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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2024.18.26

Research Article
Secretome of Hypoxia-Preconditioned Mesenchymal Stem Cells Promotes Liver Regeneration and Anti-Fibrotic Effect in Liver Fibrosis Animal Model

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Abstract

Background and Objective: Liver fibrosis (LF) is a most common pathological process characterized by the activation of hepatocytes leading to the accumulation of extracellular matrix (ECM). Hypoxia precondition treated in MSCs (H-MSCs) could enhance their immunomodulatory and regeneration capability, through expressing robust anti-inflammatory cytokines and growth factors, known as H-MSCs secretome (SH-MSCs) that are critical for the improvement of liver fibrosis. However, the study regarding the efficacy and mechanism of action of SH-MSCs in ameliorating liver fibrosis is still inconclusive. In this study, the therapeutic potential and underlying mechanism for SH-MSCs in the treatment of liver fibrosis were investigated. **Materials and Methods:** A rat model with liver fibrosis induced by CCl_4 was created and maintained for 8 weeks. The rats received intravenous doses of SH-MSCs and secretome derived from normoxia MSCs (SN-MSCs), filtered using a tangential flow filtration (TFF) system with different molecular weight cut-off categories, both at a dosage of 0.5 mL. The ELISA assay was employed to examine the cytokines and growth factors present in both SH-MSCs and SN-MSCs. On the ninth day, the rats were euthanized and liver tissues were collected for subsequent histological examination and analysis of mRNA expression. **Results:** The ELISA test revealed that SH-MSCs exhibited higher levels of VEGF, PDGF, bFGF, IL-10, TGF-β and IL-6 compared to SN-MSCs. *In vivo*, administration of SH-MSCs notably decreased mortality rates. It also demonstrated a reduction in liver fibrosis, collagen fiber areas, α-SMA positive staining and relative mRNA expression of TGF-β. Conversely, SN-MSCs also contributed to liver fibrosis improvement, although SH-MSCs demonstrated more favorable outcomes. **Conclusion:** Current findings suggested that SH-MSCs could improve CCl_4-induced liver fibrosis and decrease α-SMA and TGF-β expression.

Key words: Secretome, mesenchymal stem cells, liver fibrosis, collagen, CCl_4

Citation: Amansyah, F., B. Budu, M.H. Achmad, N.A.S. Daud and A. Putra *et al.*, 2024. Secretome of hypoxia-preconditioned mesenchymal stem cells promotes liver regeneration and anti-fibrotic effect in liver fibrosis animal model. Pak. J. Biol. Sci., 27: 18-26.

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Liver fibrosis (LF) is the most common pathological process of Chronic Liver Disease (CLD) characterized by massive disruption of hepatocytes and infiltration of immune cells in the liver leading to excessive collagenous scar[1]. The massive inflammatory condition and hepatic necrosis in LF promote multiple organ failure and sudden loss of liver function that cause high mortality[2]. The curative management for LF, such as liver transplantation could become an effective therapy for patients, however long-life use of immunosuppressive drugs, lack of resources, rejection and cost issues remain serious problems[3]. Currently, the use of mesenchymal stem cells could provide LF improvement by controlling the proinflammatory milieu, inducing tissue regeneration and inhibiting collagenous scar development[4-6]. However, low survival rates, poor engraftment and complicated technique of MSCs maintenance still become major problems[7]. Therefore, a new approach to solve these gaps is needed. A controlled hypoxia condition could enhance the production of several molecules of MSCs through a paracrine mechanism that potentially improves the LF condition[8].

The MSCs are defined as multipotent cells with plastic-adherent capacity. They are characterized by the expression of various surface markers, such as CD90, CD105, CD44, CD73 and CD29, while negatively expressing CD34, CD45, CD11b, CD14, CD19 and Human Leukocyte Antigen (HLA-DR)[9-11]. The MSCs are also capable of differentiating into several types of mature cells, such as osteocytes, adipocytes, osteocytes and nerve cells[12,13]. The hypoxia precondition treated in MSCs (H-MSCs) could enhance their immunomodulatory and regeneration capability, through expressing robust anti-inflammatory cytokines and growth factors, such as Interleukin-10 (IL10), Hepatocyte Growth Factor (HGF), Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF) and Basic Fibroblast Growth Factor (bFGF), known as H-MSCs secretome (SH-MSCs)[14-16]. These molecules are critical for the improvement of liver injury and fibrosis, through inhibiting inflammatory condition, hepatocyte apoptosis and promoting hepatocyte regeneration[4-6,9].

The prolonged injury and inflammatory condition in the liver could overactivate the hepatic stellate cells (HSCs) into myofibroblast (MFs), leading to excessive extracellular matrix (ECM) production and fibrosis[4,17]. The excessive release of proinflammatory mediators, such as Tumor Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6), Interleukin-17 (IL-17) and Transforming Growth Factor-β (TGF-β) during chronic inflammation could serve an abundant fibrosis formation[18,19]. In particular, the overexpression of TGF-β could promote MFs activation, characterized by α-Smooth Muscle Actin (α-SMA) expression through the SMAD2/3 pathway[4,16,17]. The SH-MSCs could potentially inhibit the prolonged inflammatory condition, as the abundant concentration of anti-inflammatory cytokines, including IL-10[20,21]. On the other hand, growth factors content in SH-MSCs such as HGF, PDGF and VEGF could also play a vital role in regenerating hepatocyte cells[4,9]. However, the study regarding the efficacy and mechanism of SH-MSCs in ameliorating CLD with fibrosis is still unclear.

In the recent study, SH-MSCs and secretome of normoxia-MSCs (SN-MSCs) that successfully isolated from hypoxia and normoxia MSCs culture medium, respectively using tangential flow filtration (TFF) based on a combination of Molecular Weight Cut-off (MWCO) category regarding our study by Putra *et al.*[20]. The liver fibrosis was induced in rats and a dose of SH-MSCs and SN-MSCs were injected to investigate their effectiveness and mechanism in promoting liver regeneration and fibrotic regression.

MATERIALS AND METHODS

Study area: The experimental study was conducted between December, 2022 and August, 2023 at the Stem Cell and Cancer Research Indonesia, Semarang, Indonesia. The research encompassed a total duration of 8 months, involving different phases of animal model establishment, treatment administration, sample collection and analysis.

Animals: Twenty-four of 12 weeks old adult male Wistar rats weighing 250-300 g were purchased from the Agricultural and Fishery Service of Salatiga. The rats were housed in standard laboratory cages at 22-26°C and 55-60% relative humidity with a 12/12 dark-light cycle. All rats had *ad libitum* access to food and water.

Induction of chronic liver fibrosis: To induce liver fibrosis, eighteen male Wistar rats received 16 consecutive intraperitoneal injections (1 μL g^{-1} body weight) of CCl_4:Olive oil (1:1) twice per week for 8 weeks. The injection of olive oil alone served as a control (6 rats). Furthermore, the extensive collagen deposition in the liver fibrosis model was assessed using Sirius Red staining.

Preparation, flow cytometry phenotyping and differentiation analysis of UC-MSCs: Umbilical cords (UCs) was collected from a nineteen-days pregnant Wistar rat under deep anesthesia. Isolation of UC-MSCs was performed according to Darlan *et al.*[9]. The UC-MSCs were cultured in a culture flask using Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri), supplemented with 10% fetal bovine serum (FBS) (Gibco™ Invitrogen, New York, USA), 1.5% penicillin (100 U mL^{-1})/streptomycin (100 µg mL^{-1}) (Gibco™ Invitrogen, New York, USA) and 0.25% amphotericin B (Gibco™ Invitrogen, New York, USA). The cells were maintained at 37°C with 5% CO_2 and the culture medium was refreshed every three days. After reaching 80% confluences, MSCs were passed into a new flask. The UC-MSCs from 3rd passage were used for the following studies. The UC-MSCs surface markers at 3rd passage were characterized using flow cytometry analysis according to the instruction of the manufacturer. Briefly, UC-MSCs membrane antigens were stained by subsequent incubation of rats CD90-FITC, CD29-PE, CD31-PerCP and CD45-APC antibodies (BD Bioscience, San Jose, California, USA) for 20 min in the dark at room temperature. Thus, the cells were analysed using BD Accuri C6 Plus flow cytometer (BD Bioscience) and interpreted using BD Accuri C6 Plus software (BD Bioscience). Further the adipogenic and chondrogenic differentiation analysis of MSCs at 3rd passage were performed. The UC-MSCs were cultured in a standard medium at 37°C and 5% CO_2 until 95% confluence. Subsequently, the regular medium was substituted with Rat MesenCult™ basal medium for adipogenic and osteogenic differentiation (Stem Cell Technologies, Singapore), supplemented with specific supplements, 1% L-glutamine, 1% penicillin and 0.25% amphotericin B (Gibco). The medium exchange occurred every three days. Following 21 days of incubation, staining for lipid and calcium deposition was conducted using oil red O and alizarin red staining (Sigma-Aldrich, St. Louis, Missouri).

Induction of hypoxia in UC-MSCs: After reaching 80% confluences, hypoxia condition was induced in UC-MSCs using a hypoxia chamber (stem cell technologies) until a concentration of 5% O_2. The value of Oxygen Partial Pressure (pO_2) was validated with an oxygen controller (BioSpherix, Lacona, New York, USA). Then, the cells were incubated at 37°C and CO_2 5%. The culture medium was collected after the experiment.

Preparation of secretome of UC-MSCs: The conditioned medium (CM) of hypoxia and normoxia UC-MSCs were collected from the culture condition, respectively after being centrifugated at 13,000×g for 10 min at 4°C. The isolation of SH-MSCs and SN-MSCs was employed using the tangential flow filtration (TFF) strategy (Formulatrix, Massachusetts, USA) with several molecular weight cut-off categories in previous study by Putra *et al.*[20]. The molecules from each CM were filtrated using 10-50 kDa 50%, 50-100 kDa 25% and 100-300 kDa 25% filter cassettes (Formulatrix). The SH-MSCs and SN-MSCs were then stored at -80°C before use for Enzyme-Linked Immune Sorbent Assays (ELISAs) and employed for the following experiment.

Treatment of UC-MSCs secretome: Six animals per group for this study. The research was divided into four categories. The healthy control (Sham) group and the liver fibrosis (Control) group of rats received NaCl treatment through the tail vein. In the treatment groups, liver fibrosis rats were given 500 µL of SH-MSCs and 500 µL of SN-MSCs via the tail vein. Tissue samples were collected on day 9.

Enzyme-Linked Immunosorbent Assay (ELISA): The levels of cytokines and growth factors in SH-MSCs and SN-MSCs were assessed following the manufacturer's guidelines (Invitrogen, California, USA). The VEGF, PDGF, bFGF, IL-10, TGF-β and IL-6 assays were conducted at room temperature. The outcomes were evaluated using a microplate reader (Bio-Rad, California, USA) at a wavelength of 450 nm.

Collagen histology analysis: Liver tissue samples were promptly preserved in cold 4% neutral buffer formalin at 4°C and prepared for paraffin embedding. Preceding staining, sections underwent deparaffinization and rehydration via xylene and alcohol. Subsequently, slides were exposed to a 0.1% solution of Sirius Red dissolved in aqueous saturated picric acid for 60 min, followed by rinsing in 0.5% hydrogen chloride. Afterward, the slides were dehydrated and mounted using a DPX mounting medium. This staining procedure resulted in collagen components appearing red and non-collagenous components appearing orange.

Immunohistochemical examinations of α-SMA: Liver slides embedded in paraffin were treated to remove paraffin using xylene and alcohol. Subsequently, following rehydration, the slides were subjected to an incubation process involving a primary monoclonal antibody specific to α-SMA (dilution ratio of 1:100, Abcam, Cambridge, Massachusetts United States), followed by exposure to a biotinylated secondary antibody. The detection process involved the use of streptavidin peroxidase. The expression intensity of α-SMA was semi-quantitatively assessed using ImageJ software.

Relative expression analysis of TGF-β1: Fifty milligram tissue samples collected on day 9 underwent total RNA isolation using TRI reagent following the provided guidelines (Sigma Aldrich, Dorset, UK). The synthesis of cDNA was accomplished using the Enhanced Avian First Strand cDNA Synthesis Kit as per the manufacturer's instructions (Sigma-Aldrich, Dorset, UK). The reverse transcription employed an oligo d(T) primer, with the reaction mix being incubated at 70°C for 10 min and 45°C for 15 min. Subsequently, a 2-step quantitative real-time PCR was conducted using the Eco Real-Time PCR System (Illumina Inc., San Diego California, USA). For qPCR analysis of β-actin and TGF-β, the KAPA SYBR® FAST Universal Kit (Sigma Aldrich) was utilized alongside 3 ng of cDNA template and specific primers (β-actin: forward 5'-ATTGGCAATGAGCGGTTCCGC-3' and reverse 5'-CTCCTGCTTGCTGATCCACATC-3'; TGF-β: forward 5'-TACCATGCCAACTTCTGTCTGGGA and reverse 5'-ATGTTGG ACAACTGCTCCACCTTG-3'). The thermal cycling condition comprised an initial step at 95°C for 3 min, followed by 50 cycles at 95°C for 10 sec and 60°C for 30 sec. The expression level of TGF-β was determined using the ΔΔCt formula with analysis performed via Eco Study Software (Illumina).

Ethical consideration: All procedures were approved by the ethics committee of Universitas Islam Sultan Agung.

Statistical analysis: All statistical analyses were performed using SPSS software version 24 (IBM, New York, USA). All data were measured using One-way Analysis of Variance (ANOVA), followed by a Tukey's *post hoc* test. All values are presented as the Mean±SD. A $p<0.05$ was considered as statistically significant.

RESULTS

The cell morphology, membrane marker expression and differentiation potential of UC-MSCs were assessed at the 3rd passage, following the guidelines provided by the International Society for Stem Cell Therapy. The isolated cells showed a characteristic of spindle-shaped and fibroblast-like cells (Fig. 1a). To confirm the UC-MSCs differentiation potential, we performed the adipogenic (Fig. 1b) and osteogenic differentiation analysis (Fig. 1c). After 21 days of incubation, the UC-MSCs were differentiated into adipocytes and osteocytes, characterized by the deposition of lipid and calcium, respectively marked as red color. The immunophenotyping profile of the cells also showed the positive expression of CD90.1 and CD29 and the negative expression of CD45 and CD31 (Fig. 1d).

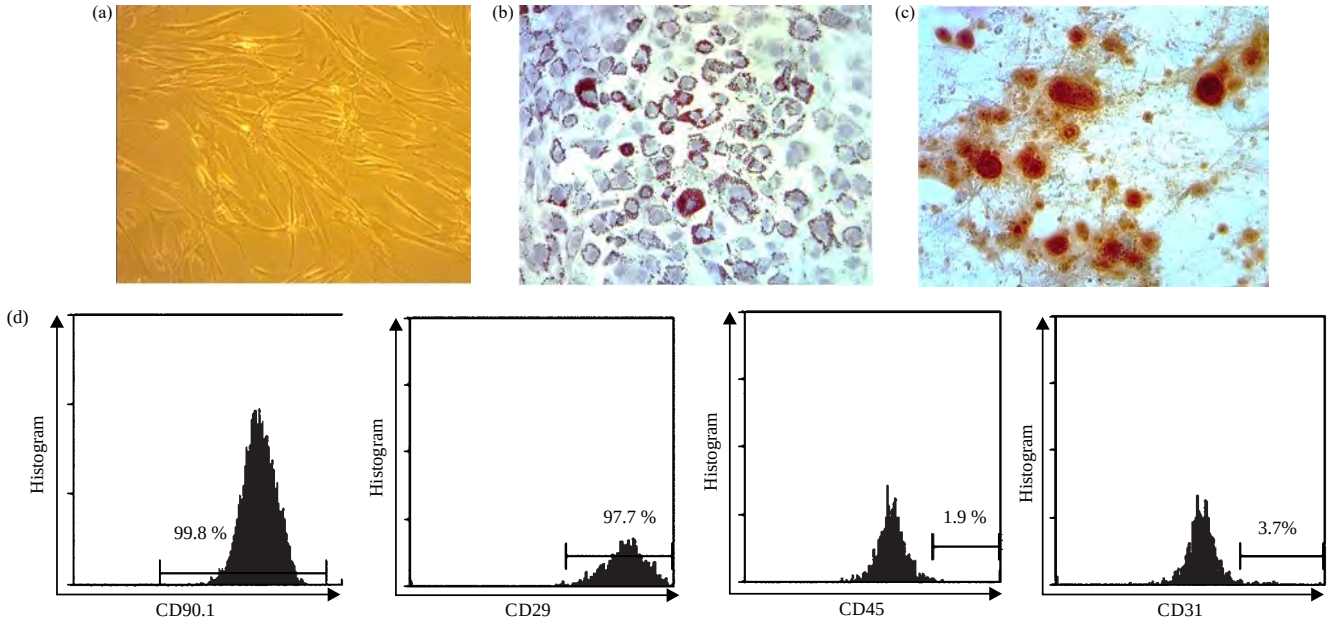

Fig. 1(a-d): Characteristic of isolated UC-MSCs, (a) At 3rd passage, (b) UC-MSCs could differentiate into adipocytes, (c) Osteocytes and (d) Phenotyping analysis

(a) UC-MSCs showed spindle-shaped and fibroblast-like characteristics (200× magnification), (b-c) Marked as red color after the incubation by oil red O and alizarin red staining, respectively (200× magnification) and (d) UC-MSCs showed positive expression of CD90.1 (99.8%) and CD29 (97.7%) and negative expression of CD45 (1.9%) and CD31 (3.7%)

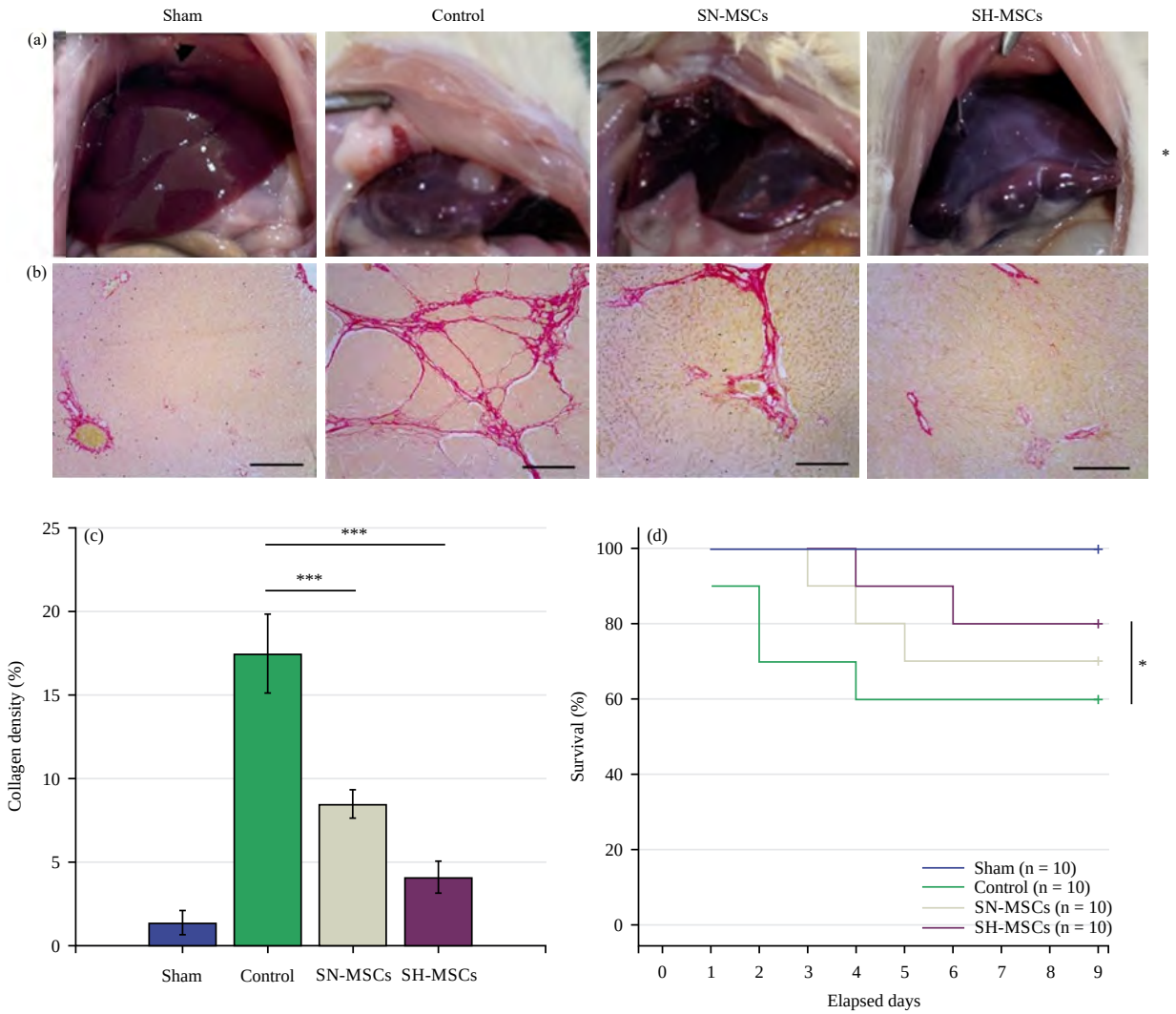

Fig. 2(a-d): SH-MSCs and SN-MSCs improve CCl_4-induced liver fibrosis, (a) SH-MSCs administration substantially repaired the gross histopathological appearance of CCl_4-induced liver with reduction of fibrosis area, (b) Sirius Red staining of liver sections from sham, control, SH-MSCs and SN-MSCs-treated rats demonstrated a massive reduction of collagen, (c) Percentage quantification of Sirius Red staining (bar indicates 400 μm) and (d) Kaplan-Meier survival analysis of rats with CCl_4-induced fibrosis

Data are shown as Mean±Standard Deviation. *p<0.05, ***p<0.001, Sham: Healthy group, Control: Negative control group of liver fibrosis without nay treatment, SN-MSCs: Liver fibrosis group treated with SN-MSCs 500 μL intravenously and SH-MSCs: Liver fibrosis group treated with SH-MSCs 500 μL intravenously

After the incubation of UC-MSCs in 24 hrs hypoxia condition, we collected the CM from H-MSCs. We isolated the cytokines and growth factors contained in H-MSC-CM to acquire the pure SH-MSCs. On the other hand, we also collected the CM from N-MSCs to isolate its cytokines and growth factors and obtain pure SN-MSCs. The TFF strategy, based on different molecular weight cut-off categories from a previous study, was employed. Molecules were isolated using filter cassettes of 10-50 kDa (50%), 50-100 kDa (25%) and 100-300 kDa (25%). Following filtration, the assessment of cytokine and growth factor levels present in both SN-MSCs and SH-MSCs was conducted using the ELISA assay. The SH-MSCs led to significantly more VEGF (1228.86±27.71 vs 255.12±6.57), PDGF (1043.06±24.49 vs 206.48±10.43), bFGF (1085.34±28.92 vs 255.70±7.98), IL-10 (415.02±7.14 vs 90.71±4.02), TGF-β (282.83±6.28 vs 213.83±6.25) and IL-6 (123.99±3.04 vs 54.45±3.57) levels than proteins in SN-MSCs (Table 1).

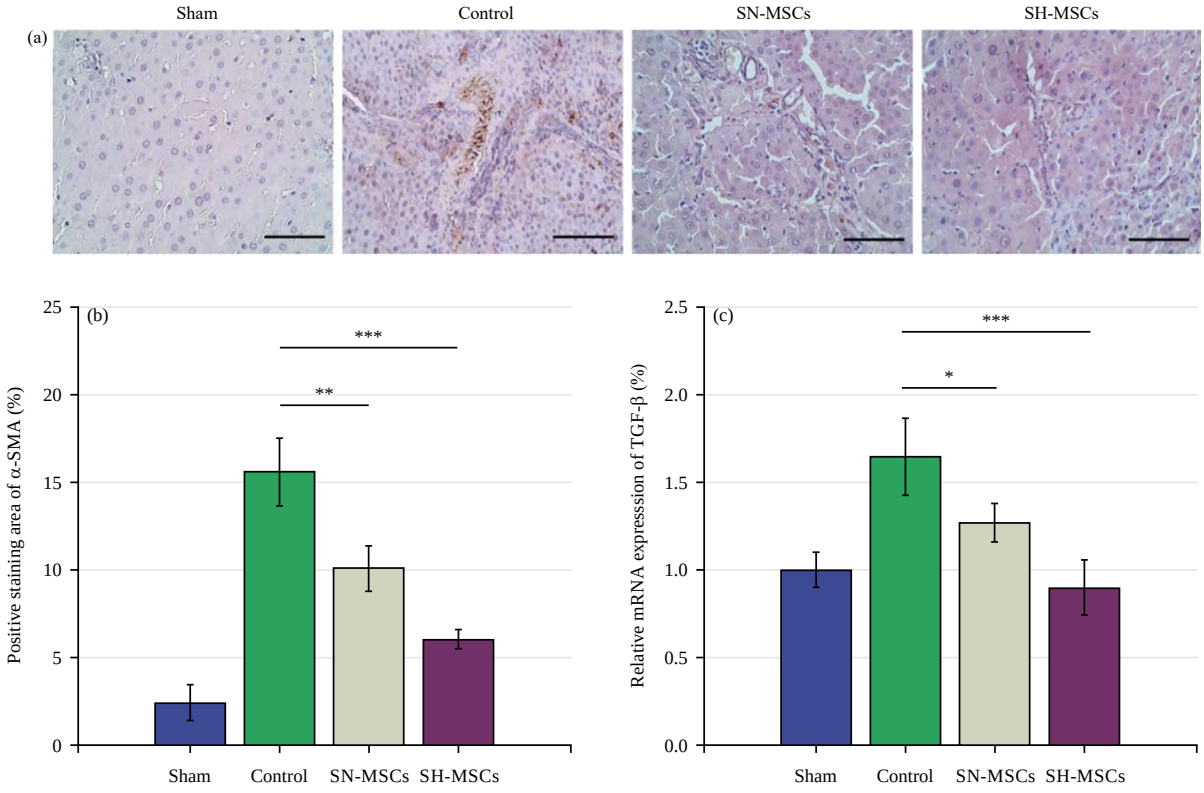

Fig. 3(a-c): SH-MSCs and SN-MSCs decrease α-SMA and TGF-β in rats with liver fibrosis, (a) Photomicrographs of liver sections showing α-SMA IHC staining (bar indicates 200 μm), (b) Quantification of α-SMA positive cells was performed by computer-assisted image analysis and (c) Relative mRNA expression of TGF-β

Data are shown as Mean±Standard Deviation. *p<0.05, **p<0.01, ***p<0.001, Sham: Healthy group, Control: Negative control group of liver fibrosis without nay treatment, SN-MSCs: The liver fibrosis group treated with SN-MSCs 500 μL intravenously and SH-MSCs: Liver fibrosis group treated with SH-MSCs 500 μL intravenously

Table 1: Concentration of cytokine on secretome

Cytokine	SH-MSCs (pg mL^{-1})	SN-MSCs (pg mL^{-1})
VEGF	1228.86±27.71	255.12±6.57
PDGF	1043.06±24.49	206.48±10.43
bFGF	1085.34±28.92	255.70±7.98
IL-10	415.02±7.14	90.71±4.02
TGF-β	282.83±6.28	213.83±6.25
IL-6	123.99±3.04	54.45±3.57

Mean±SD

Rats were sacrificed 9 days after intravenous SH-MSCs and SN-MSCs injection. Four controls, 3 SN-MSCs and 2 SH-MSCs-treated rats died before sacrifice. Six remaining control livers showed a robust collagen deposition. In contrast, none of the remaining six SH-MSCs-treated livers showed the gross histopathological appearance of fibrosis 9 days after SH-MSCs administration (Fig. 2a). To measure the improvements in liver fibrosis after rats were treated with CCl_4, SN-MSCs and SH-MSCs, liver sections were stained with Sirius Red to identify the collagen deposition (Fig. 2b-c). Six rats from sham, control, SN-MSCs and SH-MSCs were sacrificed for several microscopic measurements 9 days after the treatments. The rats from the control groups showed massive collagen deposition in livers after Sirius Red staining. The SN-MSCs noticeably decreased collagen deposition in rats with CCl_4-induced liver fibrosis (8.46±0.87%) compared with control (17.46±2.38%). Interestingly, the SH-MSCs displayed a more significant decrease in collagen deposition (4.10±0.96%), indicating a more robust improvement. During the 9 days follow-up period after treatments, four of 10 control rats died with a mortality rate reaching 40%. In contrast, improvement in survival was measured for SN-MSCs with two rats dying and more significant improvement for SH-MSCs with only a rat dying during the observation period (Fig. 2d).

To determine the mechanism of collagen reduction in liver fibrosis, the α-SMA positive cells in a liver section from all groups were evaluated by IHC. Quantification of this observation revealed that SN-MSCs significantly suppressed α-SMA positive cells (10.07±1.29% SN-MSCs group vs 15.54±1.92%). The SH-MSCs performed a more significant reduction of α-SMA positive cells (6.02±0.56%) (Fig. 3a-b). On the other hand, the analysis of α-SMA positive cells was in line with the quantification of TGF-β relative mRNA expression in the liver section of all treatment groups. The SN-MSCs showed a significant decrease of TGF-β expression in liver fibrosis (1.27±0.11) compared with control (1.65±0.22). Moreover, SH-MSCs also presented a more robust and significant decrease in TGF-β expression (0.9±0.16). These demonstrate that SH-MSCs effectively inhibit α-SMA-induced collagen in models of liver failure (Fig. 3c).

DISCUSSION

The animal model-based studies showed desirable therapeutic effects of MSCs in improving chronic liver disease with fibrosis[4-6]. In this study, the roles of SH-MSCs and SN-MSCs was investigated that were successfully isolated from hypoxia and normoxia MSC-CM, respectively using the TFF system based on a combination of MWCO categories in a rat model of CCl_4-induced liver fibrosis. Numerous studies also revealed that H-MSCs could promote more potent outcomes in liver fibrosis because of the enhancement of their immunomodulatory and regeneration capability, through expressing more robust anti-inflammatory cytokines and growth factors[8,22]. Recent investigations focusing on these molecules, using MSC-CM show several therapeutic mechanisms in improving liver injury and fibrosis[23].

Initially, the analysis of SH-MSCs and SN-MSCs revealed that SH-MSCs exhibited higher levels of VEGF, PDGF, bFGF, IL-10, TGF-β and IL-6 compared to SN-MSCs. These findings were in line with our previous study reporting the plentiful level of the concentrated secretome of H-MSCs[20]. Our previous studies also revealed that the hypoxia milieu applied in MSCs could enhance their anti-inflammatory cytokines and growth factors that congregated in their culture medium[14-16]. Several studies reported that H-MSCs potentially ameliorate the prolonged inflammatory condition through abundant concentration of anti-inflammatory cytokines, including IL-10[24]. Other studies also showed that growth factors secreted by H-MSCs such as HGF, PDGF, bFGF and VEGF also play vital roles in regenerating hepatocyte cells[25,26]. Our data indicated that the hypoxia condition is a preferable method to enlarge the anti-inflammatory and regenerative molecules secreted by MSCs that are crucial for liver fibrosis improvement.

The evaluation of SH-MSCs' efficacy aligned with our recent discoveries. In the CCl_4-induced liver fibrosis model, SH-MSCs administration notably ameliorated liver fibrosis. While SN-MSCs also contributed to considerable improvement in liver fibrosis, SH-MSCs yielded more favorable outcomes. This observation was reinforced by the reduction in collagen fiber regions following SH-MSCs administration in rats with liver fibrosis. These results are consistent with previous reports that MSC-CM could inhibit hepatocyte apoptosis and promote hepatocellular proliferation[27]. However, other previous studies also reported that several components contained in MSC-CM are toxic and could trigger other adverse events, such as phenol red[28,29]. These reports indicate that SH-MSCs are likely safer and more acceptable than MSC-CM because of their purity and concentrated molecules, even though future studies are needed.

The significant reduction of the percentage area of collagen fibers after SH-MSCs injection was also supported by our further findings. We found that SH-MSCs optimally decrease the mRNA expression of TGF-β in liver fibrosis. We also found a favourable decrease of α-SMA positive staining area in liver sections after SH-MSCs administration. These findings are consistent with previous studies that the decrease of collagen fibers is characterized by inhibited α-SMA expression[4]. Collagen fibers are produced by activated hepatocyte stellate cells (HSCs) that have a responsibility in fibrogenesis. The robust expression of TGF-β in CCL_4-induced hepatocytes activates quiescent HSCs to differentiate into myofibroblast-like stellate cells which produce plentiful extracellular matrix, such as collagen, adhesive glycoproteins and proteoglycan[30]. Regarding these findings, we suggest that abundant anti-inflammatory cytokines contained in SH-MSCs, such as IL-10 downregulate profibrotic and upregulate anti-fibrotic hepatocyte genes. A previous study supported our suggestion and reported the side role of IL-10 as a receptor-binding competitor of TGF-β. This capability allows IL-10 to play an inhibitory role in the differentiation of HSCs leading to the decrease of α-SMA and collagen[31]. Although there are several limitations, such as the analysis of liver function and SMADs intracellular proteins responsible for TGF-β-induced fibrosis progression that are still unclear, these data demonstrate that SH-MSCs treatment can optimally promote liver regeneration and alleviate fibrosis progression.

CONCLUSION

Collectively, further evidence demonstrating the potency of SH-MSCs in alleviating liver fibrosis was provided. The injection of both SH-MSCs and SN-MSCs in the chronic state of liver fibrosis had a significant therapeutic effect, in which SH-MSCs showed a more optimal effect. The SH-MSCs optimally reduced the collagen fiber deposition and inhibited α-SMA and TGF-β expression. Current findings will contribute to the development of effective treatment for liver fibrosis.

SIGNIFICANCE STATEMENT

The investigation into the therapeutic potential of hypoxia preconditioned MSCs (H-MSCs) and their secretome (SH-MSCs) revealed promising outcomes in ameliorating CCl_4-induced liver fibrosis. Notably, SH-MSCs exhibited superior efficacy compared to normoxia MSCs secretome (SN-MSCs), showcasing a remarkable reduction in liver fibrosis, collagen fiber regions, α-SMA positive staining area and downregulation of TGF-β expression. These findings underscore the significant role of SH-MSCs in improving liver fibrosis and suppressing key pro-fibrotic markers, presenting a potential avenue for advanced therapeutic interventions.

ACKNOWLEDGMENT

We would like to thank the Stem Cell and Cancer Research (SCCR) Laboratory for providing technological support for the secretome of stem cells.

REFERENCES

1. Campana, L. and J.P. Iredale, 2017. Regression of liver fibrosis. Semin. Liver Dis., 37: 1-10.
2. Sun, M. and T. Kisseleva, 2015. Reversibility of liver fibrosis. Clin. Res. Hepatol. Gastroenterol., 39: S60-S63.
3. Venturi, C., C. Sempoux, J.A. Quinones, C. Bourdeaux, S.P. Hoyos, E. Sokal and R. Reding, 2014. Dynamics of allograft fibrosis in pediatric liver transplantation. Am. J. Transpl., 14: 1648-1656.
4. Hermansyah, D., A. Putra, A.M. Muhar, Retnaningsih, K. Wirastuti and B.T. Dirja, 2021. Mesenchymal stem cells suppress TGF-β release to decrease α-SMA expression in ameliorating CCl4-induced liver fibrosis. Med. Arch., 75: 16-22.
5. Sungkar, T., A. Putra, D. Lindarto and R.J. Sembiring, 2020. Intravenous umbilical cord-derived mesenchymal stem cells transplantation regulates hyaluronic acid and interleukin-10 secretion producing low-grade liver fibrosis in experimental rat. Med. Arch., 74: 177-182.
6. Sungkar, T., A. Putra, D. Lindarto and R.J. Sembiring, 2021. Anti-fibrotic effect of intravenous umbilical cord-derived mesenchymal stem cells (UC-MSCs) injection in experimental rats induced liver fibrosis. Medicinski Glasnik, 18: 62-69.
7. García-Sánchez, D., D. Fernández, J.C. Rodríguez-Rey and F.M. Pérez-Campo, 2019. Enhancing survival, engraftment, and osteogenic potential of mesenchymal stem cells. World J. Stem Cells, 11: 748-763.
8. Kojima, Y., A. Tsuchiya, M. Ogawa, S. Nojiri and S. Takeuchi *et al*., 2019. Mesenchymal stem cells cultured under hypoxic conditions had a greater therapeutic effect on mice with liver cirrhosis compared to those cultured under normal oxygen conditions. Regener. Ther., 11: 269-281.
9. Darlan, D.M., D. Munir, A. Putra, I. Alif, N.D. Amalina, N.K. Jusuf and I.B. Putra, 2022. Revealing the decrease of indoleamine 2,3-dioxygenase as a major constituent for B cells survival post-mesenchymal stem cells co-cultured with peripheral blood mononuclear cells (PBMC) of systemic lupus erythematosus (SLE) patients. Medicinski Glasnik, Vol. 19. 10.17392/1414-21.
10. Drawina, P., A. Putra, T. Nasihun, Y.W. Prajoko, B.T. Dirja and N.D. Amalina, 2022. Increased serial levels of platelet-derived growth factor using hypoxic mesenchymal stem cell-conditioned medium to promote closure acceler-ation in a full-thickness wound. Indones. J. Biotechnol., 27: 36-42.
11. Putra, A., I. Alif, N. Hamra, O. Santosa, A.R. Kustiyah, A.M. Muhar and K. Lukman, 2020. MSC-released TGF-β regulate α-SMA expression of myofibroblast during wound healing. J. Stem Cells Regener. Med., 16: 73-79.
12. Han, I., B.S. Kwon, H.K. Park and K.S. Kim, 2017. Differentiation potential of mesenchymal stem cells is related to their intrinsic mechanical properties. Int. Neurourol. J., 21: S24-31.
13. Restimulia, L., D.A.R. Dewi, M.A. Nazar, R.C.S. Irawan, S.S. Ghaissani and E. Haryono, 2022. Downregulation of IL-6 and TNF-α expression with mesenchymal stem cells therapy in allergic rhinitis rats models. Int. J. Cell Biomed. Sci., 1: 27-34.
14. Hamra, N.F., A. Putra, A. Tjipta, N.D. Amalina and T. Nasihun, 2021. Hypoxia mesenchymal stem cells accelerate wound closure improvement by controlling α-smooth muscle actin expression in the full-thickness animal model. Open Access Maced. J. Med. Sci., 9: 35-41.
15. Prajoko, Y.W., A. Putra, B.T. Dirja, A.M. Muhar and N.D. Amalina, 2022. The ameliorating effects of MSCs in controlling treg-mediated B-cell depletion by indoleamine 2, 3-dioxygenase induction in PBMC of SLE patients. Open Access Maced. J. Med. Sci., 10: 6-11.
16. Chacko, S.M., S. Ahmed, K. Selvendiran, M.L. Kuppusamy, M. Khan and P. Kuppusamy, 2010. Hypoxic preconditioning induces the expression of prosurvival and proangiogenic markers in mesenchymal stem cells. Am. J. Physiol. Cell Physiol., 299: C1562-C1570.

17. Yao, Y., Z. Xia, F. Cheng, Q. Jang and J. He *et al*., 2021. Human placental mesenchymal stem cells ameliorate liver fibrosis in mice by upregulation of Caveolin1 in hepatic stellate cells. Stem Cell Res. Ther., Vol. 12. 10.1186/s13287-021-02358-x.
18. Rong, X., J. Liu, X. Yao, T. Jiang, Y. Wang and F. Xie, 2019. Human bone marrow mesenchymal stem cells-derived exosomes alleviate liver fibrosis through the Wnt/β-catenin pathway. Stem Cell Res. Ther., Vol. 10. 10.1186/s13287-019-1204-2
19. Berlian, M.A., I. Alif, P. Subchan, F.E. Handoyo and S.A. Husain *et al*., 2022. Topical gel of mesenchymal stem cell-conditioned medium-induced serum injury accelerates wound healing in skin excision tissue. Int. J. Cell Biomed. Sci., 1: 10-17.
20. Putra, A., A. Widyatmoko, S. Ibrahim, F. Amansyah and F. Amansyah *et al*., 2021. Case series of the first three severe COVID-19 patients treated with the secretome of hypoxia-mesenchymal stem cells in Indonesia. F1000Research, Vol. 10. 10.12688/f1000research.51191.1.
21. Putra, A., Z. Pasongka, A. Widyatmoko, A. Prasetio and B.T. Dirja, 2022. X-ray scanning reduce soluble active molecules of mesenchymal stem cells. Int. J. Cell Biomed. Sci., 1: 35-40.
22. Zhang, L., D. Zhou, J. Li, X. Yan and J. Zhu *et al*., 2019. Effects of bone marrow-derived mesenchymal stem cells on hypoxia and the transforming growth factor beta 1 (TGFβ-1) and SMADs pathway in a mouse model of cirrhosis. Med. Sci. Monit., 25: 7182-7190.
23. Huang, B., X. Cheng, H. Wang, W. Huang and Z.I.G. Hu *et al*., 2016. Mesenchymal stem cells and their secreted molecules predominantly ameliorate fulminant hepatic failure and chronic liver fibrosis in mice respectively. J. Transl. Med., Vol. 14. 10.1186/s12967-016-0792-1.
24. Liu, Y.Y., C.H. Chiang, S.C. Hung, C.F. Chian, C.L. Tsai, W.C. Chen and H. Zhang, 2017. Hypoxia-preconditioned mesenchymal stem cells ameliorate ischemia/reperfusion-induced lung injury. PLoS ONE, Vol. 12. 10.1371/journal.pone.0187637.
25. Nakamura, T. and S. Mizuno, 2010. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. Proc. Jpn. Acad. Ser. B, 86: 588-610.
26. Elpek, G.Ö., 2015. Angiogenesis and liver fibrosis. World J. Hepatol., 7: 377-391.
27. van Poll, D., B. Parekkadan, C.H. Cho, F. Berthiaume, Y. Nahmias, A.W. Tilles and M.L. Yarmush, 2008. Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration *in vitro* and *in vivo*. Hepatology, 47: 1634-1643.
28. Zhu, Y., X. Zhang, J. Zhu, Q. Zhao and Y. Li *et al*., 2012. Cytotoxicity of phenol red in toxicity assays for carbon nanoparticles. Int. J. Mol. Sci., Vol. 13. 10.3390/ijms131012336.
29. Liu, X., B. Chen, L. Chen, W.T. Ren and J. Liu *et al*., 2013. U-shape suppressive effect of phenol red on the epileptiform burst activity via activation of estrogen receptors in primary hippocampal culture. PLoS ONE, Vol. 8. 10.1371/journal.pone.0060189.
30. Györfi, A.H., A.E. Matei and J.H.W. Distler, 2018. Targeting TGF-β signaling for the treatment of fibrosis. Matrix Biol., 68-69: 8-27.
31. Dai, L.J., H.Y. Li, L.X. Guan, G. Ritchie and J.X. Zhou, 2009. The therapeutic potential of bone marrow-derived mesenchymal stem cells on hepatic cirrhosis. Stem Cell Res., 2: 16-25.