Original Article

Mesenchymal Stem Cells Combined with Bovine Colostrum Decrease the PDGF and Fibrosis Level after Hepatectomy in Liver Fibrosis Rats Model

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Abstract

Introduction: Liver fibrosis is a healing response of the liver to injury by depositing excessive extracellular matrix proteins. Although this fibrosis has a strong impact on survival following resection, there is no therapeutic guideline so far for liver fibrosis. Platelet-derived Growth Factor (PDGF) is the most potent mitogen of Hepatic Stellate Cells (HSC) which started liver fibrogenesis. Mesenchymal Stem Cells (MSCs) have an anti-fibrotic effect by controlling inflammation and HSC immunomodulation, while Bovine Colostrum (BC) has an antifibrotic effect by its antioxidant capacity and growth factor content. However, their effect was only studied in the non-resected fibrotic liver and their combination is never been studied. our study aimed to examine the effects of MSC, BC and their combination on PDGF and fibrosis level in the fibrotic liver after resection. ***Material and methods*****:** Twenty-five Sprague-Dawley rats with fibrotic liver were randomly assigned to BC, MSC, MSC+BC, control, and sham groups. Hepatectomy 50% was carried out except for the sham group. The 10^6MSCs were given intraparenchymal liver, while BC (15 µL/g) was given orally for 5 days/week until day 10. The PDGF plasma level was assessed on days 3, 7, and 10. The histopathologic examination of the liver remnant was done on day 10 using Metavir Scoring System. ***Result:*** MSC+BC significantly increased PDGF level on day 3 followed by a significant decrease on day 10 ($p<0.001$). The Metavir mean score was 1.2 in the MSC+BC group. ***Conclusion:*** MSC+BC combination can improve liver fibrosis after hepatectomy of liver fibrosis rats by preventing post-hepatectomy fibrogenesis.

Keywords: Mesenchymal Stem Cell; Bovine Colostrum; Liver fibrosis; Hepatectomy

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Introduction

Liver fibrosis is a healing response of the liver to injury by depositing excessive extracellular matrix proteins.[1] Liver fibrosis will impair the hepatocytes regeneration, and when it progresses to cirrhosis, it will become irreversible.[2,3] Liver fibrosis is the major risk for hepatocellular carcinoma, becomes a major impairing factor in liver regeneration, and also decreases survival after hepatectomy, yet there are still no therapeutic guidelines so

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far for liver fibrosis.[4–7] The previous study found that the fibrogenesis was significantly increase after hepatectomy in liver fibrotic rats model.[8] The antifibrotic effect of Mesenchymal Stem Cells (MSC) and Bovine Colostrum (BC) had been studied separately in the non-resected fibrotic liver, thus they could become an alternative therapy.[9,10] The MSC can attenuate Hepatic Stellate Cells (HSC) which plays a central role in liver fibrosis, while BC decreases fibrogenesis mainly by its antioxidant capacity. The Reactive Oxygen Species (ROS) which are produced by hepatocytes, macrophages, and inflammatory cells during liver injury will induce the increase of fibrogenesis-related gene expression thus aggravate liver fibrosis.[11] These different antifibrotic pathways can be combined to produce a stronger effect, however, their effect on liver fibrosis after resection and their combination is never been studied.

MSCs must be plastic-adherent in standard culture conditions, express several surface markers such as CD29, CD44, CD73, CD90, and CD105, whileless express CD11b, CD14, CD34, CD45, CD79a or CD19, Human Leucocyte Antigen (HLA) class II, and has multipotent differentiation potential.[12] MSCs can differentiate into osteoblasts, adipocytes, chondrocytes, and transdifferentiate into hepatocytes.[9,12] MSCs can suppress pro-inflammatory molecules and attenuate HSC through the immunomodulatory process.[13] MSCs will secrete Interleukin-10 (IL-10) and TNF-α as a dynamic response to IL-6 that is secreted by activated HSC. The anti-inflammatory cytokine IL-10 and TNF-α will inhibit HSC proliferation.[14] The decrease of active HSCs will lead to a decrease of Platelet-Derived Growth Factor (PDGF) level and ultimately decrease the fibrosis level.

PDGF is primarily produced by platelet and Kupffer Cells (KC) in physiological conditions, but it also highly expressed in macrophages, injured endothelial cells, and HSC when liver damage occurs.[1,15] PDGF is one of the markers of liver fibrosis.[1,7]PDGF is the most potent growth factor that stimulates HSC proliferation, differentiation, and migration which play a pivotal role in liver fibrogenesis. [15,16] Therefore, PDGF level shows HSC activity in liver fibrosis and correlates with the extent of fibrosis. The extent of fibrosis can also be reduced by inhibiting free radicals which have been shown in BC administration.[10]

BC was secreted in the first 72 hours after birth and has an anti-fibrotic effect in liver fibrosis induced by carbon tetrachloride by inhibiting fibrogenesis-related gene expression due to its high antioxidant capacity and radical scavenging activity.[10,17,18] Insulin-like Growth Factor I (IGF-I), which is the most abundant growth factor in BC, also has hepatoprotective and antifibrogenic effects in the liver.[18–22] The Mesenchymal Stem Cells (MSC) and Bovine Colostrum (BC) had only been studied in the non-resected fibrotic liver, while our study was conducted in the fibrotic liver after resection. The effect of MSC, BC, and their combination in resected liver fibrosis has never been studied before. This study aimed to analyze the anti-fibrotic effect of MSCs, BC, and MSCs-BC combination in the fibrotic liver on liver fibrosis animal models after resection by measuring PDGF and fibrotic level.

Materials and methods

Induction of liver fibrosis animals and experimental design

The experimental animals were male SD rats from Stem Cell and Cancer Research Laboratory, Medical Faculty of Sultan Agung Islamic University. The rats were kept in the standardized cage at 23-25°C, 40-70% humidity, with 12 hours alternating light-dark cycle. The bottom of the cage was given sawdust which was replaced every four days. The rats were fed standard food (AIN-76A) and water. The rats underwent seven days of adaptation and were observed for health and behavior changes. Liver fibrosis was induced by injecting 1 mL/kg carbon tetrachloride (CCl_4) (Sigma–Aldrich, USA) intraperitoneally, twice per week for 8 weeks. Liver fibrosis was verified with Sirius red staining and macroscopically with the presence of nodule in the liver.

Twenty-five male SD rats were then randomly divided into five groups (n = 25). Five groups that consisted of five rats were administered into the Sham group, NaCl-oral group as control group, BC group (Colostrum milk powder (Good Health, NZ) 15 µL/g orally 5 days per week for 10 days), MSC group (doses 1 x 10^6 cells dissolved in 0.5 mL NaCl via intraparenchymal injection) and MSC-BC group (MSC doses 1 x 10^6 cells dissolved in 500 µL NaCl intraparenchymal injection + Colostrum doses 15µL/g orally 5 days per week for 10 days).

All rats were operated under anesthesia using ketamine (80-100 mg/kg) and xylazine (5-10 mg/kg) and aseptic condition. Except for the sham group, the left median and left lateral lobes of the liver were resected (equal to 50% hepatectomy).[23,24]A previous

study showed low first-week survival following 75% hepatectomy in liver fibrosis rats, therefore we have chosen 50% hepatectomy in our study.[8] Two rats died after hepatectomy in the early days. This was probably caused by a surgical complication. The surgical procedure's day was counted as Day 0. After the procedure, the rats were placed in a separate standardized cage, and the sawdust in the bottom of the cage was replaced every two days. The rats were fed with the same standard food and water as before.

Isolation of MSCs

MSCs were derived from the Umbilical cord (UC) of a pregnant Sprague-Dawley (SD) rat under anesthesia. UC's blood vessels were removed before it was transferred to a T25 culture flask which contained complete Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, Louis St, MO) mixed with 10% Fetal Bovine Serum (FBS) (Gibco™ Invitrogen, NY, USA) and 100 IU/mL penicillin/ streptomycin (Sigma-Aldrich). The cells were incubated in a humidified atmosphere containing 5% CO_2,at 37℃.The medium was changed every 3 days. When these cells have reached 80% confluency, they were passaged with trypsin. This experiment used cells from the 4*th* passage.

Ethical clearance

This study has obtained ethical approval from the authorized Institutional Review Board of the Ethics Committee of the Medical Faculty of Sultan Agung Islamic University Number 289/VIII/2020, dated 30.08.2020.

Immunophenotyping of UC-MSCs

The MSCs immunophenotyping was carried with antibodies conjugated: Allophycocyanin (APC)-conjugated CD73, fluorescein isothiocyanate (FITC)-conjugated CD90, Peridinin Chlorophyll Protein Complex (PerCP)-conjugated CD105 and phycoerythrin (PE)-conjugated Lin monoclonal antibodies at 4^{O}C temperature for 30 minutes in the darkroom. The fluorescence intensity of the cells was measured with flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA).

In vitro differentiation

MSCs differentiation potential was determined to characterize the isolated cells. These cells were cultured in DMEM medium supplemented with 10% FBS, 10 mmol/L β-glycerophosphate, 10^{7}mol/L/ 0.1 μM dexamethasone, 50 μmol/L ascorbates-2-phosphate (all from Sigma-Aldrich, Louis St, MO), at 37^{O}C and 5% CO_2. The fixed cells were stained with 0.2 % Alizarin Red solution (Sigma-Aldrich) to represent calcium deposition (cells used were from the fourth passage).

Enzyme-linked immunosorbent assay (ELISA)

The plasma from each rat was drawn from the periorbital venous plexusonthe day 3,7 and 10 after the surgical procedure. The PDGF levels in plasma were measured by enzyme-linked immunosorbent assay (ELISA) kits (Fine Test, Wuhan, China).

Histologic analysis

All experimental rats were sacrificed under general anesthesia using ketamine and xylazine on day 10. The rats have confirmed death by diminishing heartbeats, respiratory rate, and acral reflexes, then the remnant liver samples were obtained. There was no sign of wound infection in each rat. Each remnant liver tissue was fixed in 10% formaldehyde. The fibrosis of the remnant liver tissues was evaluated histopathologically with Sirius-red staining (ScyTek, Utah, USA). There were 10 fields of view in each group with 100 times magnification. The liver fibrosis was graded using the Metavir Scoring System by two observers. The Metavir has five categories numbered by 0 until 4. A normal liver is categorized to 0, cirrhosis is categorized to number 4, marked fibrosis is number 3, moderate fibrosis is number 2 and mild fibrosis is number 1.

Statistical analysis

All data were presented as mean ± standard deviation with differences between groups analyzed by a one-way ANOVA with posthoc Tukey HSD test for the PDGF level, and Kruskal-Wallis test for the grade of liver fibrosis. The statistical significant value was obtained using a $p < 0.05$.The agreement between two observers for the Metavir scoring system was evaluated by kappa measurement.[25]

Results

Characteristics of UC-MSCs and differentiation test

The isolated cells showed uniform spindle-shaped morphology. We assessed the marker expression of MSC to determine and verify the cells by using flow cytometry after the fourth passages to determine and verify MSC's marker. We examined the osteogenic capabilities of MSCs to verify the multi-lineage differentiation of UC-MSC after the fourth passages (Figure 1A). We found the differentiation of MSC into osteogenic which was identified as calcium deposits

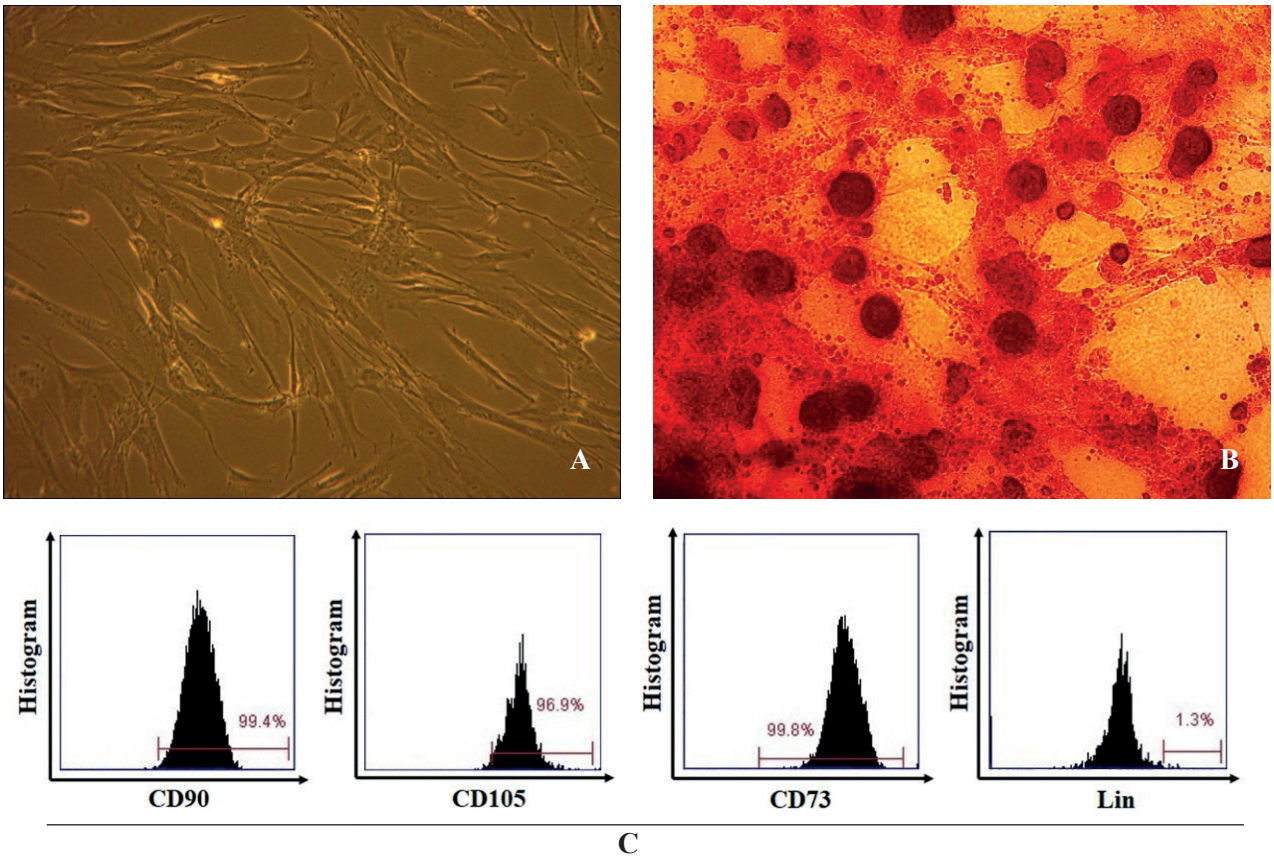

Figure 1. The characteristics and differentiation of UC-MSCs. (A) Cells with uniform spindle-shaped morphology. (B) The differentiation of MSC into osteogenic which was identified as calcium deposits by Alizarin red dye staining (red color) (10x magnification, scale bar 200 µm). (C) The immunophenotype characterization of MSC by flowcytometry analysis.

by Alizarin red dye staining (Figure 1B). Based on the flowcytometry analysis, the isolated cells showed a positive expression of CD105 (96.9%), CD73 (99.8%), CD90 (99.4%), and lack expression of Lin (1.3%) (Figure 1C).

PDGF levels

We found PDGF levels of BC, MSC, and MSC+BC groups were significantly higher (2910.83ng/mL, 2758.33 ng/ml, and 2768.33 ng/mL respectively) compared to the control group (1995.83 ng/mL, p <0.001) on day 3. This difference was diminished on day 7 caused by decreasing in the PDGF level of BC, MSC, and MSC+BC groups and increasing in the PDGF level of the control group. The PDGF level of MSC+BC groups continuously decreased and reached a significant difference compared to control groups (1744.17 ng/mL vs 2926.67 ng/mL; p <0.001), while the PDGF level of BC and MSC were similar to the PDGF level of the control group on day 10.

Histologicalexaminationofliver remnant

The Metavir Scoring System for fibrosis grade's mean of the sham group on day 10 was 2.7 or moderate to marked fibrosis, the control group was 2.6 or moderate to marked fibrosis, the BC group was 2.1 or moderate fibrosis, the MSC group was 2.1 or moderate fibrosis,and the MSC+BC group was 1.2 or mild fibrosis. There was substantial agreement (kappa 0.749) between the two observers. The Kruskal-Wallis analysis found significant differences in liver fibrosis grades among those groups.

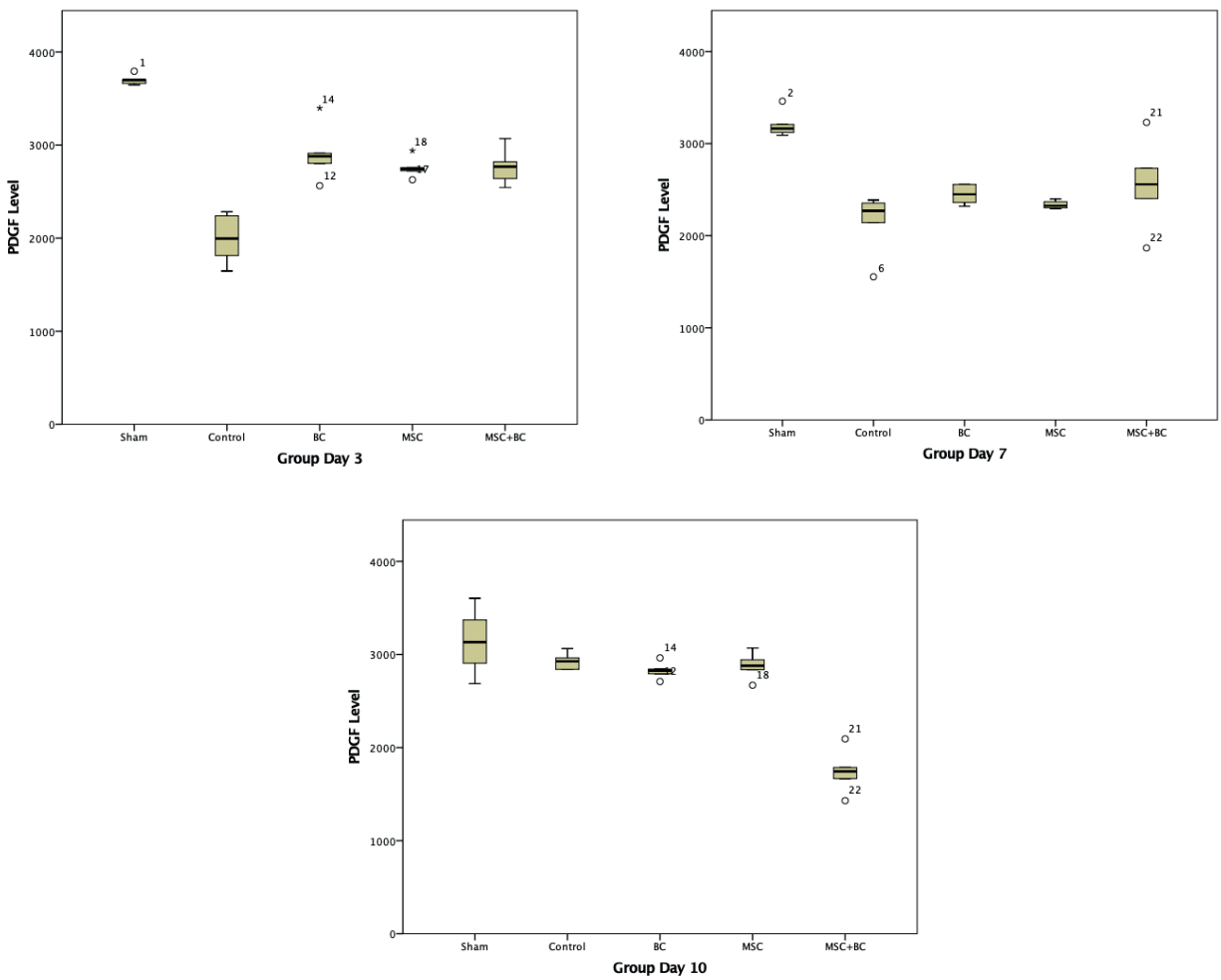

Figure 2. The PDGF levels. There were significantly different levels of PDGF of MSC, BC, and MSC+BC groups compared to the control group on day 3. There was a significant decrease in PDGF level in the MSC+BC group compared to other groups on day 10.

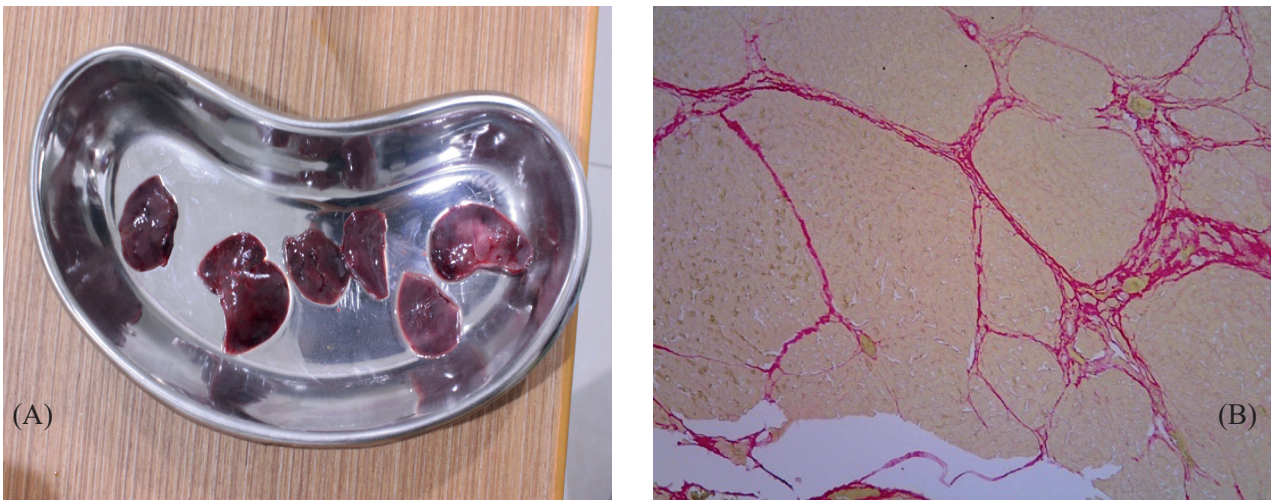

Figure 3. Fibrosis validation of liver. (A) The presence of nodule in the liver; and (B) Fibrotic liver was shown microscopically (100x magnification)

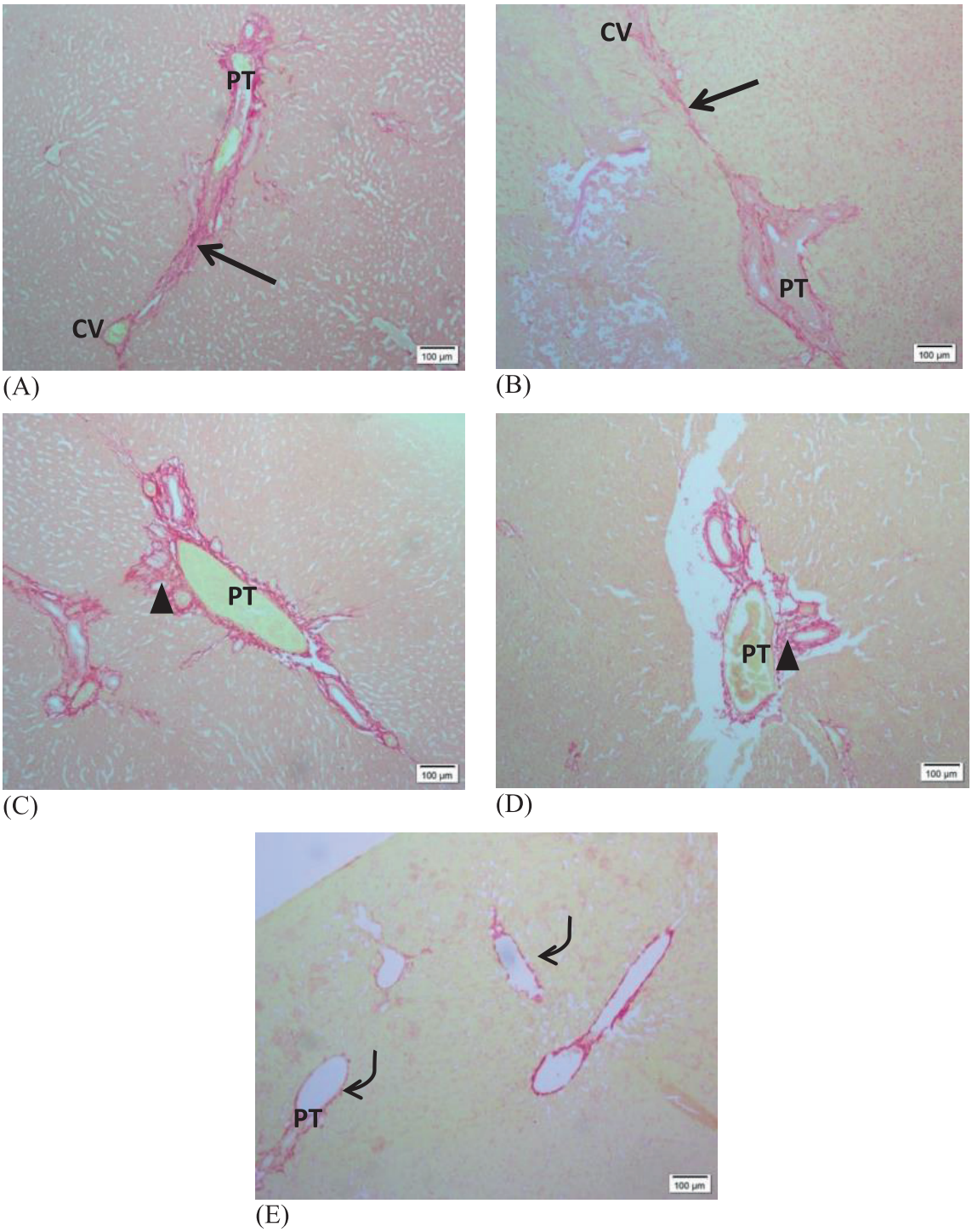

Figure 4. Metavir Scoring System showed marked bridging fibrosis (arrow) in sham (A) and Control (B) groups, moderate fibrosis (portal fibrosis with rare septa, shown in arrowhead) in BC (C) and MSC (D) groups, and mild fibrosis (portal fibrosis without septa, shown in curved arrow) in MSC+BC groups (E) (**PT**: Portal tract, **CV**: Central vein)

Discussion

Liver fibrosis is originally a healing response of the liver, but due to continuous excessive extracellular matrix protein deposition, it progresses to cirrhosis which is no longer reversible.[2,3] Liver fibrosis level has been studied to have a strong association with the liver regenerative capacity and have an impact on survival following liver resection.[5,8] PDGF is one of the fibrogenic activity markers which is the most important signaling pathway in liver fibrogenesis as being the most potent mitogen for HSC.[7,16] Recent study shows that early liver fibrosis is reversible by

eliminating the causative agent and controlling the inflammation.[26] The MSC and BC have antifibrotic effects in a different mechanisms. This study analyzed the effect of MSC, BC, and their combination to plasma PDGF and fibrosis level of the fibrotic liver of rats after hepatectomy. In our study, liver fibrosis was established on an 8-week intraperitoneal injection of CCl_4 (figure 2).

The PDGF levels were increased after hepatectomy of BC, MSC, and MSC+BC groups on day 3 due to hepatocytes replication. The replication that happened in those groups was probably allowed by controlled inflammation and fibrosis after administration of MSC's and BC. The controlled inflammation process will allow the liver to regenerate, and raised PDGF level as a growth factor.[13] While in the control group, the PDGF level was reduced due to the fibrotic environment. Our finding was supported by previous studies that showed the increase of PDGF level in the first two days after MSC administration, and the fibrotic environment impairs the hepatocytes early regenerations.[8,13] MSCs control the fibrogenesis through hepatocyte trans-differentiation, immunomodulatory of HSC, and regulation of T-reg Cells, MSC's also secrete trophic factors that enhance cell proliferation.[9,13,14] BC also has an anti-inflammatory effect, trophic factors, and IGF-1 (the most abundant growth factor in BC) which has an antifibrogenic effect.[18,19,21]

On day 7, we found that the PDGF level was similar in all groups. We suggested the fibrogenesis was continuously developed, while there was a decrease of hepatocytes proliferation after hepatectomy. In line with this study, the previous study in fibrotic liver rats showed that after 75% hepatectomy, fibrogenesis continued to develop and rose rapidly from day 5.[8] This finding was also fit a previous study that showed the effect of MSC in form of controlled inflammation in the non-hepatectomy fibrotic liver will last in seven days.[13] The BC group also show a similar pattern, therefore we suggested that the BC effect also decreased after day 3.

The PDGF level of the control group was started to increase rapidly on day 10 compared to day 7. Therefore, we assumed there was new fibrogenesis after 50% hepatectomy in the fibrotic liver around day 7 which we hereinafter call "second hit fibrogenesis". The previous study confirmed our study that new fibrogenesis occurred on day 5 after 75% hepatectomy of fibrotic liver rats, which is marked by significant periportal hepatocytes

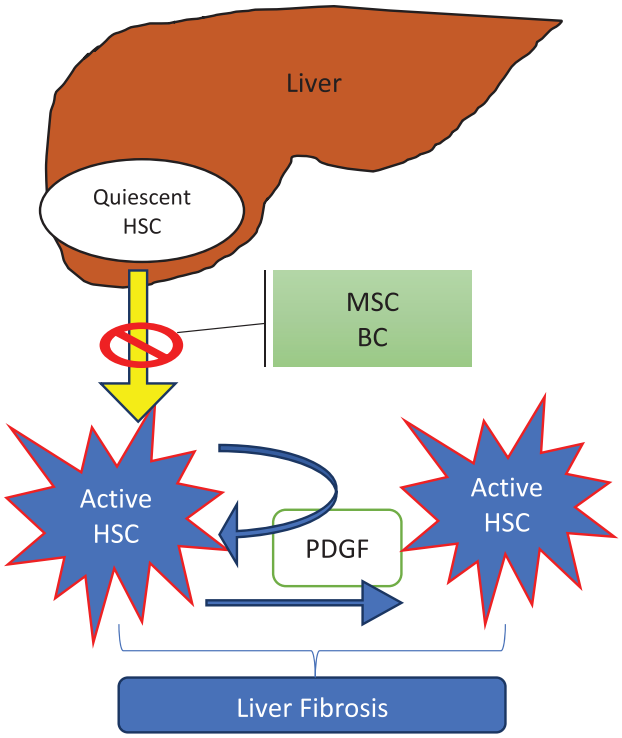

Figure 5. The MSC and BC reduce active HSCs and decrease liver fibrosis

apoptosis. The apoptosis wasn't found in the non-fibrotic liver group after 75% hepatectomy. That study also revealed that collagen production almost reached the collagen amount before hepatectomy on day 10.[8] Interestingly, only the MSC+BC group had significantly decreased PDGF level at day 10 compared to day 7. This finding suggested that MSC+BC successfully inhibited the second hit fibrogenesis, while the BC and MSC groups didn't. We assumed that there was a synergistic effect of MSC and BC so their activity was stronger and more effective than MSCs and BC alone. There might be insufficient quantities of MSCs and BC to control the second hit fibrogenesis, as a previous study of MSC and BC was only conducted in non-hepatectomy liver fibrosis with no second hit fibrogenesis.[9,10] The low PDGF level in the MSC+BC group indicated low proliferation of HSCs, as PDGF is the most potent mitogen for HSCs.

The PDGF level on day 10 was supported by histopathologic findings. Based on Metavir classification, there was only mild fibrosis in the MSC+BC group, while the BC and MSC groups had moderate fibrosis and the control and sham groups showed marked fibrosis (figure 4). This was caused by the decrease of fibrogenesis on day 7.

Liver fibrogenesis is a chronic process, so the initial fibrogenesis inhibition is important and will relate to the end process of fibrosis. The HSCs can maintain their active form and induce other HSC to proliferate through their autocrine and paracrine mechanisms by PDGF.[11,15,16] Therefore, the reduction of active HSC by MSC and BC combination will reduce the PDGF level and fibrogenesis rate (figure 5).[9,14]

Nevertheless, our finding should be verified by histopathologic evaluation on day 7, which was not done in this study. Further study with increasing of MSCs doses by bolus administration or by more than one administration will support this study. The synergistic effect of MSC and BC that was assumed to occur in the early days and prevented second hit fibrogenesis should also be proven by histopathologic and several fibrogenic marker examinations such as TGF-β, TNF-α, IL-10, and Smooth Muscle Actin.

Conclusion

The MSC and BC combination has significantly lowered PDGF plasma and fibrosis levels in the liver remnant compared to MSCs and BC alone on day 10 after hepatectomy. The combination of MSCs and BC can prevent second hit fibrogenesis after 50% hepatectomy of fibrotic liver rats, while the same amount of the MSCs and BC alone cannot. Therefore, the MSC and BC combination has a better antifibrotic effect in post-hepatectomy liver fibrosis than BC or MSC alone.

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Authors' contribution:

Data gathering and idea owner of this study:Ahmad FathiFuadi, Ignatius Riwanto, Agung Putra, Erik Prabowo

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Writting and submiting manuscript: Ahmad FathiFuadi, Agung Putra

Editing and approval of final draft: Ahmad FathiFuadi, Ignatius Riwanto, Agung Putra, Erik Prabowo

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