Therapeutic effects of mesenchymal stem cells-conditioned medium derived from suspension cultivation or silymarin on liver failure mice

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Received: 24 January 2022 / Accepted: 8 July 2022 / Published online: 12 September 2022
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Abstract
Background  Common treatments of liver disease failed to meet all the needs in this important medical field. It results in an urgent need for proper some new adjuvant therapies. Mesenchymal stem cells (MSCs) and their derivatives are promising tools in this regard. We aimed to compare the Silymarin, as traditional treatment with mesenchymal stem cell conditioned medium (MSC-CM), as a novel strategy, both with therapeutic potentialities in term of liver failure (LF) treatment.

Methods and results  Mice models with liver failure were induced with CCl4 and were treated in the groups as follows: normal mice receiving DMEM-LG medium as control, LF-mice receiving DMEM-LG medium as sham, LF-mice receiving Silymarin as LF-SM, and LF-mice receiving MSC sphere CM as LF-MSC-CM. Biochemical, histopathological, molecular and protein level parameters were evaluated using blood and liver samples. Liver enzymes, MicroRNA-122 values as well as necrotic score were significantly lower in the LF-SM and LF-MSC-CM groups compared to sham. LF-SM showed significantly higher level of total antioxidant capacity and malondialdehyde than that of LF-MSC-CM groups. Sph-MSC-CM not only induced more down-regulated expression of fibrinogen-like protein 1 and receptor interacting protein kinases1 but also led to higher expression level of keratinocyte growth factor. LF-MSC-CM showed less mortality rate compared to other groups.

Conclusions  Hepato-protective potentialities of Sph-MSC-CM are comparable to those of Silymarin. More inhibition of necroptosis/ necrosis and inflammation might result in rapid liver repair in case of MSC-CM administration.

Keywords  Conditioned medium · Silymarin · Liver failure · RIPK1 protein · MIRN122

Introduction

Liver diseases are kind of condition that have frequent rate of related mortality [1–3]. Excessive alcohol intake, obesity, viral hepatitis infections and modern lifestyle are the most important risk factors being leading to make a new epidemic wave of liver diseases [4, 5].

Treatment options for common liver diseases such as fatty liver and acute liver failure (ALF) [5, 6] are highly problematic. Liver transplantation is a one of the most common treatment of these sever diseases; however, it is associated with important challenges such as liver donor shortage, risk of immune rejection and side effects of immunosuppressive drugs [7, 8].

Botanical medicine has been used traditionally as herbal therapy worldwide for the prevention and treatment of liver disease [9]. In traditional medicine, Silybum marianum is one of the plants which is widely prescribed for the treatment of liver disorders [10–12]. Silybum marianum, also
known as milk thistle, contains a unique flavonoid complex called Silymarin which contains Silydianin, Silybin and Silychrisin, and is thought to have liver protective properties [13, 14]. Both in vitro and in vivo researches suggest that Silymarin has protective properties for liver cells against toxins [11, 13–15]. Feher and Lengyel mentioned Silymarin as an adjuvant therapy for hepatocellular carcinoma due to its anti-inflammatory effects [16].

In recent years, researchers' focus shifted from traditional medicine to mesenchymal stem cells (MSCs)-based strategies and novel cell-free strategies in term of clinical treatment [17, 18]. However, cell-free strategies with several advantages over cell-based strategies shows a promising future in the field of liver disorders [19, 20]. In this regard, the existing evidence suggests that the MSCs conditioned medium (MSC-CM) containing secreted soluble molecules, extracellular proteins, exosomes/microvesicles etc. mediate their principal therapeutic properties through paracrine effects instead of transdifferentiation mechanism in order to substitute injured cells [21–23]. Bioactive molecules of MSC-CM such as growth factors, angiogenic factors and proteases [22–25] involve in either physiological process regulation or pathological condition management [23, 26] especially in acute liver diseases [25]. According to available published studies and our experience, we aimed to compare the Silymarin, as traditional treatment with MSC-CM, as a novel strategy, both with therapeutic potentialities in term of ALF treatment.

**Materials and methods**

**Herbal extract**

Livergol, generic name and brand of Silymarin (Goldaru, Iran) was purchased from a drugstore in the form of 140 mg-tablet. The tablets were dissolved in normal saline.

**MSCs culture**

In order to prepare MSC-CM, human umbilical-derived MSCs were isolated and characterized as described previously [27]. All protocols were conducted after filling out the consent forms by parents. Next, the isolated MSCs were subjected to usual monolayer adherent or non-adherent spheroid-form cultivation as described previously [27]. PolyHEMA polymer was used to inhibit MSCs attachment on the surface of cell culture plates.

**Preparations of MSC-CM and its characterization**

MSC-CM was prepared as follows: either MSCs under adherent condition or those under non-adherent suspension condition which resulted in spheroid colony were cultured in serum-free medium for 48 h. The supernatants were aspirated gently, filtered through 0.22 μm-filter and then, transferred to Vivaspin® 500 centrifugal filter units (Sartorius, Germany). Finally, two kinds of CM, MSC-CM and spheroid-MSC-CM (Sph-MSC-CM) were centrifuged, 4000×g for 30 min at 4 °C. Bicinchoninic acid assay kit (Kiazist Life Sciences, Iran) was utilized for protein level determination in MSC-CM and Sph-MSC-CM. The concentration of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) were measured using enzyme-linked immunosorbent assay (ELISA) method (R&D Systems, USA) according to the manufacturer’s protocol.

**Animal study**

In this experimental study, 8-week-old Naval Medical Research Institute (NMRI) mice weighting 25 ± 2 g were purchased from Pasteur institute of Iran (Tehran, Iran). The mice were raised in our colony and kept in standard condition in term of light, food, and water accessibilities. Food was withheld 12–14 h before operation or death. In all experiments, the regulations of local authorities for handling laboratory animals and the National Institutes of Health (NIH) guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) were considered. This study was confirmed in ethic committee of Hamadan university of medical sciences (Ethic code No: IR.UMSHA. REC.1398.1077).

**Induction of liver failure in animals**

Carbon tetrachloride, CCl₄, (Merck, Germany) was used to produce mice models of experimental liver failure as established in our previous studies [28, 29] with some modifications in time period. 1.5 ml/kg CCl₄ dissolved in olive oil was determined as the appropriate dose for liver failure induction and administered intraperitoneally to the mice. The induction of liver failure was confirmed after 12 h with biochemical, histopathological and molecular methods.

**Different treatments of animal groups and study design**

In order to evaluate the hepatoprotective effects of Sph-MSC-CM or Silymarin, the mice were divided into four groups, each containing 10 mice as follow: normal mice receiving DMEM-LG medium or control as Cont., LF-mice receiving DMEM-LG medium as sham, LF-mice receiving Silymarin as LF-SM and LF-mice receiving Sph-MSC-CM as LF-MSC-CM. The Silymarin optimized injected dose and Sph-MSC-CM adjusted protein concentration were
determined. Their toxicity on mice fibroblast cell line and IC50 was determined using standard protocol. Then each group received 250 µl of the medium or Sph-MSC-CM (adjusted protein concentration: 200 µg/kg) or Silymarin (adjusted concentration: 100 mg/kg) at the same method according to the Institutional Animal Care and Use Committee (IACUC) recommendations intraperitoneally. All treatments were performed 12 h after the induction of liver failure with 1.5 ml/kg of dissolved CCl4 in olive oil. Subjected animals received the treatments daily for three consecutive days. Blood samples and liver tissues were collected 12 and 72 h post treatments.

**Evaluation of liver enzymes**

Blood samples were collected by cardiac puncture technique under deep anesthesia with ketamine (150 mg/kg) and xylazine (15 mg/kg) cocktail (Alfasan, Netherland). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured. Commercial kits (Parsazmoon, Iran) and automatic analyzer system (BT 3000 PL/S, Italy) were used. Argininosuccinate synthetase (ASS), as sensitive biomarker of liver injury, level was also detected using specific ELISA kit (My biosource, Canada).

**Preparation of liver tissue homogenates**

After blood sample collection, each mouse was sacrificed by cervical dislocation and placed in an ice box. Next, the body was sheared to open the abdomen. Dissection was performed and the liver tissues were collected. The liver tissue was minced to small pieces (0.5 g) and rinsed with cold phosphate buffer saline (Sigma, USA). The tissues were primed with 200–300 µl lysis buffer (Sigma, USA) and sonicated with an ultrasonic homogenizer (BioLogics, USA) three time for 20 s. The resulting suspensions were centrifuged at 10,000 g for 5 min. By harvesting the supernatant, the protein level was determined (Kiazist Life Sciences, Iran). 

**Real-time polymerase chain reaction (PCR)**

To confirm molecular level expression of intended genes and biomarkers including MicroRNA-122, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), receptor interacting protein kinases 1 (RIPK1), fibrinogen-like protein 1 (FGL-1) and keratinocyte growth factor (KGF), real-time PCR was performed on liver tissue homogenates. Total RNA was extracted (Kiazist Life Sciences, Iran). The procedure was performed according to the suggested protocols by kit manufacturer. Then, 500 ng/ml of total RNA was subjected to cDNA synthesis (BIONEER, South Korea). Quantitative-RT-PCR (qRT-PCR) was performed using SYBR green master mix (Takara, Japan) and designed specific primers (Table 1S). The LightCycler® 96 System (Roche, Bavaria, Germany) was utilized to perform reactions in triplicate. Fold change of gene of interest expression to β-actin expression was calculated after melting curve analysis using 2−∆∆Ct method.

**Evaluation of oxidative stress status**

Colorimetric method was applied to determine the level of oxidative stress in liver tissue homogenates. Malondialdehyde (MDA) as a lipid peroxidation marker and total antioxidant capacity (TAC) level was assayed by specific biochemical analysis kits (ZellBio, Germany). GSH-Px and SOD activities were also determined with commercially available kits (ZellBio, Germany) according to the manufacturer’s protocols.

**Western blot analysis**

Translational level of RIPK1, FGL-1 and KGF were detected in liver tissue homogenates. First, 12% sodium dodecyl-sulfate polyacrylamide gel (SDS-page) was prepared and sample proteins were separated according to their molecular weight by electrophoresis. Then, Semi-dry blotter (Bio-Rad, USA) was used for blotting the proteins onto the polyvinylidene difluoride (PVDF) membrane (Roche, Germany). The membranes were incubated with primary antibodies including anti-FGL-1 (Abcam, UK), anti-KGF (Abcam, UK), anti-RIPK1 (LifeSpan BioScience, USA) and anti-β-actin (Sigma, USA) after setting up the time, temperature and proper dilution separately. Afterwards, secondary antibody (Abcam, UK) was used on the surface of the membrane. Electrochemiluminescence (ECL) substrate for horseradish peroxidase (Abcam, UK) was added to the membranes, and imaging was performed using gel doc imager (Bio-Rad, USA). The density of protein bands was semi-quantified using ImageLab software.

**Histopathological assay**

To detect tissue necrosis and inflammatory cells infiltration, liver tissue sections were analyzed histopathologically. The animals were dissected and their livers were separated and fixed with formalin. After preparation of liver sections, they were stained with hematoxylin and eosin (H & E) to determine the necrosis/inflammation scores as described in our previous study [28] with some modifications. Necrosis and inflammation level were quantified by estimating the intralobular necrotic bridges, necrotic cells and
inflammatory cell numbers under high-power field (HPF) at least in 30 light microscope fields.

**Survival assay**

The survival rate of animal groups was analyzed after treatments within 2 weeks. The mice (n = 10) were observed every day in terms of their survival rate.

**Statistical analysis**

The quantitative values were given as mean ± SD. SPSS version 21 software (SPSS Inc., Chicago, IL, USA) and One-way ANOVA test was utilized to data analysis statistically. p < 0.05 was considered as statistically significant. The survival rate was analyzed by Log Rank (Mantel-Cox) test and presented via Kaplan–Meier plot. All experiments were done in triplicate.

**Results**

**Sph-MSC-CM contains more hepatoprotective growth factors than MSC-CM**

After harvesting MSC-CM and Sph-MSC-CM, the concentrations of VEGF, HGF and FGF were measured in both mentioned CMs. Protein expression levels of these growth factors were up regulated using suspension cultivation (Fig. 1S). As shown in Fig. 1S, Sph-MSC-CM contains much more amount of these growth factors especially VEGF (p < 0.001). Base on this finding, Sph-MSC-CM was used for the next step of the study.

**1.5 mg/kg CCl₄ induces liver failure in mice**

12 h after the injection of 1.5 mg/kg CCl₄, the induction of LF was assayed with different laboratory methods. Increased AST and ALT levels were confirmed in CCl₄-treated mice in comparison with controls that do not receive any CCl₄ by biochemical assay (p < 0.001) (Fig. 2Sa). The results of ELISA were indicated that ASS, as early sensitive liver injury biomarker, concentration was very high in LF-induced mice (p < 0.001) (Fig. 2Sb). More expression of MicroRNA-122, as a marker of liver injury (p < 0.001) was detected using real-time PCR in CCl₄-treated group (Fig. 2Sc). According to the results of both biochemical and molecular assays, 1.5 mg/kg of CCl₄ administration led to severe liver injury. For more confirmation, liver sections of both the CCl₄-treated mice and control groups were evaluated after H&E staining (Fig. 2Sd). Extensive intralobular necrotic bridges and multifocal diffusion lobular necrosis were detected in the sections that were accompanied by increasing number of necrotic and inflammatory cells in the portal area or around the necrotic hepatic cells (Fig. 2Sd).

**Administration of Silymarin Sph-MSC-CM decreases both the liver enzymes and MicroRNA-122 values in LF-mice**

After CCl₄ injection, animals were treated by Silymarin (LF-SM) or Sph-MSC-CM (LF-MSC-CM) as experimental groups along with the control groups. The biochemical assays indicated the alterations of both the ALT and AST levels in the serum as well as ASS levels in the experimental groups (Fig. 1a–c). 12 h after treatment, a decrease in the liver enzyme levels were detectable in LF-SM and LF-MSC-CM compared with sham especially in terms of ASS (p < 0.05). In the next 72 h, AST, ALT and ASS were significantly lower in the LF-SM and LF-MSC-CM groups compared with sham group (p < 0.001) (Fig. 1a–c). As shown in Fig. 1d, molecular assay results and decreased level of MicroRNA-122 were consistent with biochemical assay results and revealed the reduction of liver failure in both the LF-SM and LF-MSC-CM mice compared to sham (p < 0.05, p < 0.001) (Fig. 1d).

**Treatment with Silymarin or Sph-MSC-CM not only up regulates SOD, GSH-Px, FGL-1 and KGF but also alleviates RIPK1 gene expression**

In order to determine the mechanism underlying Silymarin or Sph-MSC-CM therapeutic effects, transcriptional expression levels of some genes including SOD, GSH-Px, RIPK1, FGL-1 and KGF were evaluated through real-time PCR. The expression levels of SOD, GSH-Px (anti oxidative stress enzymes) along with FGL-1 and KGF (regenerative and repairing markers) increased in LF-SM and LF-MSC-CM in comparison with sham (p < 0.01, p < 0.001) (Fig. 2a). The findings indicated that GSH-Px gene expression in liver tissue of LF-SM was a little more than that of LF-MSC-CM (p < 0.05). However, the expression of FGL-1 enhanced in LF-MSC-CM much more than LF-SM (p < 0.01) (Fig. 2a). On the other hand, the administration of Silymarin or Sph-MSC-CM led to a decrease in the level of RIPK1 (necroptosis/ necrosis marker) gene expression (p < 0.01, p < 0.001) (Fig. 2b). However, much less RIPK1 expression was detectable in LF-MSC-CM mice (p < 0.05) (Fig. 2b).

**Injection of Silymarine enhances defense mechanisms against oxidative stress a little more than Sph-MSC-CM**

For more investigation, the level of MDA and TAC as well as SOD and GSH-Px activities were assayed in the liver homogenates of animal models through specific laboratory
methods. Both treatments with Silymarin and Sph-MSC-CM resulted in the improvement of antioxidant capacity compared with no treatment condition ($p < 0.01, p < 0.001$). As shown in Fig. 3a, more increased TAC level was determined in LF-SM compared with LF-MSC-CM ($p < 0.01$). According to the data in Fig. 3b, the amount of main lipid

Fig. 1 Evaluation of Silymarin or Sph-MSC-CM effects on liver enzymes and MicroRNA122 expression 12 and 72 h after treatment. a ALT levels. b AST level. c ASS concentration. ASS reduction was significantly detected in experimental groups (LF-SM and LF-MSC-CM) 12 h after Silymarin or MSC-CM administration. d MicroRNA-122 fold change ratio related to β-actin in different groups 12 and 72 h after treatment. Early decrease in the level of MicroRNA-122 was observed only in LF-MSC-CM 12 h after Sph-MSC-CM administration. Data was shown as mean ± SD. * $p < 0.05$, *** $p < 0.001$ LF-SM and LF-MSC-CM vs. sham.

Fig. 2 SOD, GSH-Px, KGF, FGL-1 and RIPK1 expression 72 h after treatment. Real time-PCR was performed to quantified gene expression in liver homogenates. a Gene expression level of SOD, GSH-Px, FGL-1 and KGF 72 h after Silymarin or Sph-MSC-CM injection. Date indicated that SOD, GSH-Px, FGL-1 and KGF genes were up regulated in LF-SM and LF-MSC-CM in comparison with sham. Less expression of GSH-Px was detected in LF-MSC-CM compared with LF-SM. Moreover, LF-MSC-CM showed higher FGL-1 gene expression vs. LF-SM. b RIPK1 gene expression. Both LF-SM and LF-MSC-CM groups expressed down regulated level of RIPK1. The significantly lower RIPK1 expression was detected in LF-MSC-CM. All fold changes of gene expression were normalized by β-actin gene expression. Data was shown as mean ± SD. *** $p < 0.01$, *** $p < 0.001$ LF-SM and LF-MSC-CM vs. sham. # $p < 0.05$, ## $p < 0.01$ LF-MSC-CM vs. LF-SM. GSH-Px Glutathione peroxidase, SOD superoxide dismutase, RIPK1 Receptor interacting protein kinases, FGL-1 Fibrinogen-like protein 1, KGF keratinocyte growth factor, Sph-MSC-CM Conditioned medium from spheroid form of mesenchymal stem cell, LF-SM LF mice receiving Silymarin, LF-MSC-CM LF mice receiving Sph-MSC-CM.
peroxidation marker, MDA, was higher in sham against treated animal \( (p < 0.001) \). MDA content of LF-MSC-CM liver homogenates was more than that of LF-SM \( (p < 0.05) \). Similar to the molecular results for \( SOD \) gene expression, \( SOD \) enzyme activity was higher \( (p < 0.001) \) in both experimental groups compared with sham. But its level was not significantly different in both LF-SM and LF-MSC-CM groups (Fig. 3c). No significant difference was observed between LF-SM and LF-MSC-CM in terms of GSH-Px activity (Fig. 3d). Altogether, Silymarine could reinforce relative antioxidant effects against harsh conditions in LF-SM.

**LF-MSC-CM group shows more liver regeneration and less necroptosis/necrosis protein marker levels**

Using molecular assay for the quantification of mRNA amounts of \( RIPK1, FGL-1 \) and \( KGF \), western blot analysis was also performed to detect their translational expression level (Fig. 4a). Semi-quantification of protein bands density results showed increasing level of FGL-1 and KGF either in LF-SM or LF-MSC-CM group \( (p < 0.01, p < 0.001) \) (Fig. 4b). Interestingly, FGL-1 protein was expressed at high measurable level following the injection of Sph-MSC-CM \( (p < 0.05) \) (Fig. 4b). On the other hand, RIPK1 protein was down-regulated in Silymarin and Sph-MSC-CM receiving mice \( (p < 0.01, p < 0.001) \). In consistent with molecular assay results, western blot results also showed much more decreasing protein level of RIPK1 for LF-MSC-CM groups compared with LF-SM \( (p < 0.05) \) (Fig. 4b).

**Sph-MSC-CM induces relatively more liver re-construction and less liver inflammation**

The histopathological analysis was performed to evaluate liver repairing in prepared liver section. There were no intralobular necrotic bridges and diffuse necrosis zones, as severe necrosis score, in the liver sections of both Silymarin and Sph-MSC-CM-treated animals (Fig. 5a). However, either multiple intralobular necrotic bridges and diffuse necrosis zones or more inflammatory and necrotic cells were detectable in sham (Fig. 5a). Moreover, decreased inflammatory and necrotic cells were also observed in histopathological examination of liver sections of LF-SM and LF-MSC-CM \( (p < 0.001) \) (Fig. 5b) through estimating the numbers of intralobular necrotic bridge, necrotic cells and inflammatory cells. Interestingly, LF-MSC-CM group with less numbers of inflammatory and necrotic cells \( (p < 0.01) \) might be interpreted as a quick start recovery (Fig. 5b). These data
even more confirmed the data gathered from other laboratory techniques. Therefore, the administration of these mentioned treatments not only inhibited necrosis progression in liver but also repaired the damaged tissue with the quick inflammation control compared to sham.

**LF-MSC-CM shows higher survival rate**

The survival rate of different animal groups was estimated during 2 weeks. More mortality rate was seen in the sham group. Sph-MSC-CM-receiving mice, LF-MSC-CM, showed more mean of survival time compared to the sham group and even LF-SM group (Fig. 5c).

**Discussion**

In current study, we used Silymarin or MSC-CM for treatment of CCl4-liver failure-induced mice. CCl4 produces reactive oxygen species (ROS) and induces liver failure [28–30].
Our results showed that cultivation of umbilical cord-MSCs in suspension condition enhanced their growth factor secretory capacities. Employment of this strategy improved the ability of subjected MSCs in VEGF, HGF and FGF secretion. It seems MSCs culturing in non-adherent non-monolayer condition results in either enhanced secretory effects or expanded differentiation potentialities of these cells [27, 31].

Our findings indicated that both Silymarin and Sph-MSC-CM ameliorated liver injury and reduced AST, ALT, ASS and MicroRNA-122 levels after intraperitoneal injection to LF mice models. Measurement levels of ASS and MicroRNA-122 along with AST, ALT give a chance to faster confirmation of LF or therapy efficiency. The possible mechanisms behind this finding might be presented by literature reviewing. ASS levels increase more rapidly than ALT and AST and make it a reliable sensitive biomarker for the detection, progression and monitoring liver injury [32]. MicroRNA-122 is expressed in liver tissue more than other organs and play important roles in the liver injury and repair [33, 34]. Consistent with our results, Hermenean et al. reported the decreasing effects of Silybum Marianum seed oil extract on the activities of liver enzymes such as ALT, AST and gamma glutamyl transferase (GGT) [35]. They pre-treated the mice with this herbal oil extract orally at the dose of 10 g/kg during a time period of 21 days before the induction of liver failure with CCl₄ [35]. In current study, a dose of 100 mg/kg Silymarin using IP route was administrated for 3 days. Although, having a significant difference in ASS and MicroRNA-122 as mentioned above, there was no significant difference among the groups in regards to ALT and AST levels measured at 12 h after treatment which could be due to their half-life in the circulation as an average of 47 and 17 h, subsequently [32, 35]. In another study, decreased level of ALT and AST was reported 24 h after the administration of MSC-CM which was prepared by other methods [36].

The transcriptional and translational expression levels of lower RIPK1, FGL1 and KGF in LF-MSC-CM group treated with Sph-MSC-CM might explain the reasons of fast injury inhibition followed by rapid liver regeneration. Apoptosis, autophagy, necrosis and necroptosis that might be mediated by RIPK1 are different models of liver cell death under physiologic and pathologic conditions [37]. Among them, necroptosis that occur in RIPK1-dependent or/ and RIPK1-independent pathways and its proper regulation/ prevention play an important role in liver regeneration and hemostasis [38]. Necroptosis, also known as apoptosis and necrosis cross-talk, is kind of a programmed cell death that occurs on apoptosis back up and necrosis morphology [38].

FGL1, also known as hepassocin, increases hepatocyte proliferation by enhancing 3 H thymidine uptake and leads to liver regeneration [39]. Moreover, MSC-CM cytokine contents such as IL-6 could up regulate the expression of FGL-1 and result in rapid liver restoration [40]. MSCs and their CM stimulate KGF, a fibroblast growth factor family member, activation and positively influence the fate of liver diseases [41, 42]. Xagorari et al. (2013) confirmed both the anti-apoptotic and regenerative effects of MSC-CM on hepatocytes in vitro and in vivo by assessing the expression levels of annexin V/PI and FGL-1 [40].

Despite the ability of Sph-MSC-CM to address harsh CCl₄ induced oxidative stress, Silymarin administration proved to be much more effective. It not only induced an increase in TAC level, but also led to more reduction in MDA level. Silybin, the most common composition of Silymarin, is known for its different therapeutic properties. Not only it has antioxidant, anti-inflammatory and antiviral effects, but also it involves in mitochondrial function regulation [13, 14, 43, 44]. In the other word, another composition of Silybin, Taxifolin, acts as strong antioxidant [43, 44].

In another study, decreased MDA level and increased enzyme activities of SOD, GSH-Px and catalase were detected following oral administration of Silymarin [35]. Pouyandeh Ravan et al. (2019) showed the capacity of MSC-CM either to return the TAC content of hepatocytes or to control the total oxidative status and MDA production in liver fibrosis [30].

Moreover, in current study, a relatively higher survival rate was observed in Sph-MSC-CM-receiving mice. Lottinia et al. harvested the CM from embryonic stem cell-derived MSCs and assayed its therapeutic effects on thioacetamide-induced LF mice (400 µl-IP injection). Their results indicated that CM accelerates liver restoration through the induction of hepatocyte proliferation due to its immunomodulatory properties. However, it had no effect on mice survival rate [45]. On the other hand, they implied that the injection of recombinant VEGF led to more survival rate in the animal models [45]. In current study, Sph-MSC-CM derived from spheroid-form MSCs had approximately 4 times more VEGF content than that of MSC-CM derived from usual monolayer MSCs.

Many research studies have been conducted to evaluate the efficiency of herbal extracts or MSC-CM in liver failure treatment [10, 11, 13, 20, 23, 25, 26]. MSC-CM has made a breakthrough in regenerative medicine due to its safety, effectiveness, ease of delivery to the patients and no ethical complications in comparison with cell-based therapies [18–20].

An et al. (2016) administered thioacetamide or CCl₄ to induce liver fibrosis in mice. After 24 h, the animals were subjected to intraperitoneal injection of MSC-CM, 500 µg/kg [46]. We evaluate the therapeutic potentialities of MSC-CM 12 h after administration of 200 µg/kg of Sph-MSC-CM. Huang et al. infused 200 µl of MSC-CM or intact MSCs into the tail vein of both fulminant hepatic failure and liver...
fibrosis mice. Their results indicated that the anti-inflammatory property of MSCs and their CM led to a partial restoration of liver tissue while it had no effect on the animal survival rate [47].

Silymarin usage for the treatment of liver diseases was investigated in different studies [11, 14, 43]. Koçarslan (2016) evaluated hepatoprotective potentialities of 200 mg/kg Silymarin on rats [44]. Consistent with our findings; this study also reported an enhancement in TAC level and reduction in liver necrosis and inflammation. 50 mg of Silymarin flavonoids have bilirubin-mediated antioxidant effects on fibrous liver tissue [48].

Conclusions

In conclusion, both Silymarin and Sph-MSC-CM have hepatoprotective effects. However, the latter showed rapid inhibition of liver necroptosis/necrosis and inflammation. It indicates more promising results according to the survival rates. Strong hepatoprotective characteristics of Sph-MSC-CM could be due to the high content of useful growth factors including VEGF, HGF and FGF as well as anti-inflammatory, anti-apoptotic and anti-necrotic factors.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-07785-4.

Acknowledgements This work was supported by Hamadan university of medical sciences financially (No: 990202405, Ethic code No: IR.UMSHA.REC.1398.1077). Special thanks to high institute for research and education in transfusion medicine, Tehran, Iran for technical supports.

Author contributions Conceptualization: FA, Methodology: SM, SF and MB, Formal analysis: RS, Investigation and Writing—original draft preparation: SM and MB, Review and editing: FA, MB and SF. Funding acquisition: FA, RS, Supervision: FA.

Funding This work was supported by Hamadan university of medical sciences financially (No: 990202405).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent: MSCs were isolated from human umbilical cord samples after filling out the consent form by parents.

Research involving human and animal rights All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was confirmed in ethic committee of Hamadan university of medical sciences (Code No: IR.UMSHA.REC.1398.1077).

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