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ORIGINAL ARTICLE

Cumulative Effect of Mesenchymal Stem Cells and Heme Oxygenase-1 Inducer in Ameliorating Induced Liver Toxicity in Rats

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ABSTRACT: Liver diseases are most commonly occurring nowadays, that's why we are in argent need to develop new strategies in treatment. to evaluate the role of MSCs in regenerating liver cells and to clarify the anti-inflammatory role of HO-1 either alone or as a combined therapy with MSCs. 72 rats were divided into seven groups (n=10 rats/group) as follows, group1: control rats, group 2: CCL$_4$, group 3: CCL$_4$ that received MSCs group 4: CCL$_4$ that received HO-1 inhibitor, group 5: CCL$_4$ that received HO-1 inducer, group 6: CCL$_4$ that received combined MSCs and HO-1 inhibitor , and group 7: CCL$_4$ that received combined MSCs and HO-1 inducer. All groups were evaluated histopathologically with assessment of liver functions. The combined MSCs and HO-1 inducer group showed the highest significant results in ALT (p-value <0.05), albumin (p-value <0.05), HO-1 activity (p-value <0.0001), and genes expression compared to other groups. This is due to the cumulative anti-inflammatory role of both MSCs and HO-1 together with the ability of MSCs to increase the HO-1 expression with further reduction in inflammation and fibrosis. MSCs and HO-1 inducer provide promising tool in treatment of liver disease.

INTRODUCTION

Liver cell injury occurs as a result of alcoholic consumption, nonalcoholic fatty liver disease, HCV infection. Chronic inflammation causes excessive accumulation of extra cellular matrix (ECM) by activated hepatic stellate cell (HSC) among the hepatocytes with subsequent liver fibrosis [1, 2] .

Cirrhosis can result in portal hypertension with subsequent complications as ascites, varices, hepatic encephalopathy, coagulation disorders, and hepatocellular carcinoma. Cirrhosis and its complications not only impair quality of life but also decrease survival [3].

Previously it was thought that cirrhosis is irreversible but recent studies have shown that early treatments of the underlying cause can improve or even reverse the fibrosis [4].

Mesenchymal stem cells (MSCs) are multipotent cells that are capable of migration to damaged tissues and induce its healing. Inflamed tissue produces inflammatory cytokines that induce chemo taxis of MSCs. The MSCs exert their effect through immunomodulatory effects. MSCs, inhibit hepatic cell apoptosis and augment the effect of antiapoptotic factors like insulin-like growth factor (IGF-1), (hepatocyte growth factor (HGF) [5]. more over MSCs have paracrine effect through secretion of MSC-derived extracellular vesicles (EVs). EVs allow cellular communication through their nucleic acids (DNA, mRNA, miRNA, lncRNA) content [6].

Free heme released from broken RBCs can induce inflammatory process due to its oxidative properties. Thus, it is important to catabolize free hem by heme oxygenase (HO) enzyme. There are two isoforms of HO inducible HO-1 and constitutively expressed HO-2. HO catalyzes the catabolism of heme into carbon monoxide (CO), biliverdin/bilirubin, and free iron, induction of HO-1 is a protective cellular response against environmental stresses [7]. HO-1 induction can reduce hepatic inflammation by regulating immune cell infiltration, proliferation, and inflammatory cytokine signaling [8]. We suggested that combined effects of MSCs as antifibrotic and HO-1 inducer as antioxidants may be therapeutic in liver fibrosis.

The basic research of HO-1 modified MSCs has made remarkable progress, which is expected to be applied in clinical trials and provide theoretical basis and reference value for stem cell therapy [9].

We aimed in this study to explore a new method in treating liver disease, and to obtain the beneficial effect of both MSCs and HO-1 as a combined therapy.

MATERIALS AND METHODS

This study was conducted in the faculty of medicine, Cairo University in a time period from February 2018 to March 2019.

Isolation and preparation of BM-derived MSC

6 weeks old white albino female rats (Cux1: HEL1) tibiae and femurs were flushed with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) with added 10% fetal bovine serum (GIBCO/BRL) to harvest the bone marrow. Nucleated cells were isolated with a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in complete culture medium with added 1%penicillin—streptomycin (GIBCO/BRL). Cells were incubated at 37 °C in 5% humidified CO_2 for 10— 14 days until reach (80-90% confluence), cultured cells were washed and then trypsinized with 0.25% trypsin in 1mM EDTA (GIBCO/BRL) .the cells were centrifuged (at 2400 rpm for 20 minutes), then resuspended with serum-supplemented medium and incubated in 50 cm^2 culture Falcon flask) [10]. MSCs were identified morphologically by their fusiform shape and by FACS analysis using CYTOMICS FC 500 (Beckman Coulter, FL, U.S.A.) and CXP Software version 2.2 for interpretation.

Experimental animals

After obtaining an approval from Institutional Animal Care, seventy-two female 6 weeks white albino rats (Cux1: HEL1) were cared in the animal house unit of Cairo University provided the veterinary care. Liver fibrosis was induced by injection of 0.2 ml/100 g body weight of 40 ml/l CCl_4 (Sigma, St Louis, USA) dissolved in equal volume of castor oil (Sigma, St. Louis, USA) subcutaneously. The injection was given twice a week for 6 weeks [11].

Liver fibrosis was confirmed by histopathological examination of two randomly selected rats, rats were then divided into seven groups (each group 10 animals): **group 1:** a negative control; **group 2:** induced liver fibrosis (pathological control) [12].; **group 3:** induced liver fibrosis that received undifferentiated BM MSCs (3×10^6 cells/ml intraperitoneally/single dose) [13]; **group 4:** induced liver fibrosis that received zinc protoporphyrin (0.5mg/100gm /subcutaneously/ single dose) [14]; **group 5:** induced liver fibrosis that received Cobalt protoporphyrin (5mg/kg /IP/ single dose) [15]. ; **group 6**: induced liver fibrosis that received hMSCs + zinc protoporphyrin; **group 7:** induced liver fibrosis that received hMSCs + Cobalt protoporphyrin. After 4 weeks of stem cell and drugs administration, venous blood was collected from the retro-orbital vein then all rats were sacrificed and liver tissue was harvested and subjected to histopathological and molecular study.

Histopathological evaluation

Samples of liver tissues were collected into PBS and fixed in 40 g/l paraformaldehyde in PBS at 4°C overnight. Serial of the right lobes of the livers of 5-μm sections were stained with Hematoxylin and Eosin (HE) and Sirius Red (SR), for histopathological examination

Colorimetric assay

 ALT & Albumin levels were assessed in serum (Spectrum, Hannover Germany) according to manufacturer's instructions.

Assessment of heme oxygenase activity

Liver tissue samples were homogenized and then incubated in (rat liver cytosol (5 mg/mL), (heme (50 μmol/L), MgCl2 (2 mmol/L), glucose-6-phosphate (2 mmol/L), glucose-6-phosphate dehydrogenase (1 unit), and NADPH (0.8 mmol/L) in 0.5 mL of 0.1mol/L phosphate buffer saline (pH 7.4)) medium for 60 min at 37ºC. The tubes were cooled to stop the reaction and then the bilirubin product was extracted with chloroform. The spectrophotometer was used to detect the concentration of bilirubin and was monitored at 464 nm and 520 nm. The concentration of bilirubin was calculated by using an extinction coefficient 40.0 mmol/L [9].

Quantitative RT-PCR of heme oxygenase gene expression

The liver tissues from study groups were lysed for total RNA isolation with GeneJET Kit (Thermo Fisher Scientific Inc., Germany, #K0732). 5µl the total RNA from each sample were used for reverse transcription with subsequent amplification with Bioline, amedian life science company,U.K. (SensiFASTTM SYBR R Hi-ROX)One-step Kit (catalog number PI-50217 V) in a 48-well plate using the Step-one instrument (Applied Biosystems, U.S.A.). Thermal profile was as follows: 45°C for 15min in one cycle (for cDNA synthesis), 10 min at 95°C for reverse transcriptase enzyme inactivation, followed by 40 cycles of PCR amplification. Each cycle was continued for: 10 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The expression of HO gene were normalized relative to the mean critical threshold (CT) values of GAPDH as the housekeeping gene by the Ct method. Primers' sequences for HO are sense primer sequence: 5'-ACCCCACCAAGTTCAAACAG-3'; HO-1 antisense prime sequence: 5'-GAGCAGGAAGGCGGTCTTAG-3' (gene bank accession number: NM_012580.2) and primers' sequences for GAPDH are sense primer sequence: 5'-AATGGTGAAGGTCGGTGTGAAC-3'; GAPDH antisense prime sequence: 5'-AGGTCAATGAAGGGGTCGTTG-3' (gene bank accession number: NM_017008.4).

Statistical analysis

Data are expressed as mean ± SD. comparison between groups were done using analysis of variance (one way ANOVA) and post hoc tests for multiple comparisons using SPSS version 22 Computer Software. Correlation between quantitative variable was done using pearsons correlation. $p < 0.05$ is considered significant.

RESULTS

The MSCs were identified morphologically by their fusiform fibroblast like cells (Figure 1A), MSCs were

further characterized by cell surface phenotyping assessment. MSCs showed 98.3% positive expression for the β1-integrin CD29$^+$, and negative for CD 34$^-$ (Figure 1B).
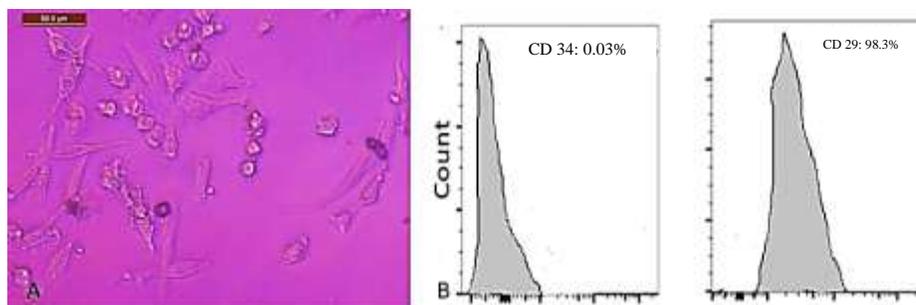

Figure 1. MSCs characterization in culture.

(A): MSCs were isolated as fibroblast-like cells (200X). (B): FACS analysis characterized MSCs; they were positive for CD29 and negative for CD34.

Histopathological results

The liver tissues were evaluated histopathologically for fibrosis, inflammation, vascular proliferation (*Figure 2*) showing that;

Control group: Figures 2A, 2B showed normal architecture, hepatocytes, central veins, normal sinusoids with regular width and portal tracts showed normal connective tissue content and no inflammation.

The fibrotic group : Figures 2C, 2D showed disturbed architecture with diffuse degenerative changes of hepatocytes, sinusoids were mostly obliterated, sinusoidal fibrosis was mild, central veins were thickened and portal tracts were markedly thickened and showed bile duct proliferation as well as sever inflammation.

The fibrotic group that received MSCs: Figures 2E, 2F showed marked improvement in the form of marked amelioration of fibrosis of the portal tract, minimal inflammation.

The fibrotic group that received HO-1 inhibitor: Figures 2G, 2H showed marked flaring of fibrosis and inflammation. *The fibrotic group that received heme oxygenase inducer:* Figures 2 I, J There was no improvement from diseased liver regarding the fibrosis but less inflammation.

The fibrotic group that received heme oxygenase inhibitor and MSCs: Figures 2K, 2L showed less improvement than the group treated by MSCs alone with marked inflammation and congestion.

The fibrotic group that received heme oxygenase inducer and MSCs: Figures 2M, 2N showed marked improvement in the form of marked decrease of fibrosis of the portal tract, no inflammation and hepatocyte appearance was near normal.
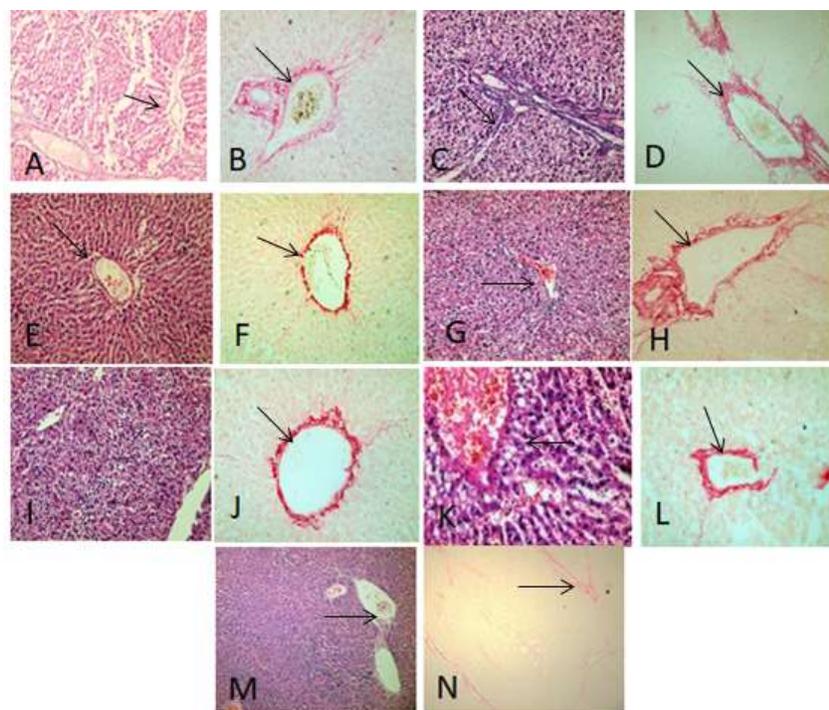
Figure 2. Histopathological assessment of liver tissues in all the studied groups (H&E and Sirius red stains): (A, B) Normal liver, (C, D) CCL4 induced, (E, F) MSCs treated only treated, (G, H) HO-1 inhibitor treated group (I, J) HO-1 inducer treated group , (k, l) MSCs and HO-1 inhibitor treated group (M, N) MSCs and HO-1 inducer treated group. Black arrows are indicative for fibrosis and congestion.

MSCs and HO-1 inducer restore liver function

Serum ALT level (Figure 3A) showed significant increase in its level in all groups in compare to the control group (p value <0.05) except the combined MSCs and HO-1 inducer showed no significant difference compared to the control. The combined MSCs and HO1 inducer group significantly showed lower ALT level in compare to the treated group with (HO-1 inhibitor, HO-1 inducer, combined MSCs and HO-1 inhibitor (p value <0.001, 0.013 and 0.002) respectively.

Serum albumin level (Figure 3B) showed significant decrease in its level in all groups compared to the control group (p value <0.05) except the combined MSCs and HO-1 inducer. The combined MSCs and HO1 inducer group significantly showed higher albumin level compared to all other treated groups and (p value =0.005, <0.001, <0.001,0.02) respectively.

MSCs potentiate HO-1 expression and activity

HO-1 gene expression (Figure 3c) showed significant decrease in its expression in all groups in compare to the control group (p value <0.001). The combined MSCs and HO1 inducer group significantly showed higher HO-1 expression compared to all other treated groups (p value <0.001) except HO1 inducer treated group showed no significant difference (p value =0.24).

HO-1 activity (Figure 3D) showed significant decrease in all groups in compare to the control group (p value <0.001). The combined MSCs and HO1 inducer group significantly showed higher HO-1 activity compared to treated groups by (HO-1 inhibitor and combined MSCs and HO1 inhibitor) (p value <0.001,0.006).
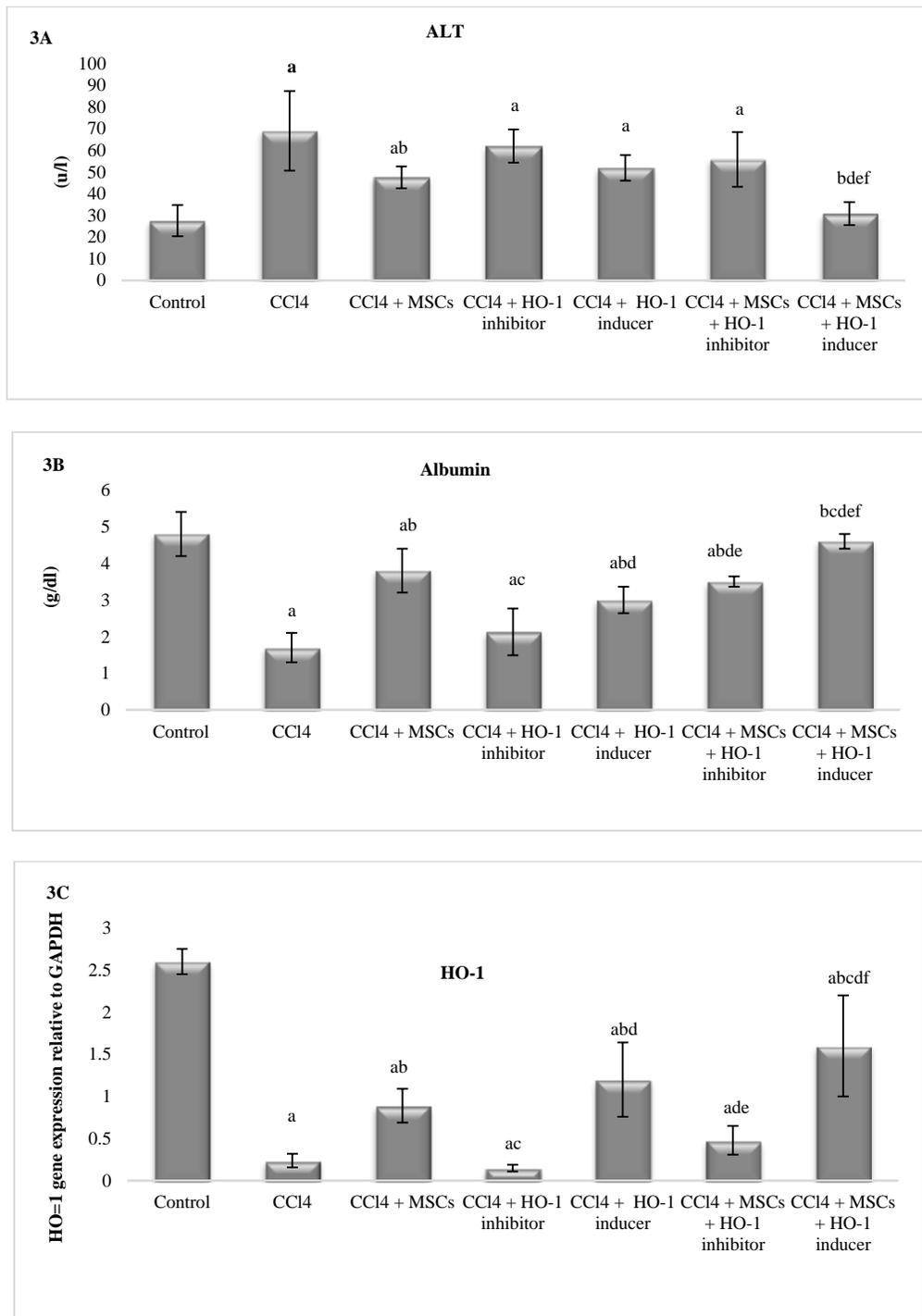
3A

ALT

3B

Albumin

3C

HO-1

Figure 3. assessed parameters among studied groups. a: statistically significant compared with corresponding value in control group. b: statistically significant compared with corresponding value in CCL4 group. c: statistically significant compared with corresponding value in MSCs treated group. d: statistically significant compared with corresponding value HO-1 inhibitor group e: statistically significant compared with corresponding value in HO-1 inducer group. f: Statistically significant compared with corresponding value in combined MSCs and HO-1 inhibitor group.
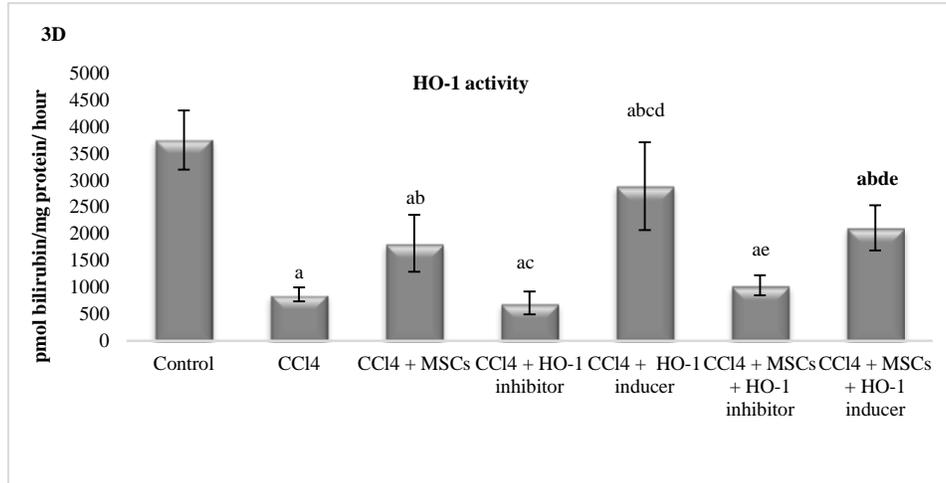
3D

HO-1 activity

Figure 3. Continued.

Liver function tests is significantly correlated with

HO-1 expression and activity

Serum ALT is significantly inversely correlated with HO-1 gene expression (Figure 4A) and inversely correlated with HO-1 activity (Figure 4B) (*p* value <0.001). Serum albumin is significantly positively correlated with HO-1 gene expression (Figure 4C) and positively correlated with HO-1 activity (Figure 4D) (*p* value <0.001). HO-1 activity is significantly positively correlated with HO-1 gene expression (Figure 4E) (*p* value <0.001).
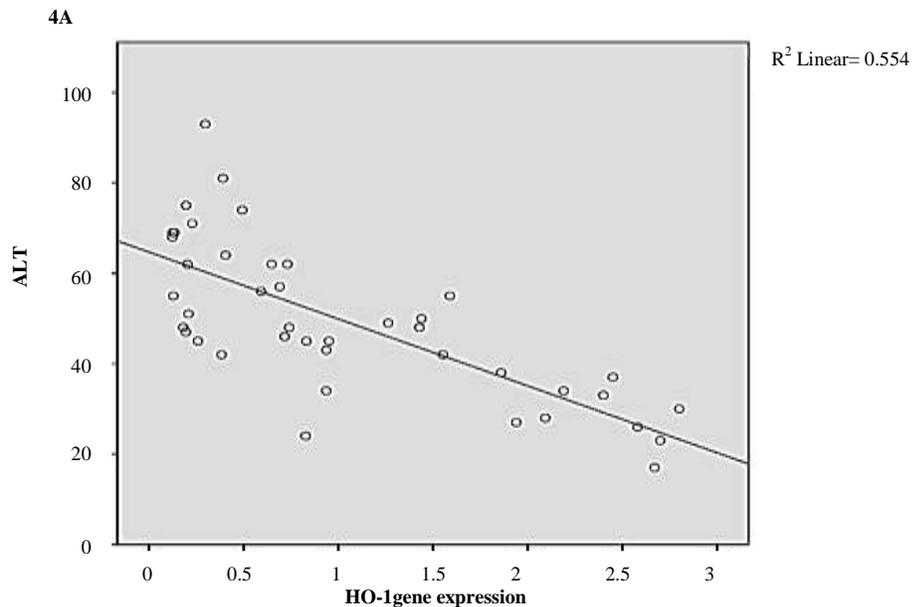
4A

R^2 Linear= 0.554

Figure 4. Correlation analysis between studied parameters showed that HO-1 activity and gene expression positively correlated with serum albumin and negatively correlated with ALT.
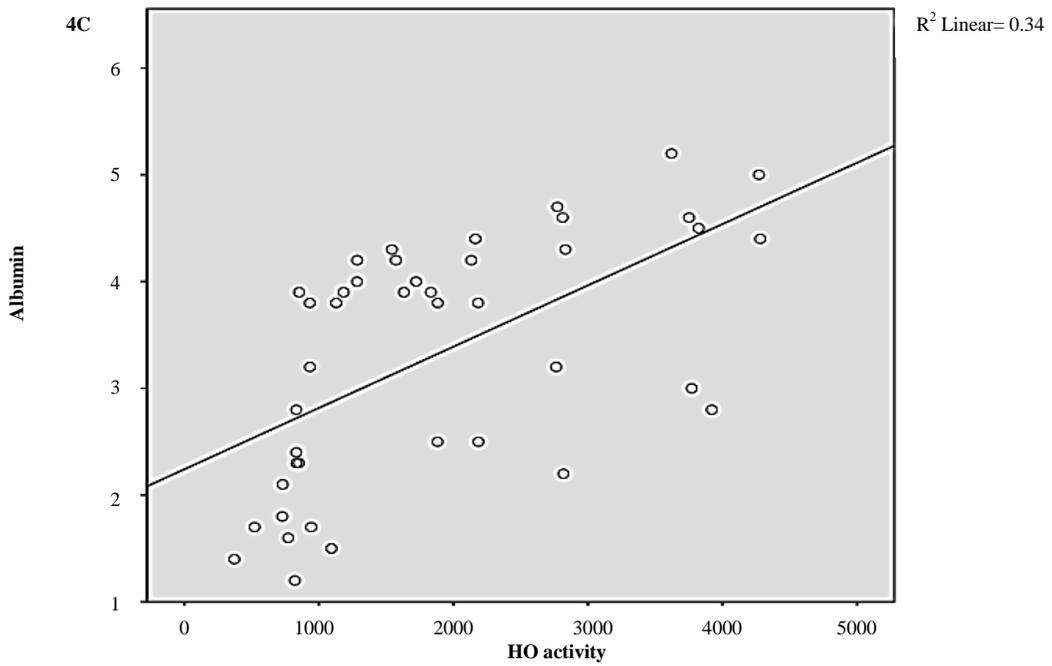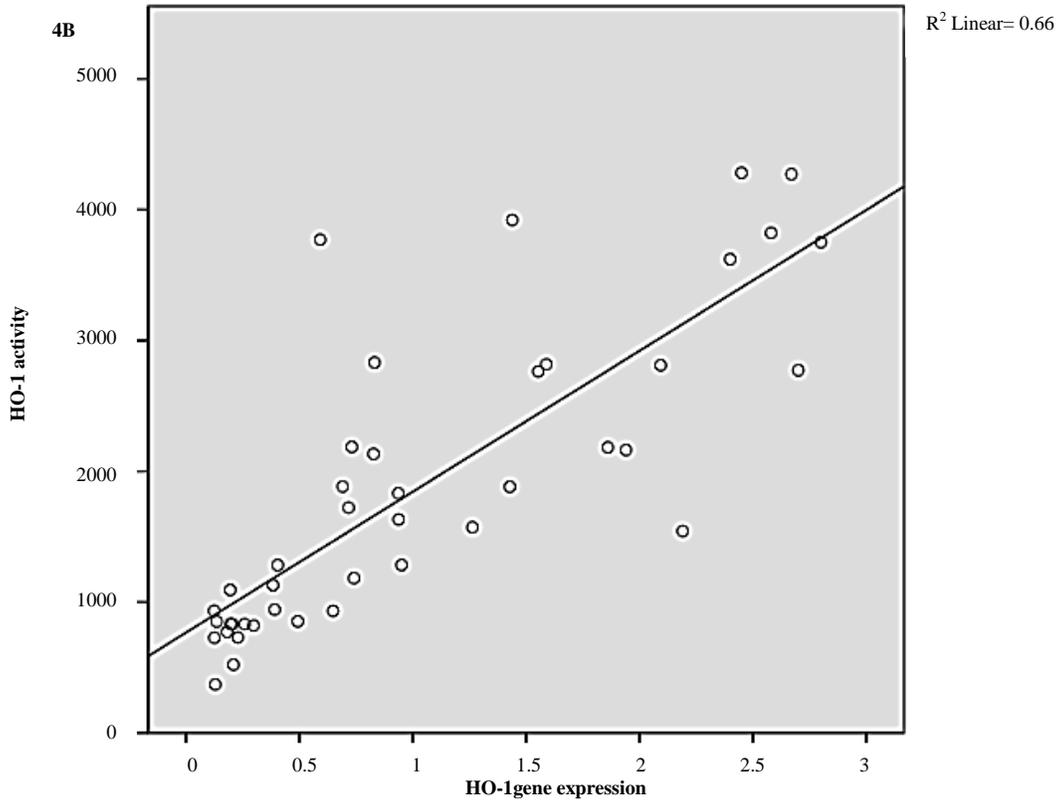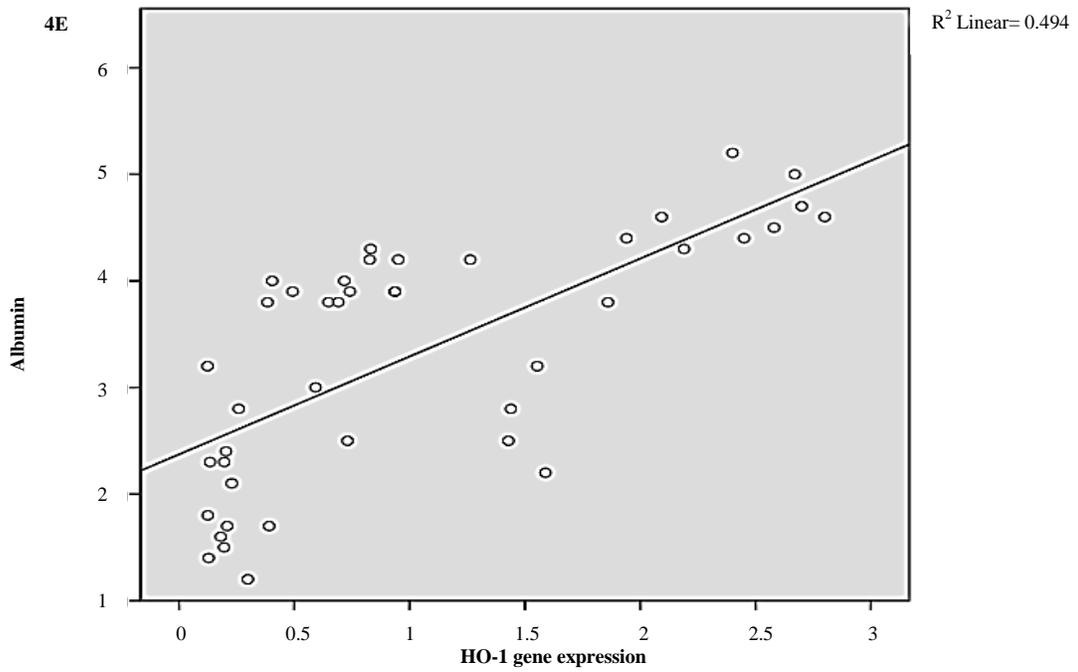
Figure 4. Continued

4D

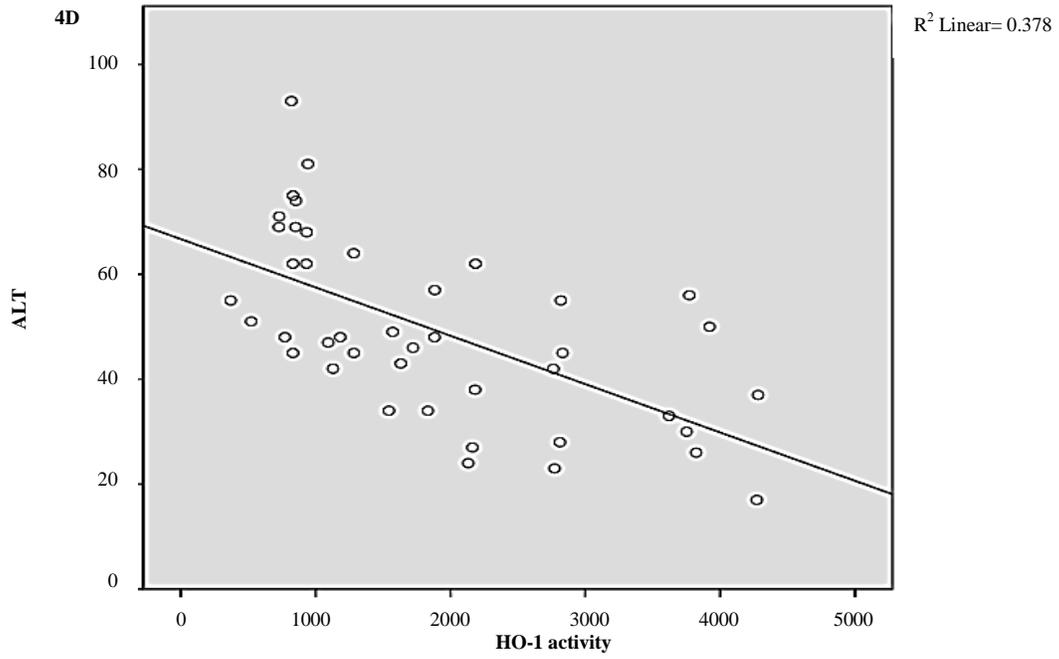R^2 Linear= 0.378

4E

R^2 Linear= 0.494

Figure 4. Continued.

DISCUSSION

The liver is the main organ responsible for protein synthesis, nutrient metabolism and detoxification of several metabolites. Liver is susceptible to various insults such as alcoholism, steatohepatitis, viral hepatitis and drug intoxication [16]. Thus, it's important to seek new strategies in treatment to save liver cells.

MSCs have a promising role in regenerative medicine. MSCs can regenerate the damaged tissue by hepatic cell trans differentiation, ameliorating inflammation and fibrosis and stimulating the angiogenesis [17].

HO-1 can be considered as antioxidant, also it has anti-inflammatory and anti-apoptotic role, thus induction of HO-1 provides a new strategy in treatment of liver disease [15].

In this study, it was found significant improvement in liver function regarding ALT and albumen in the diseased groups receiving MSCs and more improvement occurred on adding HO-1 inducer. This can be explained by the anti-inflammatory role of MSCs which further augmented by addition of HO-1 inducer. For more clarification for the role of HO-1, HO-1 inhibitor was added while deterioration of liver function even though in the group received MSCs occurred. Thus, the HO-1 and MSCs potentiate each other and work in synchronous together. This is agreed with previous studies demonstrated that induction of HO-1 in the liver, prevent the development of hepatic fibrosis by its antioxidant role [15].

Another study revealed that HO-1 causes immunomodulation through inhibition of the activation, proliferation of T helper cells and lower the level of serum ALT and proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α [13]. More over another study suggested that increasing HO-1 activity in liver cells protects them from oxidative stress-induced damage [18].

Regarding the role of MSCs in liver cell regeneration, it has been demonstrated that administration of MSCs can accelerate liver regeneration of acute liver failure through PDGF and VEGF regulation [19].

On a study about the clinical application of MSCs on hepatic patients, it revealed that MSCs improves liver function during the first 6 months after administration. A single dose of BM- MSCs administrated through the hepatic artery, improve liver function [20].

More clinical study on application of MSCs, revealed improvement of ALT, TBIL, ALB, and PT in liver cell failure patients after administration of MSCs [21].

The HO gene expression and activity went hand in hand with each other in the experimental groups. In this study, MSCs significantly increase the expression of HO-1 with no significant difference in its effect with HO-1 inducer. this is agreed with previous study that revealed that BMSCs ameliorated acute liver failure and improved liver cell function by increasing the HO-1 expression, which reduced PMN infiltration and function, MSCs have anti-apoptotic and anti-inflammatory role through reduction of inflammatory cytokines IL1-β, TNF-α, NFκB [22]. Another study reported that MSCs restore liver cell function through induction of HO-1[23].

MSCs could alleviate acute liver failure by inducing the HO-1 expression, which played an important role in activating autophagy through PI3K/AKT signaling pathway [24].

We recommended in the future work to evaluate the entire micro array genes that reducing liver fibrosis and inflammation in MSCs and HO-1 inducer group.

CONCLUSIONS

MSCs and HO-1 inducer provide promising tool in treatment of liver disease.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article contains animals' ethical approval.

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