ORIGINAL ARTICLE

Anti-fibrotic effect of intravenous umbilical cord-derived mesenchymal stem cells (UC-MSCs) injection in experimental rats induced liver fibrosis

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ABSTRACT

Aim To investigate the effect of umbilical cord-derived mesenchymal stem cells (UC-MSCs) administration among liver fibrosis experimental rat model via the regulation of angiotensin II type 1 receptor (AT1R) and platelet-derived growth factor-β (PDGF-β) due to their therapeutic potential to replace liver transplantation for advanced liver fibrosis. Yet the mechanism of action has been questionably associated with UC-MSCs fibrosis regression properties.

Methods Sprague-Dawley (SD) rats (n=18) were separated into three groups (control, untreated liver fibrosis, and UC-MSCs treated group). Serum PDGF-β level was determined by enzyme-linked immunosorbent assay (ELISA) following 14 days of UC-MSCs injection. Meanwhile, AT1R expression was interpreted based on immunoreactive score (IRS) stained using polyclonal antibody and liver fibrosis stained with hematoxylin & eosin was graded using the METAVIR score.

Results UC-MSCs were isolated successfully from rat umbilical cord. Liver fibrosis was observed following 14 weeks of CCl_4 injection concurrent with higher serum level of PDGF-β, but the UC-MSCs-treated group had lower level (980.08 ±289.41 and 606.42±109.85 for untreated liver fibrosis and UC-MSCs treated group, respectively; p=0.004). There was also a high expression of AT1R among untreated liver fibrosis group, as well as high-grade liver fibrosis versus localized fibrosis and low level of AT1R expression among UC-MSCs treated-group (p=0.001).

Conclusion UC-MSCs administration could ameliorate liver fibrosis by reducing the AT1R expression and PDGF-β serum levels, and intervention through this signaling pathway could be alternative evidence for the causative of positive outcome.

Key words: immunohistochemistry, liver cirrhosis, mesenchymal stem cell transplantation

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INTRODUCTION

Liver fibrosis is an abnormal response of hepatocyte producing collagenous composition resulting from repetitive noxious exposure as etiologic agents of chronic liver diseases (CLDs) (1). Several causative factors are associated with CLDs ranging from genetic diseases, viral infections, cholestasis, metabolic disorders, alcohol, and other hepatotoxic substance exposures (2). In severe form of liver fibrosis, so-called cirrhosis, it contributes to higher mortality and the leading cause of disability-adjusted life-years (DALYs) worldwide (3). There are more than 300 million or 5% of the World population who suffered from CLDs, with two million premature deaths predominantly caused by hepatitis B viral infection accounting for 257 million cases in 2015 (4).

The pathomechanism of liver fibrosis involves the interaction between immunologic cascade by inducing extracellular matrix production, and harmful stimuli (5). Initially, the accumulation of extracellular matrix (ECM) becomes a hallmark for fibrotic development until it finally impairs liver function. This hostile environment activates quiescent hepatic stellate cells (HSCs) to myofibroblasts (MFBs); MFBs will act as the essential source for ECM (6-8). In recent findings, platelet-derived growth factor-beta (PDGF-β) also plays pivotal roles as a potent mitogen for culture-activated HSCs as well as a chemotactic factor (9). Higher levels of PDGF-β also ramp the proliferative response in HSCs among cirrhotic liver patients compared to healthy controls (10).

In another theory subset, angiotensin II and its receptor, angiotensin II type 1 receptor (AT1R) is also involved in the HSCs activation (11). Activated-HSCs can promote AT1R expression or vice versa through the AT1R signaling pathway that activates quiescent HSCs via phosphorylation of Janus kinase-2 (12,13). The AT1R activation enhances angiotensin-II effect that perpetually insults the liver by triggering the activation of the renin-angiotensin-aldosterone system (RAAS); it will promote oxidative stress and up-regulation of proinflammatory cytokines (14,15). In a previous study, there was low-grade liver fibrosis in the AT1R deficient rat model with less inflammatory response versus markedly increased fibrosis progression in wild type rats after chronic administration of carbon tetrachloride (CCl_4) (16). Therefore, managing liver fibrosis directed against AT1R and PDGF-β inhibition could become one of the promising approaches.

The elucidation of liver fibrosis pathogenesis could unfold several accessible options to halt or reverse its progression. Meanwhile, the definitive treatment for advanced- liver fibrosis only relies on liver transplantation (LT) that is frequently overshadowed by several drawbacks, such as donor availability, long term use of immunosuppressive medication, host rejection, and high cost (17). Nevertheless, the presence of mesenchymal derived- stem cells (MSCs) could become a replacement for LT since it has successfully ameliorated liver cirrhosis in several studies but no findings of any sole mechanism that has been attributed to the positive outcomes (18). The selection of stem cells source must also be considered based on various indicators. However, umbilical cord-derived MSCs (UC-MSCs) are superior as they offer several advantages, such as high content sources for pluripotent stem cells, accessibility, and they also highly regenerate to hepatocyte (19,20).

The aim of this study was to analyze and demonstrate the fibrotic regression function of UC-MSCs administration by reducing the AT1R expression and PDGF-β serum level in experimental rats induced liver fibrosis.

MATERIALS AND METHODS

Materials and study design

This laboratory experimental study with randomized post test control group design was conducted at the Stem Cell and Cancer Research (SCCR) Laboratory and the Animal House Integrated Biomedical Laboratory facility, Faculty of Medicine, Sultan Agung Islamic University, Semarang, Indonesia, during the period April-July 2019.

A total of 18 Sprague-Dawley (SD) rats with similar characteristics, including aged 12-14-week-old and 200-250 g each, was housed under a controlled environment (12h:12h light/dark cycle, temperature 22± 2°C, 55% of humidity with accessible food and water sufficient for 104 days before the study) and umbilical cord-derived mesenchymal stem cells (UC-MSCs) were used.

Carbon tetrachloride (CCl_4) (Sigma–Aldrich, USA) was injected into the intraperitoneal site of

SD rats to induce liver fibrosis at a dose of 1 mL/kg body weight twice weekly (12 weeks of duration). The SD rats were then allocated in randomized mode into three groups with six rats in each group: group I (G1) as control group that received normal saline injection, group II (G2) as untreated liver fibrosis group, which was only administered the CCl_4 injection, and group III (G3) as UC-MSCs treated-group; this group conceived rats with CCl_4 injection as well as UC-MSCs (1×10^6 cells per rat) via the tail vein. Blood sampling and liver tissue extraction were conducted on the fourteenth day after UC-MSCs injection (Figure 1).

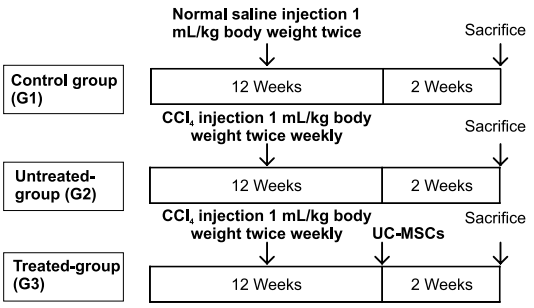

Figure 1. Flow chart of the experimental study which was divided into three groups, G1, G2, and G3; G1, control group received normal saline injection; G2, untreated liver fibrosis group (only administered the CCl4 injection); G3, UC-MSCs treated-group (rats with CCl4 injection as well as UC-MSCs of 1×106 cells per rat).

Anesthetized-rats were euthanized through cervical dislocation procedure at the end of the observation based on animal euthanasia guidelines (American Veterinary Medical Association/ AVMA) (21). The study protocol was also declared following the guidelines for the care and use of laboratory animals and approved by the Ethical Commission of Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia.

Methods

Isolation, purification, and cultivation of UC-MSCs. The UC-MSCs were developed from the umbilical tissue of single pregnant Sprague-Dawley (SD) rats (8 x 10^6 cells). Small pieces of umbilical cord were then inundated with Dulbecco's Modified Eagle's medium/ DMEM (Sigma-Aldrich, Louis St, MO) in T25 culture flask supplemented with 10% Fetal Bovine Serum (FBS) (GibcoTM Invitrogen, NY, USA). Nucleated cells were isolated in a complete culture medium, which was also supplemented with 100 IU/mL or 1% of penicillin/ streptomycin (Sigma-Aldrich, USA) and incubated at 37 °C with 5% humidified CO_2 for 14 days following manufacturer's instructions. Cell growth continuation was observed every 24 hours under an inverted microscope with replacing the medium every 2-3 days until it approached 80% of confluence. It proceeded into two times-washing processes using phosphate buffer saline (PBS) followed by trypsinization with 1 mL of 0.25% trypsin-EDTA (Gibco-BRL, NY, USA) while shaking the tube. Inactivation of Trypsin-EDTA was done using a new medium then the cells were transferred into a new conical tube for centrifugation in 1900 rpm for 10 minutes. Pellet form cells was re-suspended into complete medium and incubated in new flask disks, mentioned as first-passage cultures until it sub-cultured in the fourth passage that had been used for the experiment.

Flow cytometric analysis. The immunophenotypes of UC-MSCs were analyzed at the fourth passage using conjugated antibodies, namely fluorescein isothiocyanate (FITC) CD90, allophycocyanin (APC) CD73, peridinin chlorophyll protein complex (PerCP) CD105 and phycoerythrin (PE) lin monoclonal antibodies. After this, flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA) determined the fluorescence intensity of the cells.

***In vitro* differentiation of UC-MSCs to the osteogenic lineage.** Mesenchymal stem cells must demonstrate their ability to differentiate into mesodermal or osteogenic lineage cells. Cells of the fourth passage were used at a density of $5x10^3$ cells/cm^2 and cultured in DMEM medium supplemented with 10% FBS, 10 mmol/L β glycerophosphate, 10^{-7} mol/L/ 0.1 μM dexamethasone, 50μmol/L ascorbate 2-phosphate (all from Sigma-Aldrich, Louis St, MO) to induce osteogenic differentiation. Cell culture was then incubated at 37 °C and 5% CO_2, as well as twice a week of medium replacement. It was ultimately stained with 0.2% alizarin red solution (Sigma-Aldrich, USA); the appearance of bright red-stain was interpreted as osteocytes, which represents calcium deposition as well as its multipotency properties.

Histopathological analysis. The SD rats were anesthetized and sacrificed on days 14 after UC-MSCs injection in advance of liver extraction. The analysis was aimed at evaluating the extent of fibrosis among the experimental groups (G1, G2, and G3). Firstly, liver fixation used pot-con-

taining 10% formalin to preserve cells and tissue components. Secondly, paraffin-embedded tissue was cut into smaller pieces (3-5 µm of thickness) using a microtome. Lastly, routine hematoxylin-eosin (H&E) staining was applied to tissue samples and observed under microscope at high magnification. Liver fibrosis was graded based on the histological scale of the METAVIR score; it consists of five-point scale ranging from F0 to F4 (F0, no fibrosis; F1, localized fibrosis enclose portal area; F2, periportal fibrosis or mild portal-portal septa; F3, bridging fibrosis or septa fibrosis surrounded portal tracts and terminal hepatic vein; F4, diffuse nodule or cirrhosis) (22).

Immunohistochemistry. Immunohistochemical staining employed angiotensin II receptor type-1 (AT1R) polyclonal antibody (MyBioSource, San Diego, USA). The samples were also preserved using 10% formalin and embedded in paraffin to accommodate microtome cut. Staining was further applied to samples according to the manufacturer's instructions. The interpretation of immunohistochemistry was grouped based on immunoreactive score (IRS) from the multiplication between given score for positive cell coverage (0, no immunoreactive cells; 1, <10%; 2, 10-50%; 3, 51-80%; 4, > 80%) and colour intensity (0, no colour; 1, mild; 2, moderate; 3, intense). The IRS score was divided into four categories (0-1= negative; 2-3= mild; 4-8= moderate; and, 9-12= strongly positive) (23).

Platelet-Derived Growth Factor-β (PDGF-β) analysis. Blood was extracted from the periorbital venous plexus under general anesthesia following 14 days of MSCs administration. Platelet-derived growth factor-β (PDGF-β) serum levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA), from Rat PDGF-β ELISA kit (MyBioSource, United States), which perform under the manufacturer's instructions as well as the interpretations (formulated through the analysis of the standard curve and optical density each sample spectrophotometrically at wavelength of 450 using software).

Statistical analysis

A one-way ANOVA determined the mean difference of serum PDGF-β level with an LSD comparison post hoc test preceded by normality test. The AT1R expression was determined by Kruskal Wallis and post hoc with the Mann Whitney test. A $p<0.05$ was considered as statistically significant.

RESULTS

Umbilical cord-derived mesenchymal stem cells were expressed CD105 (95.9%), CD73 (99.2%), and CD90 (99.9%), but were negative for Lin (2%) through flow cytometric analysis (Figure 2A). The UC-MSCs appeared as long spindle-shaped cells under a microscope (Figure 2B), and it adhered to plastic in cell culture. Additionally, UC-MSCs should also demonstrate osteogenic

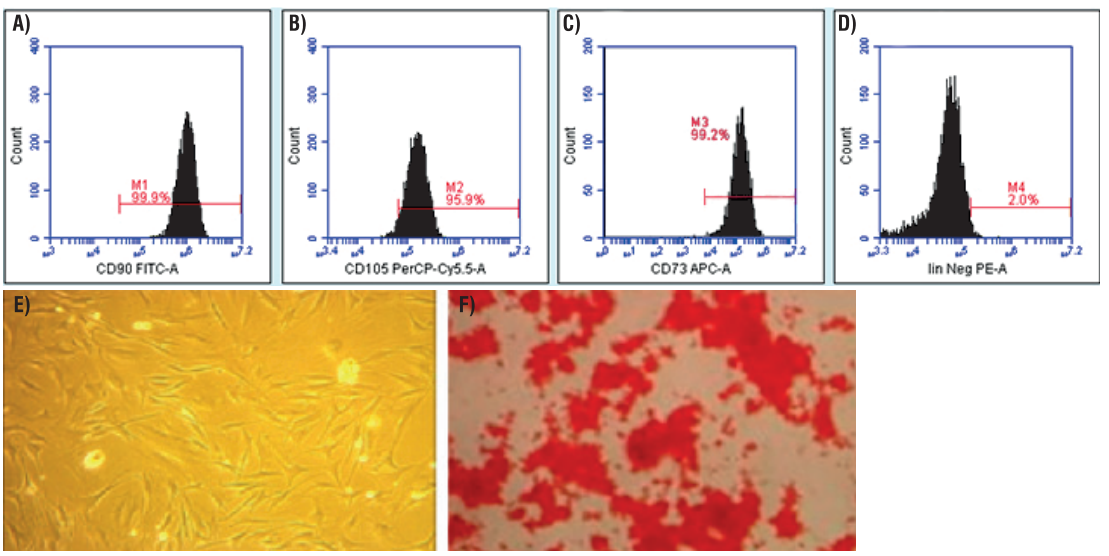

Figure 2. Characteristics, isolation, and differentiation of UC-MSCs. A-C) Most UC-MSCs expressed positive markers (CD90, CD105, and CD73) with D) negative marker Lin; E) UC-MSC candidates appear as fibroblast-like cell characteristics (magnification 10x, scale bar 200 µm); F) osteogenic differentiation (magnification 40x, scale bar 50 µm); UC-MSCs, umbilical cord-derived mesenchymal stem cells;

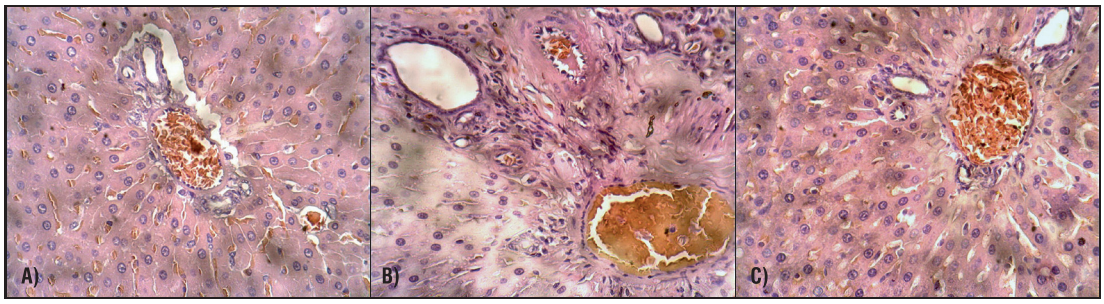

Figure 3. Histology of liver sections from rats in various groups. A) no fibrosis (G1); B) untreated liver fibrosis group (G2) was positive with a fibrous expansion of the most portal areas with marked bridging (F3); C) UC-MSCs treated-group (G3) had eliminated the effect of CCl4 injection with mild fibrosis (F1) (magnification 400x); UC-MSCs, umbilical cord-derived mesenchymal stem cells; G1, control group received normal saline injection; G2, untreated liver fibrosis group (only administered the CCl4 injection); G3, UC-MSCs treated-group (rats with CCl4 injection as well as UC-MSCs of 1×106 cells per rat);

differentiation capability, which was noticeable through the appearance of calcium deposition stained red by Alizarin red dye (Figure 2C).

CCl_4 successfully induced fibrosis in the experimental rat model; it demonstrated significant histological alteration encompassing from septal fibrosis that expanded to portal tracts and terminal hepatic vein until conspicuous bridging fibrosis (F3) (Figure 3). It was also notable that the UC-MSCs-treated group (G3) had low-grade hepatic fibrosis; specifically, it included isolated fibrosis surrounding the portal area (F1) compared with the untreated liver fibrosis group (G2). In other words, UC-MSCs injection was associated with a lower accumulation of collagenous scar.

UC-MSCs injection into fibrosis-induced rat group remarkably reduced serum PDGF-β level compared to control (G1) and untreated liver fibrosis group (G2) with a significant mean difference at day 14 (606.42±109.85 vs. 762.11 ±235.12 vs.

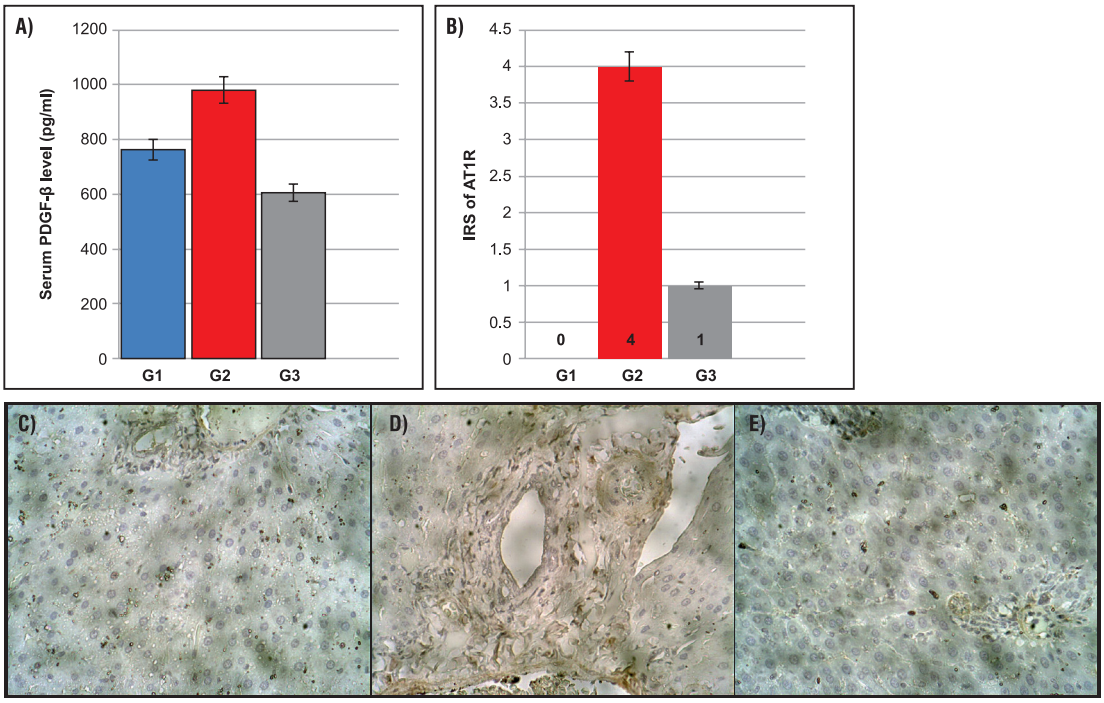

Figure 4. The effect of UC-MSCs injection on serum level of PDGF-ß and AT1R expression. A) Lower serum level of PDGF-ß following 14 days of UC-MSCs administration compared to untreated liver fibrosis group (G2) (p=0.003); B) AT1R expression also significantly reduced in UC-MSCs treated groups versus untreated liver fibrosis group (p=0.003); C-E) immunohistochemistry analysis showed that UC-MSCs attenuated AT1R expression, as well as fibrosis up-regulated AT1R abundantly (left to right G1-G3) (magnification 400x); UC-MSCs, umbilical cord-derived mesenchymal stem cells; PDGF-ß, platelet-derived growth factor-beta; AT1R, angiotensin II type 1 receptor; G1, control group received normal saline injection; G2, untreated liver fibrosis group (only administered the CCl4 injection); G3) UC-MSCs treated-group (rats with CCl4 injection as well as UC-MSCs of 1×106 cells per rat);

980.08 ±289.41, respectively) (p=0.004). Post hoc analysis using LSD also demonstrated significant mean differences between each group (Figure 4A). Low immunoreactive score (IRS) of AT1R in UC-MSCs treated group (G3) compared to G2 (1 (1-2) vs. 4 (4-6), p=0.001) was also successfully proved (Figure 4B). The G2 showed a strong expression of AT1R, which was represented by the appearance of dark-brown colour through polyclonal antibody-targeted AT1R staining, but no AT1R expression was demonstrated in G1. Immunohistochemistry interpretation verified low expression of AT1R among UC-MSCs treated group as well as fibrosis induced up-regulation of AT1R expression (Figure 4C). The post hoc analysis of immunoreactive scores of AT1R expression and PDGF-β level for three groups is depicted in Figure 4.

DISCUSSION

Repetitious administration of CCl_4 establishes liver fibrosis; it initially triggers an acute response, including interstitial edema, focal or centrilobular necrosis, and inflammatory cell infiltration followed by HSCs activation that increases the production of extracellular matrix (ECM), and it ultimately transforms normal liver parenchyma into well-established liver fibrosis (24-27). In the presented study, CCl_4 induction produced pathological changes consisting of high-grade fibrosis among experimental rats.

The major problem relating to chronic liver diseases, as well as liver fibrosis, is the continuation of collagenous and ECM deposition in the liver tissue (28). The immune arm would react, subsequently resulting in the activation of quiescent HSCs that directly play pivotal roles in the progression of hepatic fibrosis per se. Several growth factors and cytokines will also overwhelm liver milieu to turn on dormant HSC (29). Therefore, experimental studies have shown that suppression of HSCs proliferation could be beneficial against liver fibrosis. The PDGF is one of the efficient mitogens that could up-regulate supportive protein expression for HSCs proliferation and migration. Several reports have investigated PDGFRβ up-regulation during HSCs activation and positively correlated with liver fibrogenesis (25,30,31). In general, mesenchymal stem cells (MSCs) can produce cytokines and signaling molecules, which finally produce an immunomodulatory and paracrine effect, hepatocyte proliferation, and restoration (32). The role of MSCs has been studied for their activity to restrain myofibroblast differentiation, and they act in deterring quiescent HSCs transformation to myofibroblast in several stages, thus reducing fibrosis progression (33,34). The presented study showed considerable reduction of PDGF-β level following UC-MSCs injection intravenously to the experimental rats indicating inhibitory effects of UC-MSC to prevent catastrophic implications of PDGF/PDGFR signaling pathway; on the 14th day, PDGF-β level decreased in the UC-MSCs-treated group, but there was no significant difference between the control group and UC-MSCs treated-group. This phenomenon is relatable to the fact that MSCs will actively engage in the induction of endogenic stem cells to trigger hepatocyte regeneration via paracrine effect during the initial phase of administration (35).

The renin-angiotensin system (RAS) is also involved in the process of fibrogenesis. Several investigations reported that RAS re-distributed only in chronically injured livers and activated HSCs de novo, which then generates angiotensin II (36). Subsequently, angiotensin II accumulates at the sites of parenchymal injury and binds to angiotensin II type 1 receptor (AT1R) in myofibroblasts to promote the recruitment of inflammatory cells, secretion of extracellular matrix proteins, and inhibition of collagen degradation (37). Albeit angiotensinogen is the only component of the RAS expressed in the healthy rat liver, the expression of angiotensin-converting enzyme (ACE) and AT1R are evident in the fibrotic rat livers. In humans, there was up-regulation of ACE and chymase, a serine protease, in the liver with severe fibrosis, whereas AT1R expression is re-located to fibrotic areas (38). In the previous study, the administration of human adipose-derived MSCs down-regulated AT1R expression in addition to α-SMA, TGFβ1, Col I, and Col III reduction in cardiac myofibroblast (39).

Regarding the direct anti-fibrotic effects of MSCs against HSCs, MSCs can inhibit the proliferation of HSCs as well as its apoptosis inducer (40). In our study, prominent pathological abnormalities among the untreated liver fibrosis group occurred in linear with the strong expression of AT1R; in contrast, UC-MSCs treated group showed weak

expression of AT1R. Therefore, the primary outcome relating to the UC-MSCs administration is to down-regulate the AT1R expression that subsequently halts fibrosis progression.

CCl_4 injection in our study was administered biweekly via intraperitoneal for 12 weeks until it developed liver fibrosis, as confirmed through histopathological examination. Liver fibrosis was significantly mild in rats which received UC-MSCs and CCl_4 injection, acknowledging the anti-fibrotic effect of UC-MSCs specifically through the reduction of PDGF-β serum level and AT1R expression; the amalgamation of the processes synergistically decreased or inhibited HSCs activation from proliferating.

Our study did not escape some limitations, including several markers for HSCs activation that were not investigated; the HSCs activity was purely based on the final results of the induction and UC-MSCs transplantation. In addition, some transcription factors associated with AT1R upregulation have not yet been elucidated, but measuring AT1R expression was still acceptable to determine the linkage between its overexpression and fibrosis development.

In conclusion, the attenuation of liver fibrosis occurred following UC-MSCs administration via AT1R down-regulation and PDGF-β serum level reduction. This supportive mechanism directly implicated the disease progression of the experimental rat model. The study results indicated that UC-MSCs might have a potential as anti-fibrotic treatment through downregulating PDGF-β and AT1R signaling pathways.

FUNDING

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The study approved by the Ethical Commission of Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia (No: 542/TGL/KEPK FK USU-RSUP HAM/2019).

TRANSPARENCY DECLARATION

Conflict of interest: None to declare.

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