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Research Article
Role of Hepatocyte Differentiated Mesenchymal Stem Cells in Treatment of Experimentally Induced Canine Liver Cirrhosis

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Abstract

Liver cirrhosis represents the final pathway of all chronic hepatic injury. Replacement the damaged hepatocytes through cell-based therapies have been a promising therapeutic agent for tissue regeneration. This study aimed to assess the effect of treatment with Hepatocyte Differentiated Mesenchymal Stem Cells (HD-MSCs) in experimentally induced canine liver cirrhosis. Experimental induction of liver cirrhosis was done on 18 skeletally mature mongrel dogs using carbon tetrachloride (CCl_4) for 16 weeks; dogs were evaluated for cirrhosis through clinical, Liver Function Tests (LFTs), ultrasonography with Portal Vein (PV) and Hepatic Vein (HV) diameter estimation and histopathological examination. Dogs were randomly allocated into one of the two study groups; Sham group: CCl_4 with no HD-MSCs treatment and group II: CCl_4 with HD-MSCs treatment then dogs were evaluated for the efficacy of cell treatment through clinical, LFTs, ultrasonography with PV and HV diameter estimation, postmortem examination, histopathological examination of liver tissue and relative quantitative expression of collagen Iα and albumin were measured by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The HD-MSCs administration restored liver function that was confirmed by clinical, LFTs, histopathology and RT-PCR. Liver tissue ultrasonography, PV and HV diameter were improved. The study proves the ability of HD-MSCs to potentially improve cases of liver cirrhosis.

Key words: Dog, CCl_4, liver cirrhosis, mesenchymal stem cells, RT-PCR, liver function test, HD-MSCs

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Liver is the largest metabolic organ that performs several vital functions including immune defense, metabolism of carbohydrates, lipids, proteins, hormones, xenobiotics, in addition to secretion of plasma proteins and bile. Hepatocytes are the main liver function unit (Ren *et al*., 2015). Functional disorders of these cells are related to a variety of pharmacological agents, hepatitis viruses, alcohol abuse, autoimmune inflammation and exposure to metabolic metals as iron and copper leading to inflammation of the liver (acute and chronic liver failure) ending with cirrhosis and hepatocellular carcinoma (HCC) (Wu and Tao, 2012; Ren *et al*., 2015). Cirrhosis develop as liver slowly deteriorates, malfunctions and scar tissue replaces the healthy hepatocytes (Passos *et al*., 2010). The liver has the amazing potential to regenerate by its own hepatocytes when mild liver damage occurs but due to persistent and severe damage, hepatocytes can no longer proliferate (Kung and Forbes, 2009; Puglisi *et al*., 2011) and the only option for patients with end-stage liver cirrhosis is liver transplantation. Nevertheless, liver transplantation is limited by a lack of donors, long waiting lists, cost of surgery, immune rejection, revival of the original disease in transplant recipients and the lack of antifibrotic therapeutic agents (Ryder, 2007; Iredale, 2007). Hence, extensive studies are being conducted to develop new treatments such as stem cell based therapy that has been suggested as an alternative treatment strategy for patients who suffer from various hepatic diseases (Iredale, 2007; Kisseleva *et al*., 2010; Piscaglia *et al*., 2010).

Stem cells are biological cells found in all multicellular organisms that can divide and differentiate to produce more stem cells including the hepatocytes (Zhang and Wang, 2013; Shrihari, 2011). Bone Marrow (BM) is a major source of hematopoietic multipotent stem cells, as well as Mesenchymal Stem Cells (MSCs) (Le Blanc and Ringden, 2005). The transplanted MSCs help the survival of endogenous cells through direct contact, decreasing tissue inflammation and increasing cell proliferation (Navarro-Alvarez *et al*., 2009). This takes place either by fusion between the transplanted MSCs and local hepatocytes or providing cytokines and growth factors in their microenvironment (niches) that promote hepatocyte functions by paracrine mechanisms (Wang *et al*., 2015).

The induction of liver cirrhosis in an animal model becomes necessary to investigate the possible therapeutic treatments for chronic liver diseases. The dog is one of the commonly used animals in experimental research as the metabolic and immunologic reaction of the dog is close to human beings (Ryder, 2007). Carbon tetrachloride (CCl_4) is a potent hepatotoxin that has been used most frequently to induce experimental liver regeneration in mouse, rat, pig, monkey and dog (Palmes and Spiegel, 2004). The free radicals (CCl_3^-) generated by CCl_4 induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This lipid peroxidative degradation of biomembranes (Fatty acids degeneration) is a principle cause of hepatotoxicity of CCl_4 (Kaplowitz *et al*., 1986). It is associated with centrolobular fatty necrosis (Recknagel *et al*., 1989; Ryder, 2007).

The objective of this study was to assess the effect of treatment of hepatocyte differentiated mesenchymal stem cells (HD-MSCs) in experimentally induced canine liver cirrhosis.

MATERIALS AND METHODS

Eighteen dogs 2-2½ years old weighing 15-20 kg of both sex (11 male and 7 female). Before enrollment in the study, dogs were acclimated to the kennel environment for at least 2 weeks. Complete clinical, Liver Function Tests (LFTs) and ultrasonographic examinations were conducted on each dog to exclude evidence of hepatic diseases. All study procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Cairo University (Cu F Vet/F/SUR/2013/16). During the study, dogs were fed standard dry food twice daily and were allowed a free access to drinking water.

Dogs were randomly divided into two groups; Sham group (control group), non HD-MSCs treated (n = 9) and group II, HD-MSCs treated (n = 9).

Induction phase: Liver cirrhosis was induced chemically by oral administration of 1 mL kg^{-1} of CCl_4 (98%) (Liu *et al*., 2007) using orogastric tube 2 times/week for 16 weeks; the administration was on the same day of the week and at the same time of the day under light sedation (Lemke, 2007). The liver was evaluated for cirrhosis through clinical, LFTs, ultrasonography with Portal Vein (PV) and Hepatic Vein (HV) diameter estimation and histopathological examination. At 13th week bone marrow was aspirated from iliac crest for preparation of MSCs.

Treatment phase: An ultrasonography guided intravenous catheter (16G×20 mm) was directed to the portal vein at the end of the 16th and 18th weeks to inject 3 mL of sterile saline for Sham group and 3 mL of 6×10^6 HD-MSCs for Group II.

The liver of both groups was evaluated for efficacy of HD-MSCs through clinical, LFTs, ultrasonography, PV and HV diameter, histopathology, post-mortem evaluation and finally

RT-PCR for evaluation of collagen Iα and albumin genes from the end of the 18 week till the 30 week. At the end of the study, dogs were humanly sacrificed with a lethal I/V dose of T61 (Bertol *et al*., 1983).

Preparation of BM-derived MSCs: Bone marrow was harvested throughiliac crest aspiration at 13th week with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL) (Abdel Aziz *et al*., 2011). Cells were incubated at 37°C in 5% humidified CO_2 for 12-14 days as primary culture or upon formation of large colonies. When large colonies developed (80-90% confluence), cultures were washed twice with phosphate buffer saline (PBS) and the cells were trypsinized with 0.25% trypsin in 1 mMEDTA (GIBCO/BRL) for 5 min at 37°C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 50 cm^2 culture flasks (Falcon). The resulting cultures were referred to as first passage cultures (Abdel Aziz *et al*., 2007). On day 14, the adherent colonies of cells were trypsinized and counted. The adherent cells incubated with FITC labeled anti-dog CD45, CD34, CD29 and CD105 for 30 min. Positive cells were counted by flow cytometry with FACscan flow cytometer (Beckman coulter, Fl, USA) and analyzed using CXP software version 2.2 (Aurich *et al*., 2009). Hepatocytes differentiation had been carried out by Gordon *et al*. (2006) and induced for 14-21 days after treatment with hepatocyte growth factors. The HD-MSCs was assessed by immunofluorecsent detection of albumin using Abcam albumin immunocytochemistry.

Clinical manifestation of liver failure examination: Every dog went through routine physical examination that included, activity, general body condition (hair, skin and body weight), appetite and mucous membrane.

Assessment of LFTs: Blood samples were collected at two weeks interval along the study period for assessment of alanine aminotransferase (ALT), aspartate aminotransferase (AST), Total Protein (TP), albumin (ALB), Total bilirubin (T. bili), Direct bilirubin (D. bili), Alkaline Phosphatase (AP), Hemoglobin (HB), platelets (PLT) and Prothrombin Time (PT) (Lilford *et al*., 2013).

Assessment of liver ultrasonography: Liver ultrasonography and biopsy were applied on four weeks interval till the end of the study. The degree of liver fibrosis was evaluated semi-quantitatively according to the Laennec system. Site of ultrasonography and measurement techniques of PV and HV using Doppler ultrasonography was applied according to Wanless *et al*. (2002) and Szatmari *et al*. (2004)

Analysis of liver histopathology: Liver samples were taken every four weeks, fixed in 10% buffered formalin and embedded in paraffin. For processing, sections were cut in 4-5 μm thickness and stained with Haematoxylin and Eosin (HE) and Masson's Trichrome (MTC) for collagen fibers (Jones, 1996).

Post mortem examination: Necropsies were performed at the end of 30th week. The excised livers were washed in sterile saline then photographed. Representative sections from all lobes of the liver were collected for histopathological and morphological examinations (cut section, borders, colour and lobes consistency).

Relative quantitative reverse transcription PCR (RT-PCR) of albumin and collagen 1 alpha detection: Total RNA was extracted from cultured cells using RNeasy purification reagent (Qiagen, Valencia, CA). The cDNA was generated from 5 μg of total RNA extracted with 1 μL (20 pmol) antisense primer and 0.8 μL superscript AMV reverse transcriptase for 60 min at 37°C. For PCR, 4 μL cDNA was incubated with 30.5 μL water, 4 μL 25 mM $MgCl_2$, 1 μL dNTPs (10 mM), 5 μL 10×PCR buffer, 0.5 μL (2.5 U) Taq polymerase and 2.5 μL of each primer containing 10 pmol. The following oligonucleotide primers were used: Albumin (Forward,5'-GG CAGGGCTCAGTCAGTAATGA-3'; Reverse,5'-AGGCCTACCCCAGC CAGTAG-3'), collagen 1-α (Forward, 5'-TCCTGAATGGGAGAGGT CC-3'; Reverse, 5'-TCTTGGCCAAAGGAGACG-3'). Amplification reactions were performed at 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec for 30 cycles. The PCR product yielded a 163 bp fragment on 1.5% agarose gel electrophoreses

Statistical analysis: Data of LFTs and diameter of PV and HV were expressed as Mean±SD. Significant differences were determined by using independent-samples T test on SPSS version 17.0 computer Software. Results were considered significant at $p<0.05$.

RESULTS

MSCs culture and identification: The MSCs were isolated, propagated and identified by phenotypic analysis. The MSCs were negative for CD45, CD34 and positive for CD29 and CD105 (Fig. 1a-e). Isolated and cultured undifferentiated

MSCs reached 70-80% confluence at 14 days and differentiated to hepatocytes (Fig. 2a-c).

Clinical symptoms: At the first 4 weeks, no obvious clinical symptoms appeared on dogs, at the 6th week, the weight loss, fatigue and dullness were noticed in all animals, from the 8th week, decreased activity and manifested by depression and dullness and severe weight loss were observed in all animals. Furthermore, mucous membrane started to become pale in color and progressed to deep yellow discoloration at the end of the 16th week follow up period. This icteric color was also noticed on the mucous membranes of the eye and gum.

Sham group showed the previous clinical signs with no evidence of improvement from the 16th week till 30th week while dogs of group II showed progressive

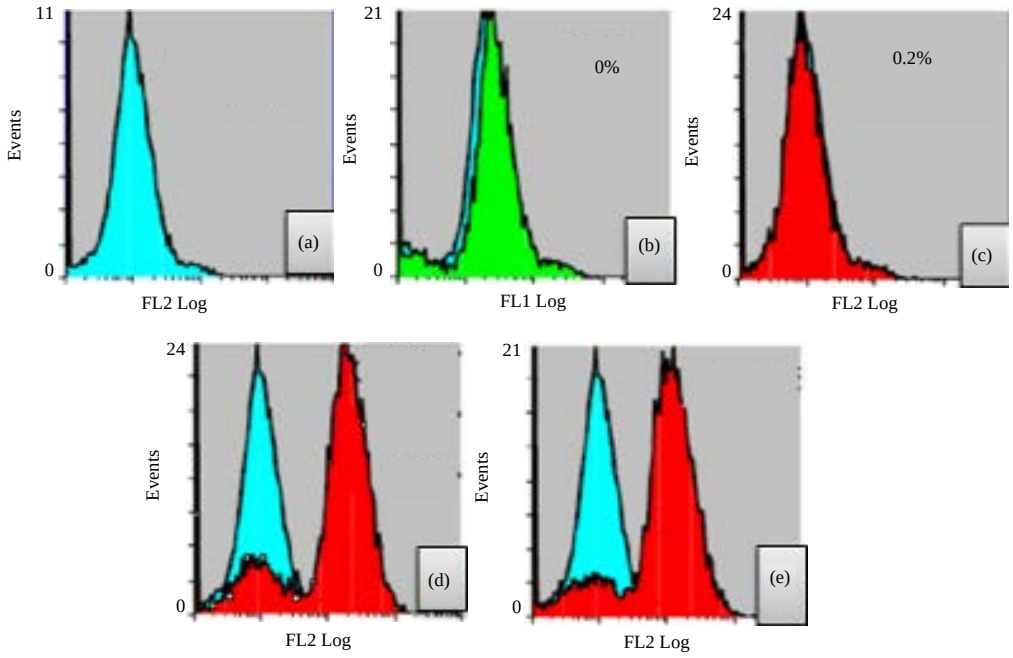

Fig. 1(a-e): A flow-cytometric analysis of dog MSCs, (a) MSCs negatively expressed hematopoietic stem cell markers including (b) CD45 and (c) CD34 but positively expressed MSC specific markers (d) CD29 and (e) CD105

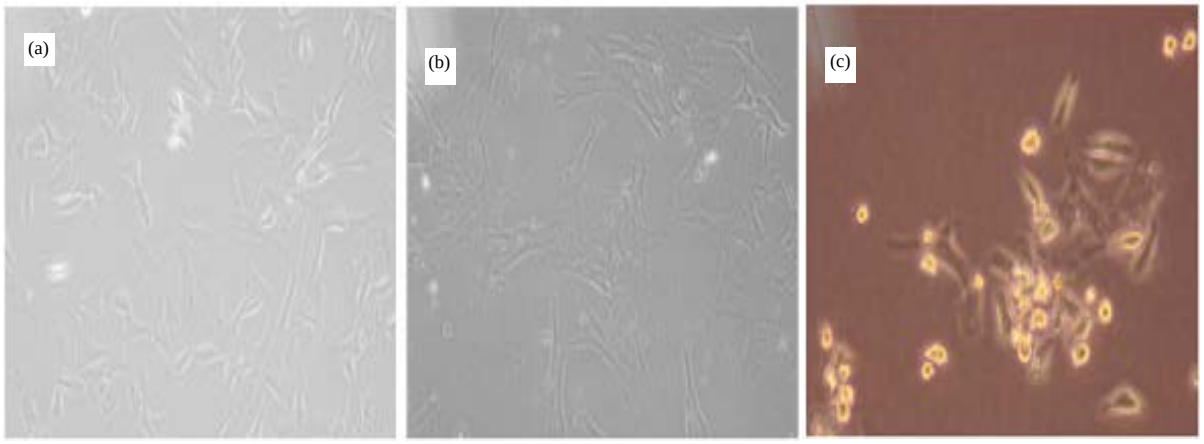

Fig. 2(a-c): (a) BM-MSCs at two weeks of culture (70-80% confluent), (b) Differentiated BM-MSCs into hepatocytes like cells and (c) Immunoflourescent albumin antibodies in differentiated hepatocytes

improvement with healthy mucous membrane. Appetite and weight returned approximately to normal at the 30th week.

Effect on LFTs: The biochemical and hematological test values varied from the base line, some values went higher such as ALT, AST, T. bili, D. bili, AP that significantly increased till 16th week while others went lower such as ALB, TP, HB, PLT, PT that had significantly decreased throughout the follow up period. Sham group at the end of the study showed significant increase in ALT, AST, T. bili, D. bili, AP levels as levels of theses enzymes were 157.91±9.08, 150.15±14.61, AP 379.45±55.06, 4.22±0.33 and 3.77±0.78, respectively while group II levels of enzymes at the end of the study, were 51.6±4.39, 49.47±6.16, 136.09±20.36, 0.92±0.11 and 0.82±0.21, respectively. Treatment with HD-MSCs significantly decreased ALT, AST, T. bili, D. bili, AP levels (p-value 0.05). Sham group at the end of the study showed significant decreased in ALB, TP, HB, PLT, PT levels as their levels were 1.01±0.13, 2.46±0.65, 5±0.47, 140±17.82 and 8.07±0.15, respectively while these levels in group II were 4.25±0.49, 6.52±1.02, 11±0.47, 371.74±38.43 and 12.04±0.96. Treatment with HD-MSCs significantly increased ALB, TP, HB, PLT levels (p-value 0.05) as shown in Fig. 3(a, b).

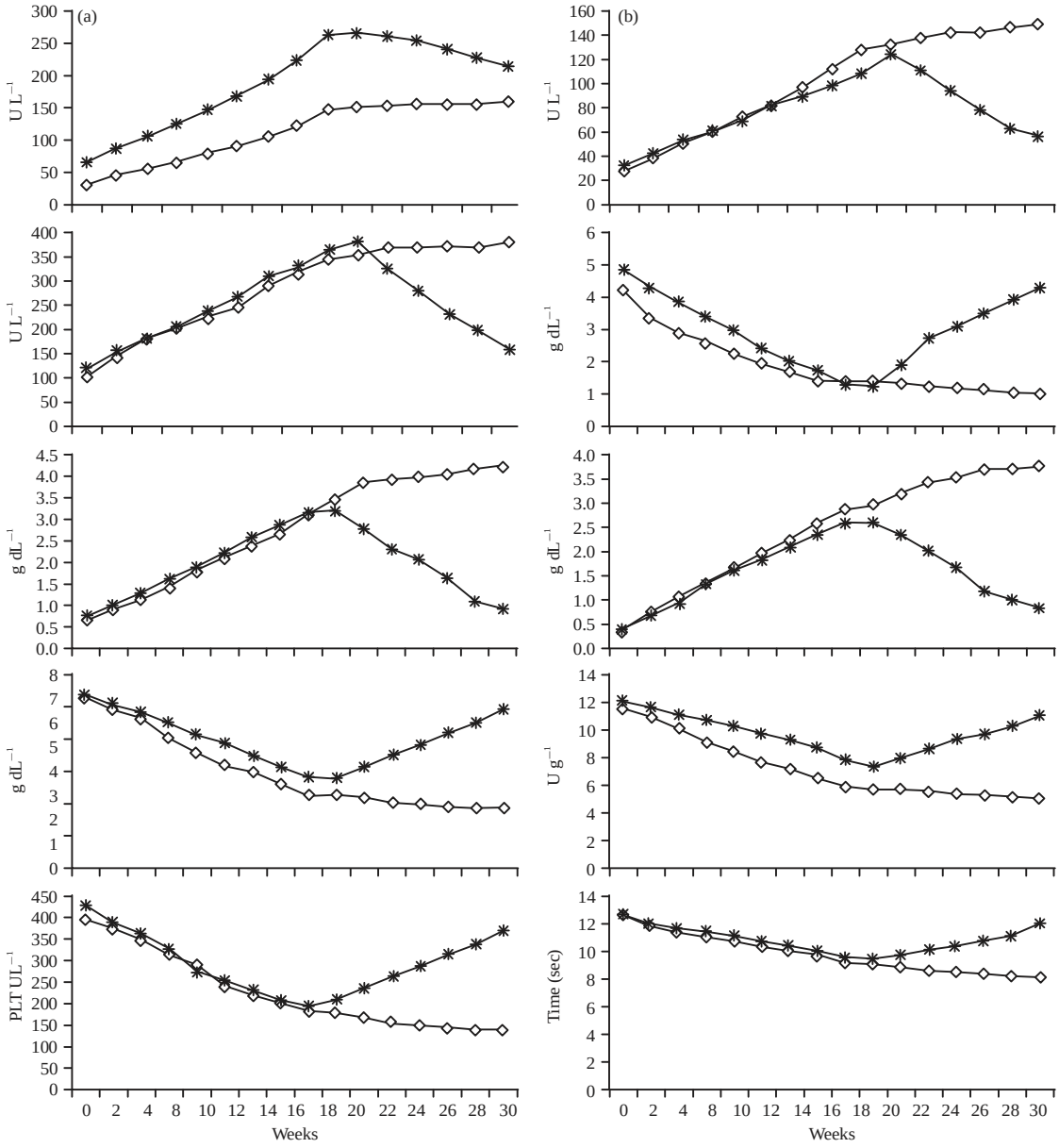

Fig. 3(a-b): Differences of liver function tests between (a) Sham and (b) Group II

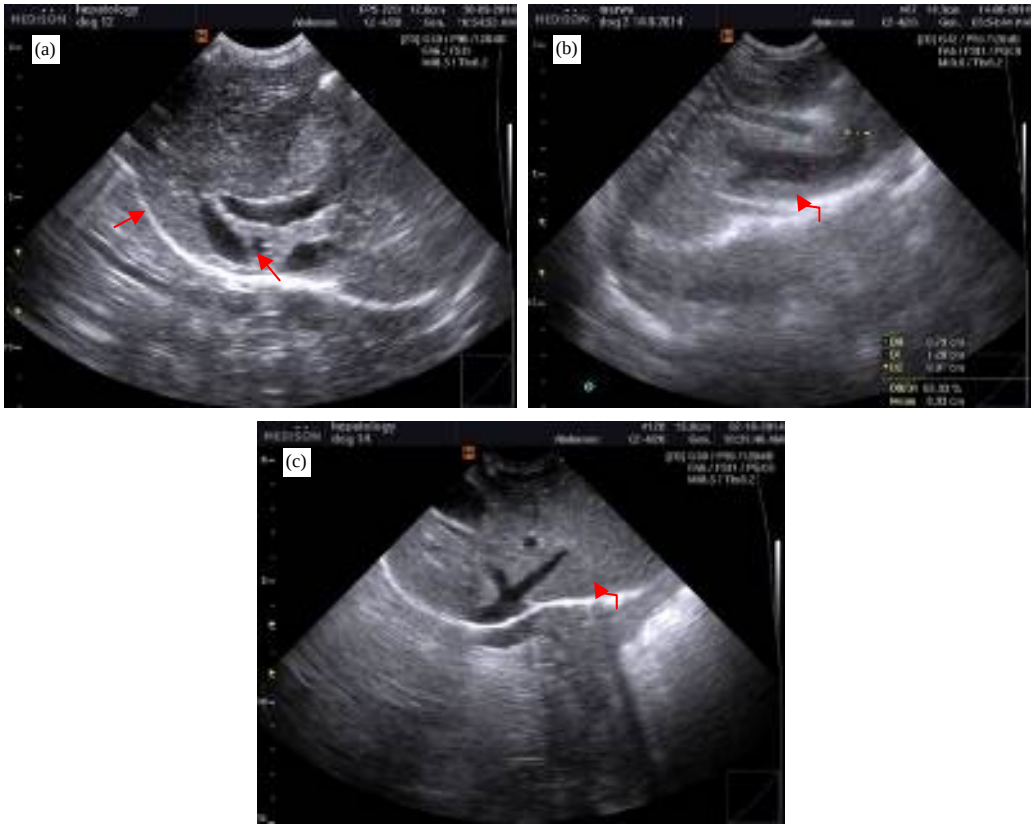

Fig. 4(a-c): (a) Sonography of mongrel dog's liver at 16th week showed serrated capsule (arrows), increased echogenicity of the liver, diameter of PV = 0.73 cm, diameter of HV = 0.50 cm, (b) Sonography of mongrel dog's liver without stem cell treatment (sham group) at 30th week showed a marked thickened liver capsule (arrow), coarse hyperechoic region in the liver parenchyma, diameter of PV = 0.79 cm, diameter of HV = 1.2 cm and (c) Sonography of mongrel dog's liver after stem cell treatment (group II) at 30th week showed a slightly thickened liver capsule (arrow), fine granular hypoechogenic regions in the liver parenchma, diameter of PV = 0.45 cm, diameter of HV = 0.54 cm

Ultrasonographic examinations: Ultrasonographic examinations of the liver differed from the normal one as the liver had a thickened capsule, echogenicity of the liver increased, Portal Vein (PV) and Hepatic Vein (HV) were dilated (0.73±0.03 and 0.64±0.11 cm, respectively) at the 16th week (Fig. 4a). In sham group, the liver didn't changed but thickening of capsule increased and became coarse hyperechoic and diameter of PV and HV were significantly increased (0.78±0.01 and 1.12±0.08 cm, respectively p-value 0.05) at the 30th week (Fig. 4b). While in group II, the liver showed significant improvement as the liver capsule slightly increased with presence fine granular hypoechogenic regions in the liver parenchyma and diameter of PV and HV were significantly decreased (0.43±0.03 and 0.52±0.02 cm consequently, p-value 0.05) in the 30th week (Fig. 4c). The change in PV and HV diameters is recorded in Fig. 5a, b for both sham and group II.

Histopathology of liver tissues: Histopathological examinations of the liver differed significantly from the normal and continued to change with the progression of fibrosis as presence of fibrous connective tissue proliferated in the portal area with septal fibrosis associated with severe vacuolar degeneration of hepatic cells with infiltration of inflammatory cells at different periods along the 16 weeks. In sham group, no improvement was noticed as presence of portal fibrosis associated with severe vacuolar degeneration at the end of 30 weeks (Fig. 6a, b) while group II showed good improvement of liver histoarchitecture, marked reduction in the fibrous connective tissue with deep eosinophilic cytoplasm around the portal area at the end of 30 weeks (Fig. 6c, d).

Post mortem findings: Liver of sham group was yellowish with mottled appearance, hard in texture and blunt edges

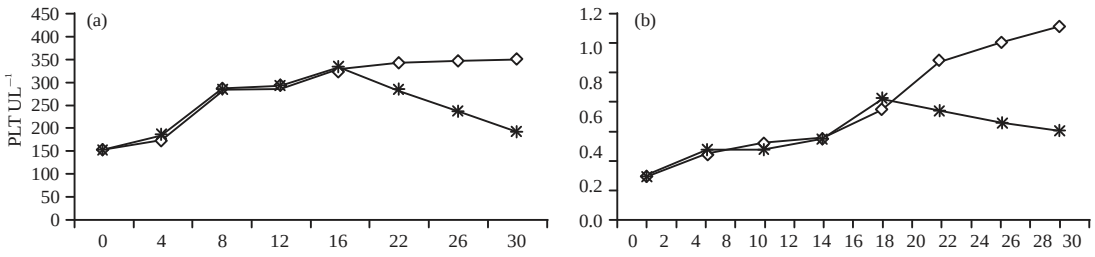

Fig. 5(a-b): Effect of MSCS on diameter of (a) PV and (b) HV

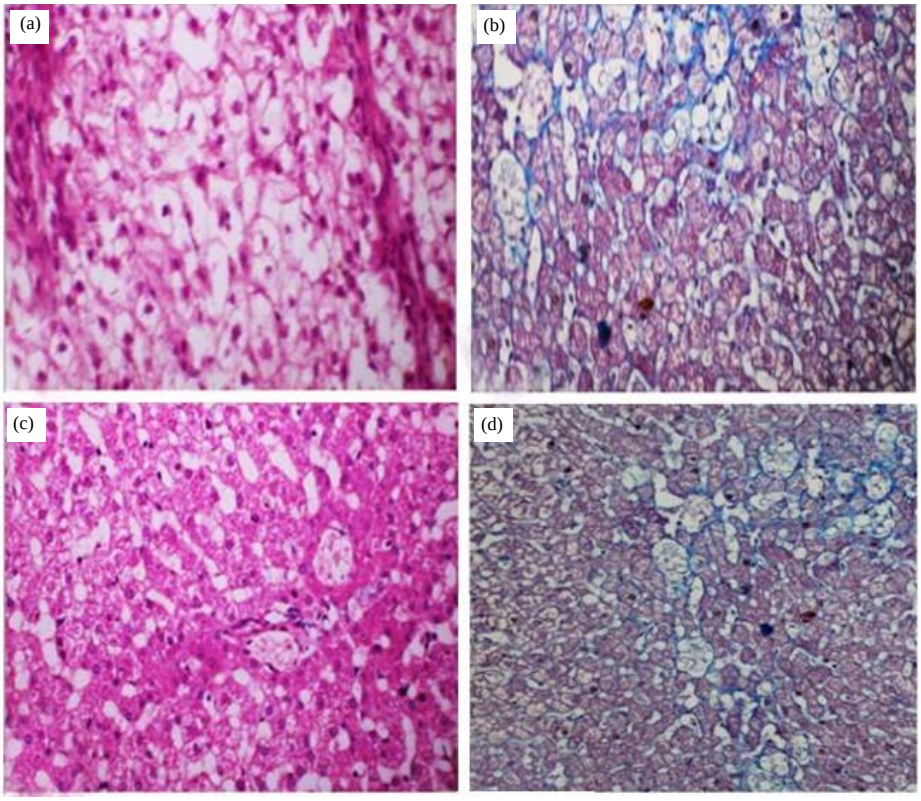

Fig. 6(a-d): Photomicrograph of dog's liver at 30th week, (a) sham group showing showing proliferation of fibrous connective tissue in the portal area with septal fibrosis and vacuolar degeneration of most hepatocytes with H and E×600, (b) MTC×400, (c) group II showing marked reduction in the fibrous connective tissue in the portal area with H and E×400 and (d) MTC×200

while the liver of group II was rosy red, soft texture with sharp edges (Fig. 7a-c).

Relative quantitative RT-PCR of collagen 1 alpha and albumin: Sham group showed significant increase in gene Collagen Iα gene expression (1.90±0.75) compared to group II (0.64±0.2) at 30th week ($p<0.05$). Sham group showed significant decrease in albumin gene expression (0.97±0.36) compared to group II (3.13±0.78) at 30th week ($p<0.05$) (Fig. 8a, b).

DISCUSSION

End-stage liver cirrhosis is an irreversible disease, characterized by loss of hepatocytes and increased deposition of the scar tissue in the liver parenchyma (Szatmari *et al*., 2004). Orthotopic liver transplantation was the only treatment option for these patients. However, the shortage of donors and the possibility of organ rejection are considerable limitations. Thus, there is urgent need for new and more available technologies for long-term support of patients with

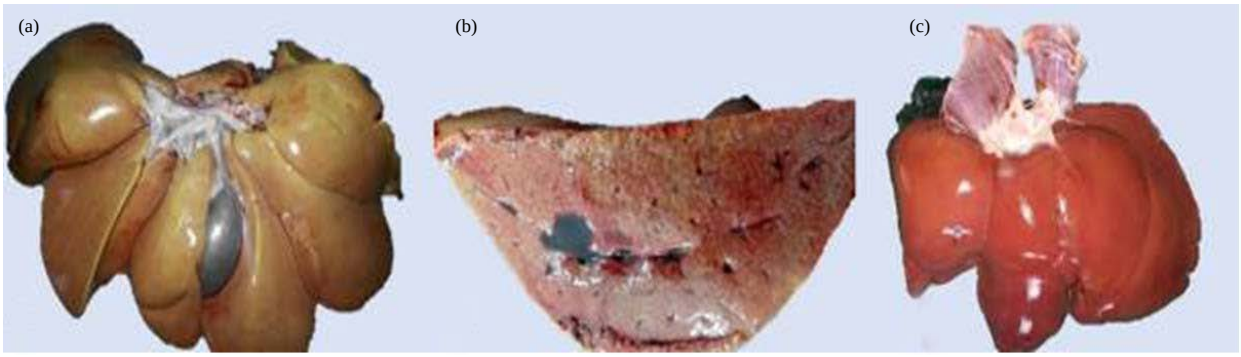

Fig. 7(a-c): Post mortem of dog's liver at 30th week after CCl_4 administration, (a) sham group showed yellowish liver, blunt and rounded edges, (b) Icteric color, icteric mottled appearance in cut section and (c) Group II showed sharp edges, soft texture and rosy red color

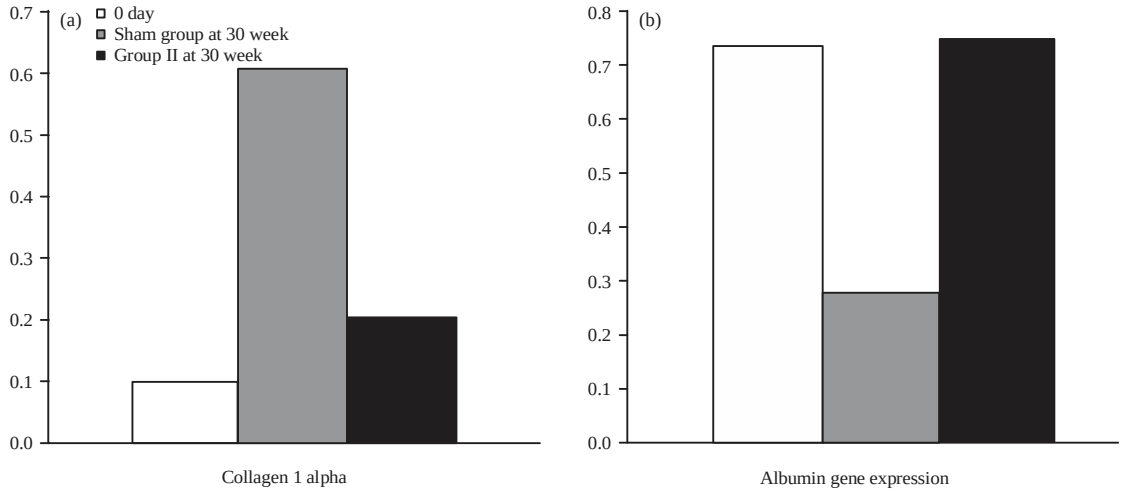

Fig. 8(a-b): Difference between collagen Iα and albumin gene expression between group-I and group-II at 0 day and 30th week, (a) Collagen Iα gene expression significant increase in sham group while significant decreased in group II and (b) Albumin gene expression significant decreased in sham group while significant increase in group II

liver failure. In the last years, several experimental studies demonstrated beneficial effects of using MSCs as an alternative approach in the treatment of cirrhosis (Palmes and Spiegel, 2004). The MSCs have also been proved to be a potential solution in severe liver injury such as ischemia-reperfusion injury, acute and chronic liver failure or HCC (Bataller and Brenner, 2005). The potential for MSCs to differentiate into hepatocytes and their immunomodulatory capabilities make MSCs an attractive source in the therapy of acute or chronic liver diseases. Previous studies have shown that transplanted MSCs replace the damaged cells and repopulates in the injured tissues or organs (Sakaida *et al*., 2004). However, the number of long-term substituted MSCs is disputable because those numbers differed depending on the injury models, the transplanted route and time and so on (Sun *et al*., 2012). The CCl_4 is affect adversely on the liver function as it causes lipid peroxidative degradation of biomembrane of endoplasmic reticulum rich in polyunsaturated fatty acids, which is one of the principle causes of hepatotoxicity. This leads to the formation of lipid peroxide which intern gives toxic radical species such as trichloromethyl (CCl_3*), trichloromethylperoxy ($OOCCl_3$*) and chlorine (Cl*) free radicals as well as phosgene and aldehydic products of lipid peroxidation that causes damage to liver (Wu and Tao, 2012). This radical can also react with oxygen to form a highly reactive species, trichloromethylperoxy radical (CCl_3OO*) that affects the permeability of mitochondrial endoplasmic reticulum and plasma membranes resulting in

the loss of cellular calcium sequestration and homeostasis leading to severe cell lysis so the cytoplasmic enzymes of the liver are released into the blood stream leading to an increase in their serum levels (ALT, AST, ALP and bilirubin). Moreover, hepatocytes damage lead to impairment of metabolism of bilirubin in the liver with subsequent excessive breakdown of red blood cells in the blood, bilirubin increase in the blood (hyperbilirubinemia) leading to increase serum levels of total and direct bilirubin (Meier *et al*., 2013). This causes the development of the yellowish discoloration of the skin, gum and mucous membrane of the eye (icterus) similar findings agrees with Bigoniya *et al*. (2009). On the other hand, the total protein and albumin levels had significant decreased (p-value 0.05). This could also be explained through the adverse toxic effect of CCl_4 on hepatocytes that leads to the impairment of protein synthesis, reduced absorption of amino acids and loss protein in urine. All these factors lead to the observed emaciation conditions in all intoxicated dogs. This finding is in agreement with Bigoniya *et al*. (2009). As a result of hepatocellular damage, there is impairment in the secretion of coagulation factors which was indicated by the significant decrease in PT level and PLT count. These results agree with Sourabie *et al*. (2012).

Serum collagen Iα gene expression levels in the group II were significantly higher than in the Sham group, albumin gene expressions levels were significantly lower than in group II suggesting that the biological activities of injected hepatocytes induced liver regeneration in cirrhotic dogs.

As a result of replacement of hepatocytes with fibrous connective tissue in Sham group, the liver appeared hyperechoic, non-homogenous and serrated capsule on ultrasonography similar to Al-Fartosi *et al*. (2012). Where as in group II, the liver appeared homogenous with normal echogenicity as cirrhotic cells were reduced as that suggested that the biological activities of injected hepatocytes induced liver fibrous connective tissue in cirrhotic dogs.

Cirrhosis is often complicated with portal hypertension. Endothelin (ET) is the most powerful vasoconstrictor peptide; it is also a damage-inducible factor produced during ischemia, hypoxia and chronic liver diseases (Mammen, 1992). The ET-1 is the most important member of the ET family and is one of the primary inducers of portal hypertension (Kuroda *et al*., 2006). It catalyzes the phosphorylation of amino acid residues in many kinases through the G-protein complex-phospholipase C-protein kinase C signaling pathway, regulates gene expression, the synthesis of collagen and matrix proteins, as discussed by Kuroda *et al*. (2006). After ET-1 binds to endothelin receptor, it activates voltage-dependent calcium channels, promoting calcium influx and leading to vasoconstriction leading to increased portal vein pressure (portal hypertension). In our study, ultrasound results demonstrated that the Sham group had a significant portal hypertension while group II showed significant reduction in portal and hepatic vein diameter suggesting that treatment of hepatic cirrhosis with MSCs is able to regulate the expression of ET.

Histopathology of cirrhosis (Sham group) was characterized by presence of fibrosis, with destruction of other normal liver tissues including; the sinusoids and other vascular structures, which in turn increased resistance to blood flow in the liver and portal hypertension arise. This finding is congruent with the results obtained by Guo *et al*. (2004) Collagen fiber hyperplasia was significantly reduced in dogs treated with hepatocytes stem cells. A small number of collagen fibers around the lobules were observed in treated livers (group II) and the majority of hepatocytes in the lobules were morphologically normal.

Liver was firmer in consistency than normal with blunt edges in Sham group as a result of deposition of fibrous connective tissue between the lobes of the liver. The developed icterus could be attributed to the toxic damage of CCl_4 on hepatocytes, both findings agree with Raja *et al*. (2009) on the other hand, liver in group II regained normal color and consistency indicating presence of renewed functional hepatocytes.

CONCLUSION

All these results demonstrate that hepatocyte-differentiated MSCs have significantly improved liver morphology and functions in dogs with hepatic cirrhosis. These cells have also effectively inhibited hepatic fibrosis and promoted normal hepatocyte proliferations and functions.

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