the current study provided an experimental evidence for the ability of AT-MSCs-derived differentiated hepatocytes to alleviate liver fibrosis than undifferentiated one. This observation could be explained by the ability of differentiated AT-MSCs to home into the injured liver tissues more than the undifferentiated AT-MSCs and the effectiveness of these cells to express and secrete liver specific proteins, factors, and cytokines enhancing the regeneration of tissue. The present work suggest to further proceed in investigating other culture media and conditions to make it feasible for application in bioreactors. Proceeding with this approach will benefit a vast majority of end-stage liver disease patients, where remove the burden of waiting list for liver transplantation.

ACKNOWLEDGMENTS

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Abbreviations

Adipose tissue derived mesenchymal stem cells (AT-MSCs), Alanine transaminase (ALT), Albumin (ALB), Alpha fetoprotein (AFP), Cytochrome P450,7A1 (CYP7A1), Cytokeratin 7 (CK7), Epidermal growth factor (EGF), Fibrinogen (FBG), Fibroblast growth factor-4, Forkhead box 2 (FOXa2), Hepatic nuclear factor (HNF)-4α, Hepatocyte growth factor (HGF), Interleukin-8 (IL-8), Thioacetamide (TA), Transforming growth factor-β1 (TGF-β1), Tumor necrosis factor-α (TNF-α) and β-5-tubulin (β-5-Tub).

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