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Original article

Comparative study on the effect of low intensity laser and growth factors on stem cells used in experimentally-induced liver fibrosis in mice

Eman Naguib [a,*], Ashraf Kamel [b], Osama Fekry [c], Gamal Abdelfattah [a]

[a] Department of Laser Sciences and Interactions, National Institute of Laser and Enhanced Sciences, Cairo University, Cairo, Egypt
[b] Department of Histology, Faculty of Medicine, Cairo University, Cairo, Egypt
[c] Department of Medical Laser Applications, National Institute of Laser and Enhanced Sciences, Cairo University, Cairo, Egypt

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ABSTRACT

Background and study aims: The therapeutic effects of human umbilical cord-derived mesenchymal stem cells (UC-MSCs) exposed to diode laser and/or hepatocyte growth factor (HGF) were compared in mice with experimental liver fibrosis induced by carbon tetra chloride (CCl_4).

Material and methods: Animal model of liver cirrhosis was induced by intraperitoneal injection of CCl_4 in a dose of 0.4 ml/kg, twice a week for 6 weeks. UC-MSCs were obtained from normal full term placentas and were exposed to diode laser and/or HGF. Before treatment, UC-MSCs were labelled with red fluorescent PKH26. Fifty four male mice weighing 25–35 g were randomly divided into four groups control, stem cells, CCl_4, and treated groups. After the experimental period, body and liver weights were recorded, and the liver specimens were processed for histological examination using haematoxylin and eosin, Periodic Acid-Schiff (PAS), and Masson's Trichrome staining (MT).

Results: Results showed that administration of UC-MSCs stimulated by diode laser and/or HGF improved body and liver weights, reduced vascular dilatation and congestion, reduced mononuclear cellular infiltration, reduced hepatocyte vacuolation, eosinophilia, and pyknosis. Furthermore, periportal fibrosis was minimized and PAS reaction was increased. These effects were maximum when UC-MSCs were exposed to both diode laser and HGF.

Conclusion: UC-MSCs stimulated by both diode laser and HGF proved to be an effective therapeutic option in experimental liver fibrosis induced by CCl_4 in mice.

Introduction

Liver diseases constitute a major health problem in many parts of the world [1]. The endpoint of the progressive damage to the liver is loss of 80–90% of hepatic functional capacity with 70–95% mortality from hepatic failure [2]. Fibrogenesis is characterized by excessive accumulation of extracellular matrix (ECM) components. The net accumulation of the ECM connective tissue results from enhanced synthesis, or diminished breakdown of the matrix, or both. Collagens, predominately types I and III, are the major fibrous proteins in ECM and their synthesis increases in the liver exposed to carbon tetrachloride (CCl_4) [3].

While transplantation is currently an accepted therapy for liver disease, there remain many challenges as shortage of donors and the harshness of the invasive procedures [4,5]. The use of stem cells for future tissue engineering and regenerative medicine to replace conventional therapeutic methods has been the subject of growing interest in different areas. These cells have self-renewing properties and are able to differentiate into one or many different specialized cell types [6]. Stem cell research has expanded well due to their usefulness in regenerative therapies for improving the life of patients suffering from various genetical and neurological diseases [7].

Mesenchymal stem cells (MSCs) can be found in various locations in the body, including the bone marrow [8], adipose tissue [9], and peripheral blood [10]. MSCs can differentiate into several types of mature cells, including neurons, adipocytes, cartilage, skeletal muscle, hepatocytes, and cardiomyocytes, under appropriate conditions [9,11].

Umbilical cord derived mesenchymal stem cells (UC-MSCs) isolated from human placenta can differentiate into hepatocytes in the normal liver and in some pathologic environments. Stem cells are undifferentiated cells that divide to replenish dying cells and regenerate damaged tissues. [12]. Bone marrow derived mesenchymal stem cells (BM-MSCs) have the capacity to differentiate into hepatocyte-like cells, as well as their ability to reduce fibrogenesis [13–15].

* Corresponding author.
E-mail address: emansherief1@hotmail.com (E. Naguib).

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UC-MSCs ameliorated liver fibrosis in rats with carbon tetrachloride (CCl_4)-induced cirrhosis. However, these results showed some variation across different animal models, CCl_4 treatment protocols, and in vitro conditions [14,15].

Although BM-MSCs represent an attractive therapeutic alternative for treating degenerative diseases, their use is limited by several factors, including low cell yields from donor BM, dependence on donor age, limitations to autologous use, and difficulty in recruiting donors [13,16–18].

UC-MSCs, which have received much research attention, exhibit characteristics similar to those of BM-MSCs, but they bear several advantages [19]. UC-MSCs display multi-lineage differentiation potential, and they are free of ethical concerns, easily accessible, abundant, and strongly immunosuppressive [17,20,21]. Minimal criteria for defining UC-MSCs are (i) foetal origin; (ii) generation of fibroblast colony-forming units; (iii) specific patterns of surface antigen expression; and (iv) potential to differentiate into one or more lineages [22]. UC-MSCs have the potential to differentiate in vitro into hepatocyte-like cells and insulin-positive cells, as well as mesodermal lineages [22–24].

There is increasing evidence that the age of the donor tissue affects several properties of mesenchymal stem cells and this fact cause decreased repair capacity and increased susceptibility to degenerative diseases [18,25].Wharton's jelly derived MSC, in their short, prenatal life do not have proaging factors.

In comparison to MSC from adult tissues, UC-MSCs at such an early embryonic state retain telomere at highest possible length, which protects them from premature loss of viability [26].

Laser phototherapy has been used to treat pathological tissue conditions, to control inflammatory processes and also to promote tissue healing. The mechanism by which low intensity lasers induce biomodulation of cell activity has been well described by Karu [27]. Laser irradiation is postulated to intensify the formation of a transmembrane electromechanical proton gradient in mitochondria. Thus, the efficiency of the proton-motive force (pmf) is increased and more calcium is released into the cytoplasm from the mitochondria. At low laser doses, this additional calcium transported into the cytoplasm triggers mitosis and enhances cell proliferation. [6].

Hepatocyte growth factor (HGF) is a polypeptide that affect a number of cellular processes such as proliferation and differentiation both *in vivo* and in vitro [2]. It has potent cytoprotective action on hepatocytes, besides enhancing liver regeneration. HGF also stimulates the migration and proliferation of activated hepatic stem cells into the liver parenchyma, where the cells differentiate into mature hepatocytes [29].

The aim of the present study was to compare the therapeutic effects of human umbilical cord derived mesenchymal stem cells (UC-MSCs) exposed to diode laser and/or HGF in mice with experimental liver fibrosis induced by carbon tetra chloride (CCl_4).

Material and methods

Drugs and chemicals

Carbon tetrachloride (CCl_4): CCl_4 and PKH26 Red Fluorescent Cell Linker kit were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hepatocyte growth factor was purchased from Koma Biotech, Seoul, Korea.

Animals

Animals: fifty-four male mice weighing 25–35 g, 2–3 months old, were obtained from the Holding Company for Biological Products & Vaccines (VACSERA), Cairo, Egypt. The animals were divided into 8 groups each with 6 animals. They were bred and maintained on standard laboratory rodent diet. The animals were maintained at 12-h light/dark cycles at constant temperature (20 ± 1 °C) and humidity (50 ± 5%). The study complied with the Guide for the Care and Use of Laboratory Animals [30].

Experimental design

Animals were randomly divided and treated in the following way:

Group I – Control Group: subdivided into:

Subgroup I-a: Negative control group: six mice; received no treatment.
Subgroup I-b: Vehicle group: six mice; each received intraperitoneal (i.p.) injection of olive oil twice a week for 6 weeks, at the same volume as CCl_4-treated mice.

Group II – Stem cells group: subdivided into:

Subgroup II-a: 6 mice; each received UC-MSCs treatment without exposure neither to diode laser nor HGF
Subgroup II-b: 6 mice; each received UC-MSCs irradiated with diode laser.
Subgroup II-c: 6 mice; each received UC-MSCs exposed to HGF.

Group III – CCl_4-treated group: 6 mice; each received i.p. CCl_4.
Group IV – Experimental group: subdivided into:

Subgroup IV-a: 6 mice; each received CCl_4 for 6 weeks followed by UC-MSCs irradiated with diode laser.
Subgroup IV-b: 6 mice; each received CCl_4 for 6 weeks followed by UC-MSCs exposed to HGF.
Subgroup IV-c: 6 mice; each received CCl_4 for 6 weeks followed by UC-MSCs exposed to both diode laser irradiation and HGF.

Induction of liver fibrosis

Mice of groups III and IV were given CCl_4 intraperitoneally (i.p.) at a dose of 0.4 ml/kg, dissolved in olive oil, twice a week for 6 weeks [31]. In group IV, UC-MSCs were injected i.v. after the end CCl_4 treatment.

Isolation and cultivation of UC-MSCs from Wharton jelly

The collection of placenta samples was done in Cairo University Hospitals and their subsequent utilization for research purposes was approved by the Institutional Review Board of Faculty of Medicine, Cairo University. All participating women provided written, informed consent prior to the collection of samples. Placentas were collected from women who were free of medical, obstetrical, and surgical complications and who delivered at term (37 gestational weeks). Briefly, the umbilical cords (UCs) were collected in a transfer medium of phosphate-buffered saline (PBS) and 50 IU heparin, and were maintained at 4 °C until processing, which was within 24 h of collection. UCs were washed three times in PBS, and the umbilical veins were rinsed with PBS to remove contaminating red blood cells. UCs were cut into 1 cm segments, and UC arteries, veins and amnion were removed. The gelatinous tissue was excised, and minced into 0.5–1 mm^3 pieces. Equal volumes of PBS were used to swell the tissue pieces with constant shaking at 4 °C for 48 h, and the tissue pieces were subjected to 5 freeze–thaw cycles (−80–37 °C). Samples were ruptured by high-speed dispersion on ice for 10 min, and homogenized with a glass homogenizer on ice. Tissue homogenates were centrifuged at 10,000*g* for 30 min

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to remove debris. Supernatants that were Wharton's jelly extract (WJE) were collected and protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). WJE was diluted to 1% (w/v) with PBS and used to coat plastic culture plates at 4 °C overnight, after which excess liquid was discarded. WJE-coated plates were washed two times with PBS before use [32,33].

Laser irradiation of UC-MSCs

Laser irradiation was delivered with a diode laser as a light source. UC-MSCs were radiated with 650 nm diode laser with power 200 mW. The laser beam expanded to 20.72 cm^2 area by a concave lens. In order to cover all the specimen area, this allows the laser to be applied on the whole specimen once at a time and to be sure that all cells receive the same dose of laser. This also decrease the risk of contamination. We have taken in consideration the absorption due to thickness of the flask wall containing media. For this reason the actual energy density is 0.7763 J/cm^2 in 90 s time exposure. Where the absorption coefficient of the flask material is measured and found to be 0.9453 cm^{-1}.

A plane mirror at 45° angle was used to change the direction of the beam by 90° angle to ease the handling of the specimen. Cells were irradiated on a clean bench at room temperature.

UC-MSCs irradiated with laser were injected i.v. to the mice of groups II-b, IV-a and IV-c in the dose of 1×10^4 cells/cm^2 [34,35].

Application of hepatocyte growth factor (HGF) to UC-MSCs

Passage-five UC-MSCs were plated at a density of 1×10^4 cells/cm^2 in basal medium (60% DMEM-LG, 40% MCDB201, 2% FBS, and 1% P/S) containing 10 ng/ml HGF. Cells were grown to 60% confluence and then incubated for 7 days in basal medium supplemented with 2% FBS, 10 ng/ml HGF [15,28]. These cells were injected i.v. to the mice of groups II-c, IV-b and IV-c in the dose of 1×10^4 cells/cm^2.

Fluorescent labelling of UC-MSCs

Before injection in mice, UC-MSCs were labeled using PKH26 Red Fluorescent Cell Linker kit according to the manufacturer's instructions [36]. After that, the PKH26-labeled UC-MSCs were injected intravenously into the tail vein of mice of groups II and IV as a single dose of 1 x 10^4 cells/cm^2 with or without exposure to laser and/or HGF. Liver tissue was examined by fluorescent microscope to detect the cells stained with PKH26 to confirm homing of UC-MSCs.

Body weight (BW) analysis

The BWs of all mice were recorded at the beginning of the experiment, and just before sacrifice. The percentage of BW gain was calculated according to the method of Chapman et al. [37] using the following formula:

$$\frac{\text{Final BW} - \text{initial BW}}{\text{Initial BW}} \times 100$$

Histopathological analysis

Mice were sacrificed by decapitation under light isoflurane anesthesia two weeks after the injection of UC-MSCs.

The abdomen of animal was cut open by a middle line incision and the liver was perfused with isotonic saline, excised, blotted dry, weighed, and divided into small samples. Liver specimens were fixed in 4% phosphate buffered formalin, embedded in paraffin, and cut to sections of 5 μm thickness.

Fluorescence detection (PKH26-labeled MSCs) by fluorescent microscope in unstained paraffin sections to confirm homing of UC-MSCs.

Paraffin embedded tissues were subjected to the following staining techniques:

1. Haematoxylin and Eosin (H & E) staining [38].
2. Masson's Trichrome staining (MT) to examine liver collagen deposition [39].
3. Periodic Acid-Schiff (PAS) to assess glycogen storage [40].

Calculation of liver index:

The liver index was calculated according to the formula [41]:

$$\frac{\text{Mouse' liver weight}}{\text{Mouse' final BW}} \times 100$$

Quantitative morphometric study

Images were processed and analysed using computer-based image analysis software (Leica QWin 500; Imaging Systems, Cambridge UK) to measure:

1. Area percent of fibrous tissue as detected by MT stain
2. Mean grey (optical density) of PAS stained liver sections

Statistical analysis

Data collected (body weights, mean grey of PAS sections and area percent of fibrous tissue in MT stained sections) were subjected to statistical analysis using Statistica 5 software (StatSoft, Inc.) where analysis of variance (ANOVA) was used for an overall comparison between the study groups and the post hoc Tukey honest significant difference (HSD) test was used for pairwise comparisons. Differences were considered significant when probability (p) was equal to or less than 0.05. Data was tabulated and represented graphically [42].

Results

It was found that the body and liver weights, as well as the histological and morphological results of the stem cells group (subgroups II-a, II-b and II-c) and the vehicle group (subgroup I-b) were indistinguishable from those of the negative control group (subgroup 1-a) and thus they were all grouped together and will be collectively referred to as the control group.

Body weights

CCl_4 administration significantly decreased ($P < 0.05$) the percentage of gain in BW as compared to the control group. Administration of stem cells (in subgroups IV-a, IV-b and IV-c) with CCl_4 significantly increased the percentage of gain in body weight ($P < 0.05$) as compared to the CCl_4-treated group. Comparison of the percentages of gain in BW of subgroups IV-a, IV-b and IV-c showed insignificant variations. (Table 1).

Liver index

CCl_4 administration significantly decreased ($P < 0.05$) the liver index as compared to the control group. Administration of stem cells (in subgroups IV-a, IV-b and IV-c) with CCl_4 significantly increased the liver index ($P < 0.05$) as compared to the CCl_4-treated group. Comparison of the liver index values of subgroups IV-a, IV-b and IV-c showed insignificant variations. (Table 2).

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Table 1
Mean values ± SD of initial and final body weights of mice in the different groups and the percentage of gain in body weights.

Group	Mouse BW		BW Gain% (mean ± SD)
	Initial BW (g)(mean ± SD)	Final BW (g) (mean ± SD)	
Control Group	29.40 ± 2.82	41.31 ± 2.52	41.20 ± 11.64
Group III	30.66 ± 2.73	36.06 ± 2.42	17.97 ± 7.28●
Group IV-a	30.09 ± 3.08	41.00 ± 3.43	36.52 ± 4.29*
Group IV-b	29.54 ± 3.37	39.73 ± 2.86	35.06 ± 7.06*
Group IV-c	29.50 ± 2.74	40.48 ± 2.38	37.65 ± 7.12*

BW = Body weight, SD = standard deviation.
● Significant as compared to control group ($p < 0.05$).
* Significant as compared to CCl_4-treated group (Group III) ($p < 0.05$).

Table 2
Mean values ± SD of liver weights and Liver indices of mice in the different groups.

Group	Liver weight (g) (mean ± SD)	Liver Index (g) (mean ± SD)
Control Group	3.70 ± 0.39	8.96 ± 0.71
Group III	2.17 ± 0.28	6.03 ± 0.91●
Group IV-a	3.60 ± 0.21	8.84 ± 1.05*
Group IV-b	3.34 ± 0.28	8.43 ± 0.88*
Group IV-c	3.63 ± 0.47	8.94 ± 0.77*

BW = Body weight, SD = standard deviation.
● Significant as compared to control group ($P < 0.05$).
* Significant as compared to CCl_4-treated group (Group III) ($P < 0.05$).

Histological results

Light microscopic examination of liver sections from the control group showed the characteristic architecture of the classic hepatic lobules with hepatocytes radiating outwards from the central vein towards the periphery of the lobules that enclose the portal areas (Fig. 1a). The hepatocytes displayed an eosinophilic granular cytoplasm with vesicular nuclei and several binucleated cells were spotted. Narrow blood sinusoids were seen between the cords of hepatocytes and displayed Von Kupffer cells in their walls (Fig. 1b).

PAS reaction was strongly positive in the hepatocytes (Fig. 1c), whereas MT staining revealed minimal traces of collagen (Fig. 1d).

Concerning CCl_4-treated (group III); liver sections presented congestion of the central veins and blood vessels of the portal areas (Fig. 2a and b). Mild dilatation and congestion of the blood sinusoids was noticed (Fig. 2b). Extensive mononuclear cellular infiltration in the portal areas was frequently seen (Fig. 2c and d). Many hepatocytes displayed vacuolations in their cytoplasm (Fig. 2e–g); others exhibited pyknotic nuclei, while some other hepatocytes were anucleated; showing cell outlines only without nuclei (Fig. 2f). Moreover, some hepatocytes showed ballooning (Fig. 2e–g), whereas others exhibited increased eosinophilia and hyalinization (Fig. 2e and g).

Weak PAS reaction was noticed (Fig. 2i), and extensive fibrosis especially in periportal regions was seen in the MT stained sections (Fig. 2j).

Liver sections from mice of subgroup IV-an in which liver fibrosis was induced by CCl_4 and then received Laser-stimulated stem cells showed reduced pathology compared to group III in the form of dilatation of central veins and periportal vessels, mild congestion and moderate mononuclear cellular infiltration (Fig. 3a and b). Hepatocytes exhibited moderate vacuolation and apoptosis (Fig. 3c), PAS reaction was only moderately reduced (Fig. 3d), while MT stained sections showed moderate fibrosis in some periportal areas (Fig. 3e).

Liver sections from mice of subgroup IV-b in which liver fibrosis was induced by CCl_4 and then received HGF-stimulated stem cells showed even more reduced pathology compared to group III in the form of mild dilatation of central veins (Fig. 4a), slight congestion, mild mononuclear cellular infiltration and mild vascular congestion (Fig. 4b and c). Furthermore, hepatocytes displayed less vacuolation and less apoptosis (Fig. 4c). PAS reaction was mildly reduced

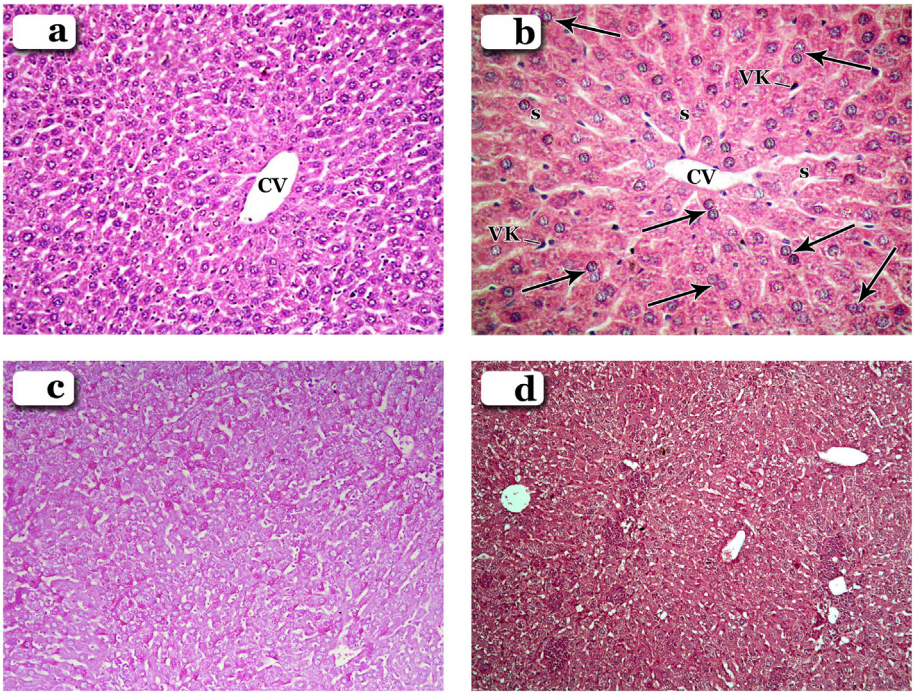

Fig. 1. Photomicrographs of sections in the mice livers of the control group showing (a) characteristic architecture of the classic hepatic lobules with hepatocytes radiating outwards from the central vein (CV) towards the periphery of the lobules that enclose the portal areas [H & E, x200]. (b) hepatocytes radiating from the central vein (CV) and displaying an eosinophilic granular cytoplasm, vesicular nuclei and several binucleated cells (arrows). Narrow blood sinusoids (s) are seen between the cords of hepatocytes with Von Kupffer cells (VK) in their walls [H & E, x400]. (c) A strongly positive PAS reaction in the hepatocytes [PAS, x100]. (d) Minimal traces of collagen [MT, x100].

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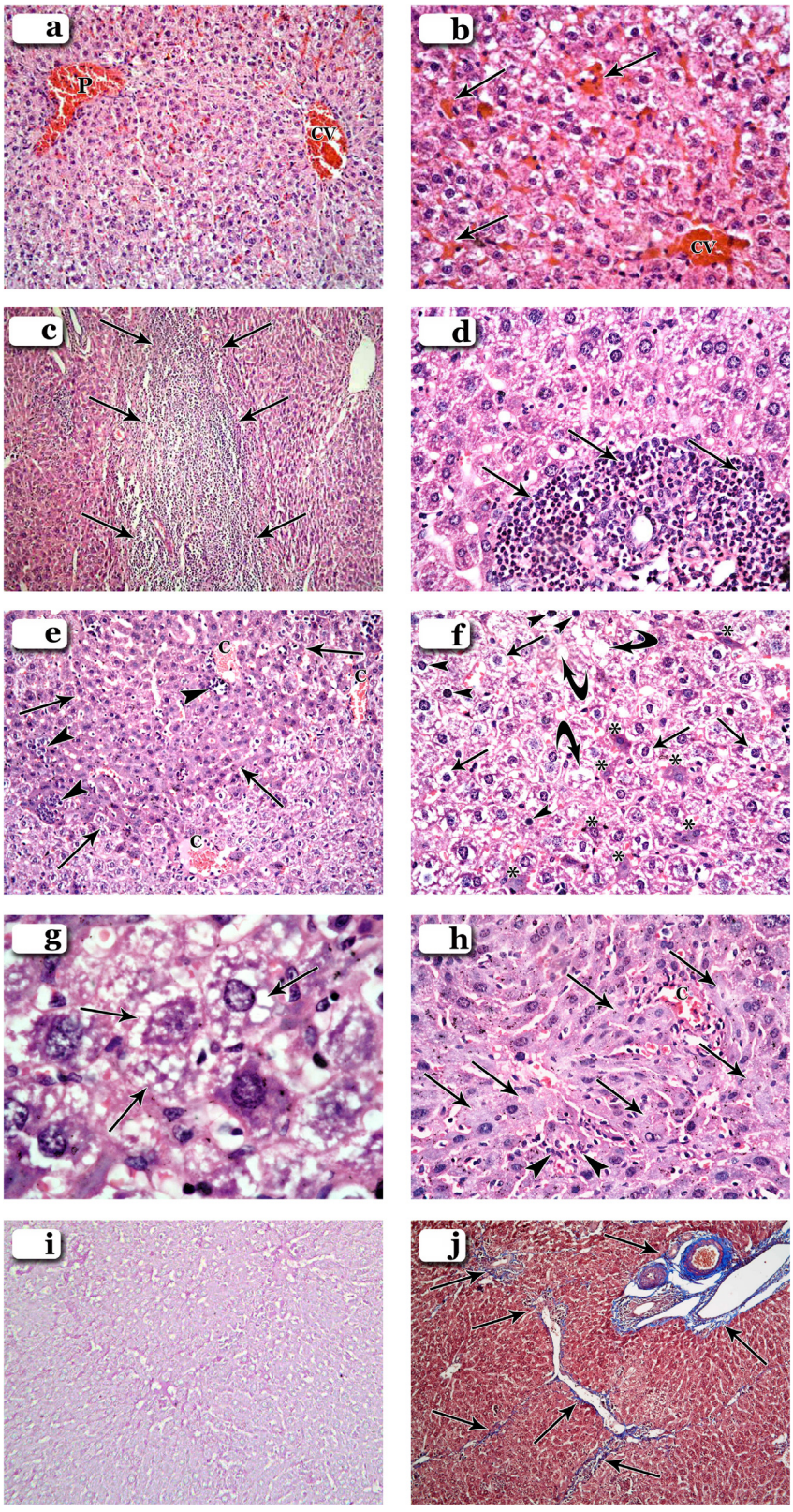

Fig. 2. Photomicrographs of sections in the livers of a CCl_4-treated mice in group III showing (a) congestion of the central vein (CV) and blood vessels of the portal areas (P) [H & E, x200]. (b) Congestion of the central vein (CV) with mild dilatation and congestion of the blood sinusoids (arrows) [H & E, x400]. (c) Extensive mononuclear cellular infiltration in the portal areas (arrows) [H & E, x200]. (d) Mononuclear cellular infiltration in the portal area (arrows) [H & E, x400]. (e) Increased eosinophilia of hepatocytes (arrows), mononuclear cellular infiltration (arrowheads) and vascular congestion (C) [H & E, x200]. (f) hepatocytes show cytoplasmic vacuolations (arrows), pyknotic nuclei (arrowheads), and anuclear cells (curved arrows) exhibiting cell outlines only without nuclei. Some hepatocytes show increased eosinophilia and hyalinization (*) [H & E, x400]. (g) hepatocytes showing cytoplasmic vacuolations (arrows) and increased eosinophilia (*) [H & E, x1000]. (h) Increased eosinophilia and hyalinization of hepatocytes (arrows), mononuclear cellular infiltration (arrowheads), and vascular congestion (C) [H & E, x400]. (i) A weak PAS reaction in the hepatocytes [PAS, x100]. (j) Extensive fibrosis (arrows) especially in the periportal regions [MT, x100].

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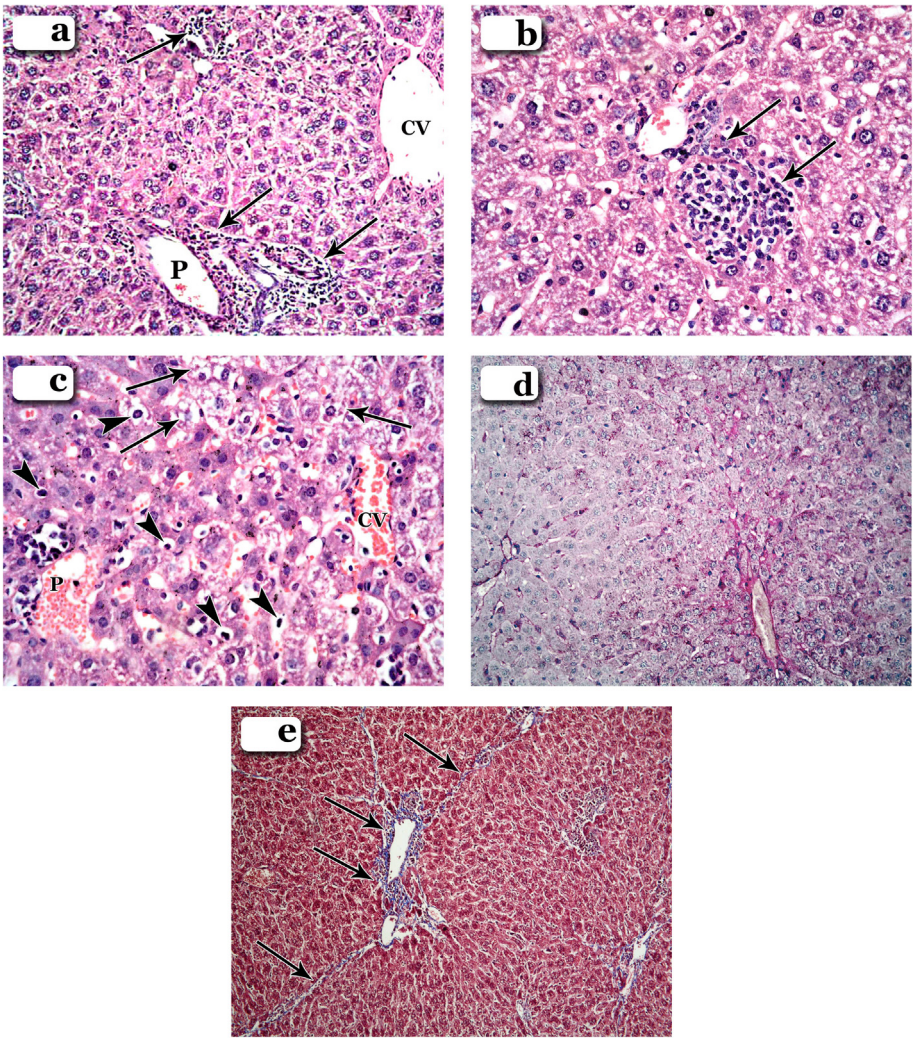

Fig. 3. Photomicrographs of sections in the liver of mice in which liver fibrosis was induced by CCl_4 and then received Laser-stimulated stem cells (subgroup IV-a) showing (a) Dilatation of central vein (CV) and periportal vessel (P), and moderate mononuclear cellular infiltration (arrows) [H & E, x200]. (b) Moderate periportal mononuclear cellular infiltration (arrows) [H & E, x400]. (c) Congestion of the central vein (CV) and a blood vessel in the portal area (P). Some hepatocytes exhibit moderate vacuolation (arrows) and apoptotic nuclei (arrowheads) [H & E, x400]. (d) Moderately reduced PAS reaction [PAS, x100]. (e) Moderate fibrosis in the periportal areas (arrows) [MT, x100].

(Fig. 4d), while MT stained sections showed mild fibrosis in few areas (Fig. 4e).

Liver sections from mice of subgroup IV-c in which liver fibrosis was induced by CCl_4 and then received stem cells stimulated by both Laser and HGF showed very much reduced pathology compared to group III with mild dilatation of central veins and portal vessels, mild mononuclear cellular infiltration, and mild vascular congestion (Fig. 5a–c). PAS reaction was slightly reduced (Fig. 5d), while MT stained sections showed minimal fibrosis (Fig. 5e).

Confirmation of homing of PKH26-labelled UC-MSCs using fluorescence microscope

Homing of UC-MSCs into liver tissue of mice was confirmed by detecting the labelled UC-MSCs with the PKH26 dye. These cells showed red fluorescence, distributed uniformly in the livers of the different groups confirming that these cells were actually seeded into the liver tissue (Fig. 6).

Morphomertic and statistical results

The mean optical densities (mean grey) of the PAS reaction: The mean optical densities (mean grey) of the PAS reaction in control sections was 0.59 ± 0.03. It was significantly reduced in group III to 0.21 ± 0.04. In group IV sub divisions, compared to group III, the mean optical densities (mean grey) of the PAS reaction significantly increased to 0.35 ± 0.04 in subgroup IV-a, 0.42 ± 0.07 in subgroup IV-b, and 0.55 ± 0.02 in subgroup IV-c (Table 3).

The mean area percent of MT staining: The mean area percent of MT staining in control sections was 1.45 ± 0.41. It significantly increased in group III to 31.47 ± 7.07. In group IV sub divisions, compared to group III, the mean area percent of MT staining was significantly reduced to 17.19 ± 4.88 in subgroup IV-a, 11.88 ± 5.92 in subgroup IV-b, and 8.54 ± 2.54 in subgroup IV-c (Table 4).

Discussion

Liver fibrosis is a common chronic hepatic disease. It is caused by a variety of factors, such as viral infections, alcohol, drugs and chemical toxicity [43]. It is characterized by inflammation and increased deposition of extracellular matrix (ECM) with fibrillar collagens [3].

To investigate the effects of UC-MSCs on liver fibrosis, CCL_4 was used in this study to induce liver fibrosis because it was established that CCL_4 was the best hepatotoxin to produce the nearest

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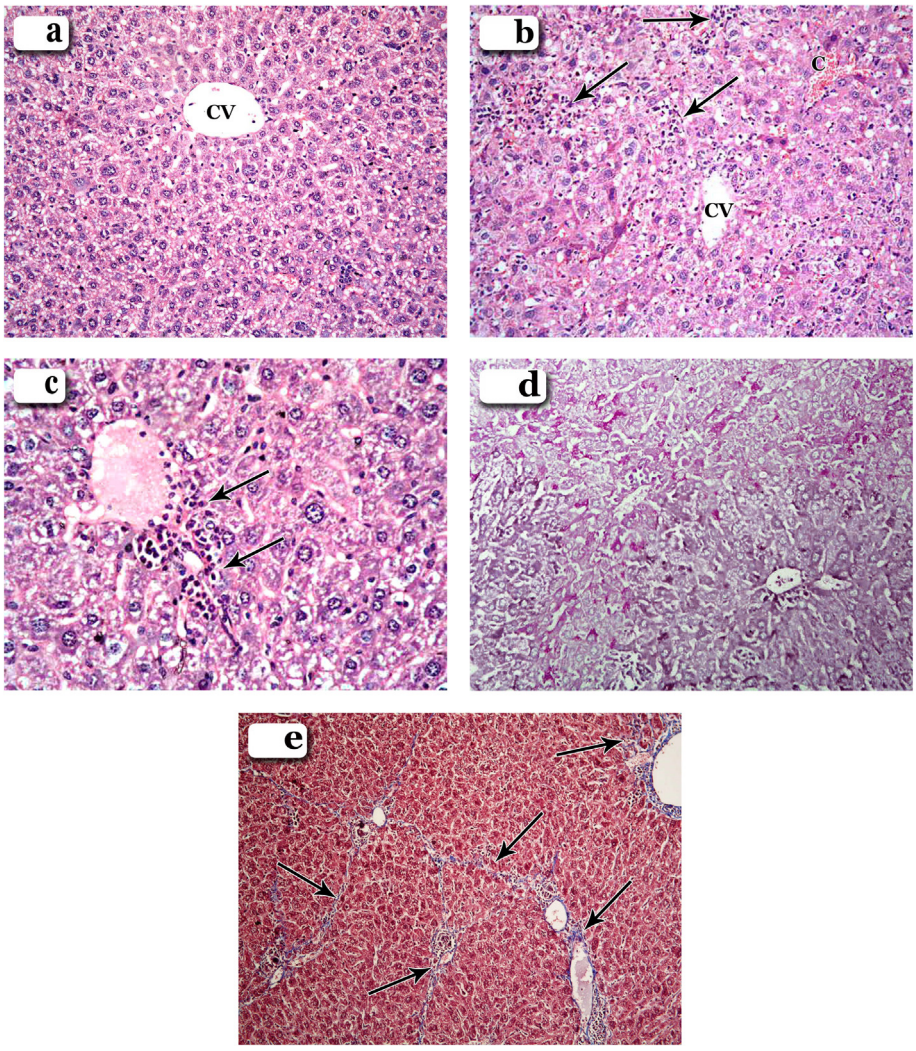

Fig. 4. Photomicrographs of sections in the livers of mice in which liver fibrosis was induced by CCl_4 and then received HGF-stimulated stem cells (subgroup IV-b) showing (a) mild dilatation of central vein (CV) [H & E, x200]. (b) Mild dilatation of central vein (CV), mild congestion of a blood vessel in the portal area (C) and mild mononuclear cellular infiltration (arrows) [H & E, x200]. (c) Mild mononuclear cellular infiltration (arrows) [H & E, x400]. (d) Mild reduction in PAS reaction [PAS, x100]. (e) Mild fibrosis in the periportal areas (arrows) [MT, x100].

model to human liver cirrhosis [44]. This model had been extensively used to examine the pathogenesis of cirrhosis. [45]. Mice were injected with CCl4 at a dose of 0.4 ml/kg, dissolved in olive oil, twice a week for 6 weeks according to Domitrović, et al. [31].

Major advances have been made in the prevention, diagnosis, and treatment of liver cirrhosis, including the use of liver transplantation and artificial liver. However, the number of patients suffering from liver disease is still increasing, in conjunction with a shortage in the availability of livers from suitable donors. Morbidity and mortality from liver cirrhosis continue to be enormous. Effective therapies to replace liver transplantation are clearly required. [46].

The use of mesenchymal stem cells (MSC) in tissue engineering and regenerative medicine to replace conventional therapeutic modalities has been the subject of growing interest in different areas. The main principle of stem cell therapy is the replacement of damaged and dead cells in injured tissues and organs with new healthy ones. They have the property of self-renewal and differentiation into specific functional cell types. MSCs are useful cells due to their therapeutic potentiality. Animal experimentation has shown that MSCs have a migratory property and can reach to the site of injury. Mesenchymal stem cells MSCs can be obtained from many tissues such as bone marrow (BM), dental pulp, adipose tissue and umbilical cord [47,48].

In this study, human UC-MSCs had been investigated in experimentally induced liver fibrosis in mice.

As UC-MSCs are more accessible and easier to get in comparison with BM-MSCs, the umbilical cord/placenta vessels can serve as a rich source of MSCs which may be used in experimental and clinical demands and these cells may therefore prove to be a new source of cells for cellular therapies involving stromal tissue and, potentially, cardiac muscle repair. This will avoid the ethical and technical issues involved in the use of cells from other origins. Such studies may have a significant impact on the cell therapy, functional genomics, pharmacological testing, and tissue engineering and potentially help to avoid worrying ethical issues [49].

It has been postulated that stimulation of UC-MSCs could give better results. Differentiation is commonly induced by combinations containing a selection of powerful, stimulating factors [50]. It has been shown that exposure to low intensity laser can accelerate the growth of fibroblasts and osteoblasts [51]. Moreover, there is a potent antiapoptotic and mitogenic effect of Hepatocyte growth factor (HGF) on hepatocytes during liver injury, as it is used to direct the MSCs to differentiate in the hepatocyte lineage and it

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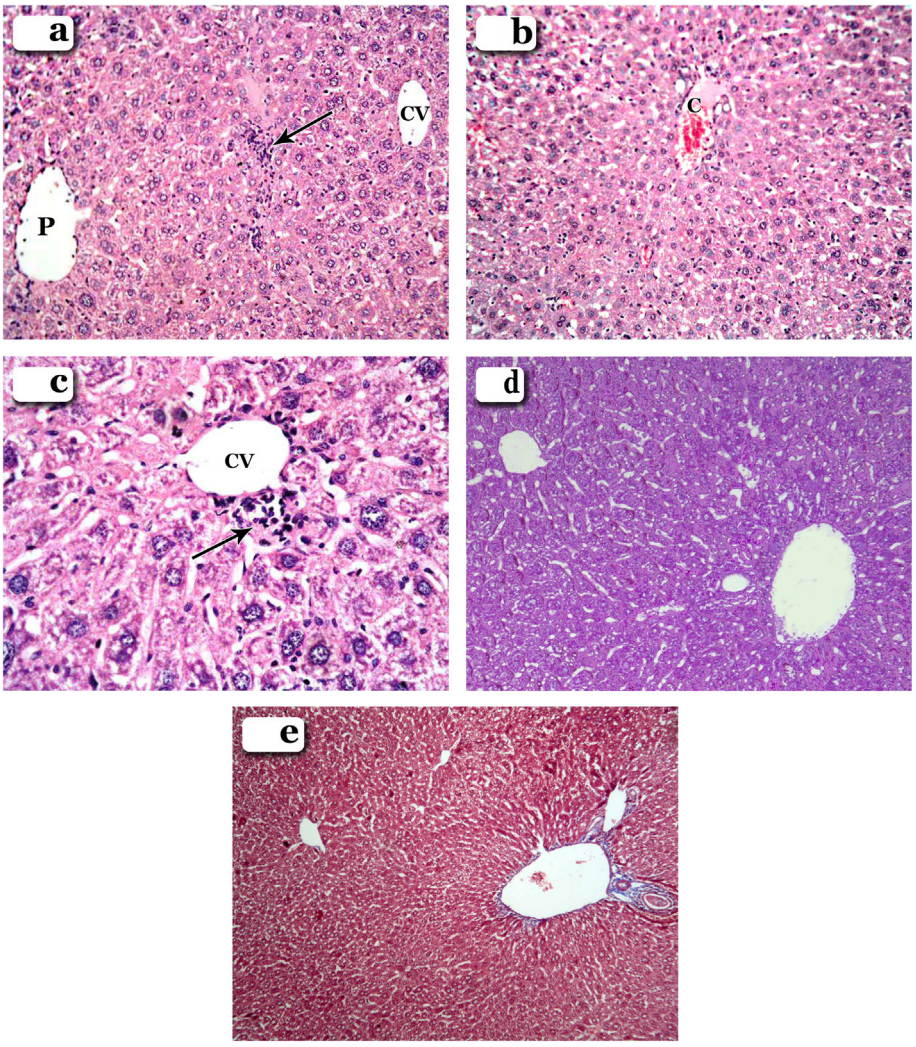

Fig. 5. Photomicrographs of sections in the livers of mice in which liver fibrosis was induced by CCl_4 and then received stem cells stimulated by both Laser and HGF (subgroup IV-c) showing (a) Mild dilatation of central vein (CV) and portal vessel (P) mild mononuclear cellular infiltration (arrow) [H & E, x200]. (b) Mild vascular congestion [H & E, x200]. (c) Mild mononuclear cellular infiltration (arrow) [H & E, x400]. (d) Slight reduction in PAS reaction [PAS, x100]. (e) Minimal fibrosis in the periportal areas [MT, x100].

has been utilized in many experimental and clinical applications. Moreover, it stimulates the migration of the activated hepatic stem cells into the place of injury and differentiate into mature hepatocytes. This enhances liver regeneration [15,29,52].

In this study the possible therapeutic effect of UC-MSCs exposed to diode laser and /or HGF were compared in CCl_4 induced liver fibrosis in mice. The Diode laser wavelength used in this study was 650 nm with a power of 200 mW. The power density (fluence) which is the actual power applied on a certain area was 0.77 J/cm^2 and the time exposure was 90 s. [15,29]. UC-MSCs were exposed to HGF with a dose of 10 ng/ml which is lower than the dose used by other studies who used 20 ng/ml [15,28].

UC-MSCs are capable of homing, differentiation and stimulation of a local repair response and could restore the liver structure in the experimental model of liver fibrosis [53]. In the present study, homing of MSCs into liver tissue of mice treated with UC-MSCs was confirmed by detecting the labelled MSCs with the PKH26 dye. These cells displayed red fluorescence, confirming that these cells were actually seeded into the liver tissues. These results were similar to Park et al., [54] who showed that the MSCs homed into the CCl_4-damaged liver tissue and differentiated into hepatocyte-like cells.

The percentage of gain in body weights (BW) and liver indices of mice significantly decreased after CCl_4 administration as compared to the control group, while the administration of stem cells exposed to laser and HGF significantly restored the percentage of gain in BW and liver indices. These results showed improvement of liver status caused by the stem cells exposed to laser and HGF and these results coincided with previous studies [55,56].

Liver toxicity due to CCl_4 was clearly evidenced, in the present work, in liver sections stained by H & E by congestion of the central veins and blood vessels of the portal areas, mild dilatation and congestion of the blood sinusoids, and extensive mononuclear cellular infiltration in the portal areas. Many hepatocytes displayed vacuolations in their cytoplasm, others exhibited pyknotic nuclei, or were anucleated, some hepatocytes showed ballooning, whereas others exhibited increased eosinophilia and hyalinization. PAS reaction appeared significantly weaker than the control group. Liver fibrosis was clearly evidenced by a significant increase in area percentage of the collagen fibers. The CCl_4-induced liver fibrosis improved by treatment with UC-MSCs stimulated by Laser and/or HGF. Maximum improvement was observed in the group where the UC-UMCs were exposed to both laser and HGF where most hepatocytes appeared nearly as those of the control group. This was in agreement with Ahmed et al. [53].

PAS which is a very useful technique to show glycogen storage ability of differentiated cells toward hepatic lineage. In the current study, it was observed that PAS and MT staining of CCl_4-induced

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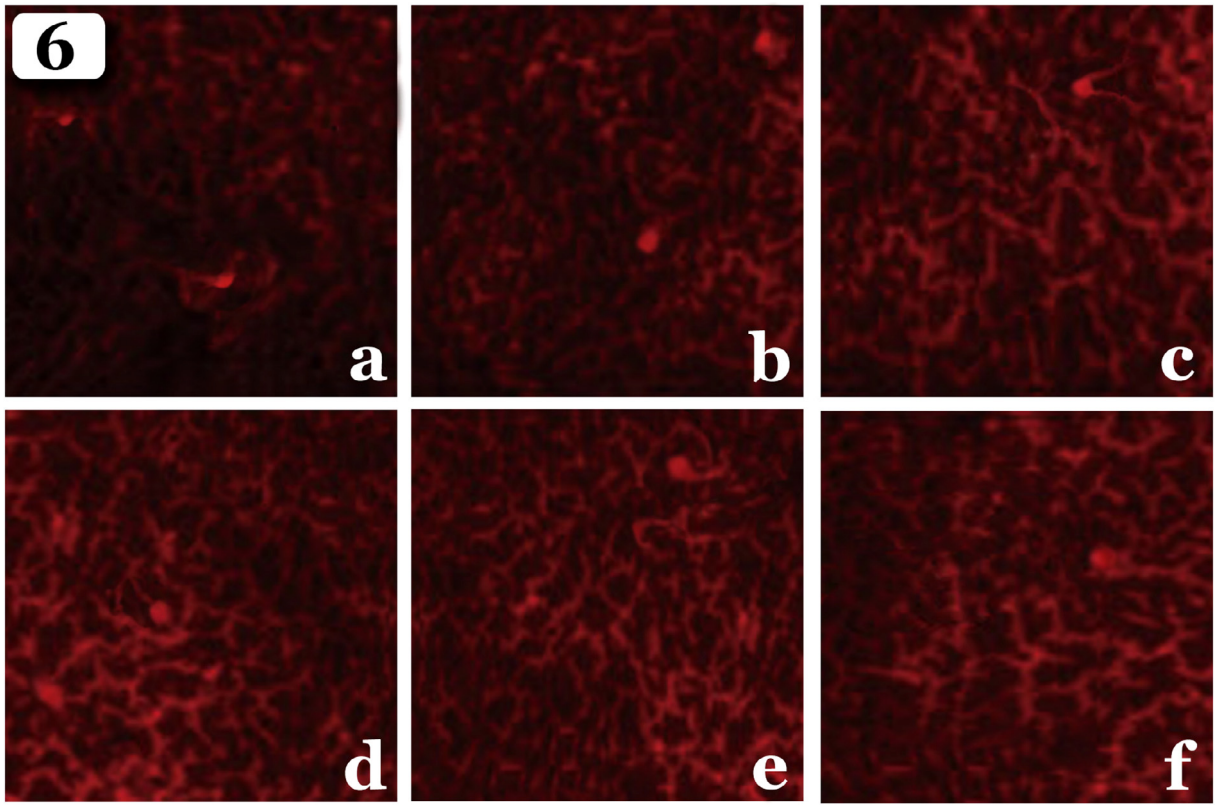

Fig. 6. A photomicrograph of sections in the liver showing red fluorescence of PKH26-labeled UC-MSCs distributed uniformly in the liver sections of the different groups. (a): subgroup II-a, (b): subgroup II-b, (c): subgroup II-c, (d): subgroup IV-a, (e): subgroup IV-b, and (f): subgroup IV-c). (PKH, x100].

Table 3
Mean values ± SD of optical densities (mean grey) of the PAS reaction in the different groups.

	Control Group (mean ± SD)	Group III (mean ± SD)	Group IV-a (mean ± SD)	Group IV-b (mean ± SD)	Group IV-c (mean ± SD)
Optical Density of PAS reaction	0.59 ± 0.03^{A}	0.21 ± 0.04^{B}	0.35 ± 0.04^{C}	0.42 ± 0.07^{D}	0.55 ± 0.02^{A}

Different superscript letters indicate significant differences ($p \leq 0.05$) between groups.
Groups sharing the same superscript letter are not significantly different at $p \leq 0.05$.

Table 4
Mean values ± SD of area percent of MT in the different groups:

	Control Group (mean ± SD)	Group III (mean ± SD)	Group IV-a (mean ± SD)	Group IV-b (mean ± SD)	Group IV-c (mean ± SD)
Area percent of MT	1.45 ± 0.41^{A}	31.47 ± 7.07^{B}	17.19 ± 4.88^{C}	$11.88 \pm 5.92\ C^{D}$	8.54 ± 2.54^{DE}

Different superscript letters indicate significant differences ($p \leq 0.05$) between groups.
Groups sharing the same superscript letter are not significantly different at $p \leq 0.05$.

liver fibrosis significantly improved by treatment with UMCs stimulated by Laser and/or HGF and that the maximum improvement was when the UMCs were exposed to both laser and HGF.

These findings agreed with other studies [57,58] who demonstrated that high glycogen levels were restored in animals liver tissue transplanted with pretreated MSCs with cytokines. Likewise, the MT staining results agreed with Ahmed et al., [53] and Truong et al., [59] who found few collagen fibers in liver sections of mice treated by UC-MSCs following CCl_4 induced liver fibrosis.

Many previous studies in the literature used laser to stimulate stem cells like Gasparyan et.al., [60] who reported that red and infrared laser irradiation can activate the migration of stem cells and can up-regulate the rate of stem cell migration towards higher SDF-1α gradient.

The wavelength of laser irradiation is a crucial parameter in biostimulation, since the photobiological effects of laser light is based on the light absorption by primary endogenous chromophores (mitochondrial enzymes, porphyrins, flavins, and cytochromes). One important consideration should involve the optical properties of tissue. There is a so-called "optical window" in tissue, where the effective tissue penetration of light is maximized. This optical window runs approximately from 650 nm to 1200 nm. In this study diode laser of wavelength 650 nm is used to be optimized to this optical window. The absorption and scattering of light in tissue are both much higher in the blue region of the spectrum than the red, because the principle tissue chromophores (hemoglobin and melanin) have high absorption bands at shorter wavelengths [61].

Effects of LLLT on proliferation of cell cultures depend also on consumed energy density (fluence), diameter of beam or irradiated area and the exposure time. Reviews suggests that an energy density value from 0.5 to 4.0 J/cm^2 are very helpful in enhancing the

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proliferation of various cell lines [62]. The power density used in this study was 0.77 J/cm^2 in 90 s time exposure. The actual power is calculated to know the exact power that the cells received from laser and to eliminate any absorption taking place through the plastic walls of the container. The laser beam expanded to 20.72 cm^2 area by a concave lens [35,63,64].

Mvula et al., [63] studied irradiation of human Adipose derived stem cells hADSCs and they stated that is dependant on the wavelength as well as a fluence. The cells irradiated in the visible range (680 nm) and low fluence (5 J/cm^2) responded better and showed more proliferation than cells irradiated in the infra-red range (830 nm) and higher fluencies (10 and 15 J/cm^2).

Other studies investigated biochemical and electrophysiological effects of a diode laser treatment (635 nm) on the differentiation of stem cells. They offered a novel experimental evidence on the mechanisms of action of the diode laser, may provide a promising therapeutic perspective for the treatment of tissue fibrosis extending the potential clinical application of the low level laser therapy [65].

Giannelli et al., [35] studied that diode laser, operating at a wavelength of 635 nm stimulates the proliferative potential of MSCs. The enhancement of cell growth after irradiation is dependent on the activation of physiological processes including membrane ion channel modulation. These results assured the proliferative potential can be increased by low-level laser irradiation (LLLI), but the mechanisms involved remain to be clarified.

The photobiological effect of laser light on cells and tissues has strong relation with the exposure time. Laser has a significant effect on the proliferation rate but when the exposure time increased after a certain time seems to be a significant decrease in proliferation/viability, perhaps due to damaging thermal effects [64].

In this work, liver sections of mice treated with UC-MSCs exposed to HGF showed enhanced regeneration in the damaged regions and it was even more effective than low-level laser irradiation (LLLI) alone. But there was a greater regenerative and healing capacity of the liver sections of mice treated with UC-MSCs exposed to both LLLI and HGF.

The dose of the HGF used in other studies was 20 ng/ml [15,63]. However, in this study we used 10 ng/ml HGF because it was observed that UC-MSCs did not tolerate the 20 ng/ml dose.

Methods of inducing MSCs differentiation included several chemicals but with limitations because the toxicity of these chemicals made them too risky to be applied in humans. However, HGF used in this study are secreted naturally in human body, and can be safely iccorporated to induce MSCs differentiation. Bae et al., [52] studied the BM-MSCs, ability to proliferate and differentiate into neuron-like cells which indicated a possibility for their application to cell therapy for a wide range of diseases such as neurodegenerative diseases.

In a similar study, Mvula and Abrahamse [28] investigated the role of diode laser at wavelength 636 nm 5 J/cm^2 and growth factors (retinoic acid and TGF-β1) on the differentiation of adipose derived stem cells that were co-cultured with smooth muscle cells (SMCs). The study showed that LILI increased the proliferation of cells in vitro. In contrast, our study was able to show the positive regenerative effect of LILI and HGF stimulated UC-MSCs in liver fibrosis model *in vivo*.

From the previously discussed results, it can be concluded that the diode laser in association with HGF together caused proliferation of UC-MSCs which markedly decreased the liver fibrosis induced by CCl_4 in mice. The combined effect of both diode laser and HGF together on the UC-MSCs was more effective than the effect of the diode laser or HGF alone. These results can give hope to patients especially those awaiting liver transplantation.

Conflict of interest

The authors declared that there is no conflicts of interest.

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