

The Effectiveness of Mesenchymal Stem Cell and Colostrum Bovine Combination in Post Hepatectomy Liver Failure with Liver Fibrosis Animal Model

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Abstract: Post hepatectomy liver fibrosis (PHLF) is the most common cause of morbidity and mortality after liver surgery. Recently, mesenchymal stem cells (MSCs) and bovine colostrum (BC) have been studied to exert an anti-fibrotic. However, the effect of MSCs, BC, and their combination of tissue regeneration after PHLF remains unclear. This study aimed to evaluate the effect of MSCs, BC and its combination in regulating transforming growth factor- β (TGF- β) and serum glutamic pyruvic transaminase (SGPT) associated with liver destruction inhibition on PHLF animal models. Eighteen Sprague-Dawley were injected with CCl₄ for eight weeks and 50% liver resection (LR). Mice were divided into three groups: group NaCl 0.9 % with parenchymal injection, group MSC with parenchymal injection, and a group of MSCs with parenchymal injection and BC oral. After administration, the level TGF β and SGPT were collected on the 3rd and 7th days. This study showed that the TGF- β levels nit significant decrease under combination treatment compared to the MSCs group until 34,11pg/mL \pm 4,18 and 34,5 pg/mL \pm 2.94, respectively, on day 7th. Besides, SPGT levels of combination treatment also did not significantly decrease compared to the MSCs group until 93,6 U/L \pm 23,8 and 163,2U/l \pm 73,62, respectively, on day 3rd. In conclusion, the combination therapy is no better than single MSCs treatment in regenerating liver tissue on PHLF.

1 INTRODUCTION

Hepatocellular carcinoma (HCC) is the liver's primary cancer, accounting for most liver cancers. HCC is one of the leading causes of cancer-related death worldwide and has high evidence in 2012. The incidence of HCC found fourteen million patients, and it grew to twenty-two million patients in the last two decades. HCC causes infection of hepatitis B virus infection, often accompanied by cholestasis. It makes inflammatory processes that encourage liver fibrosis. On normal liver can perform liver resection

up to 75% of the total liver volume by maintaining 25% of the remnant liver on large HCC. HCC cases with fibrosis and less than 40% of liver remnants after resection mostly progress into small for size liver syndrome (SFSS) and liver failure (William and Janagin, 2017; Xia, Lu, and Wang. 2008)

The progression of SFSS into liver failure is not determined by the size of the liver remnant only but also the hemodynamic liver circulation (Golriz et al.,2015). There was a disruption of normal hepatocyte regeneration due to various events, including parenchyma loss and hepatic vascular bed reduction. The increasing portal pressure makes shear stress on

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the sinusoid endothelial cell. Nitric oxide (NO) is then released by liver sinusoidal endothelial cells (LSECs), and a continuous pro-inflammatory mediator exposure produced by the inflammatory cell, particularly Kupffer cell (Golriz et al., 2015, Ray et al., 2015). During chronic inflammation, Kupffer cells induce the prolonged release of TGF- β 1 leading to LF formation that contributed to liver failure following liver resection (Hoffmann et al., 2020). Considering this complicated process, finding an effective treatment in resolving post hepatectomy liver fibrosis (PHLF) could be very challenging. Only a few methods to prevent SFSS in the preoperative, perioperative, and post-operative due to liver transplantation as the lack of liver donors (Kim et al., 2019).

On the other hand, previous studies reported that MSC has the robust capability to suppress the release of TGF secreted by inflammatory cells leading to wound healing acceleration (Yo et al., 2013; Putra et al., 2020). Other studies revealed that BC containing growth factor and anti-oxidant also has an essential role in liver regeneration (Sinn et al., 2017). MSCs are multipotent cells characterized by the high expressions of several surface markers such as CD105, CD73, CD29, CD90, and CD44 and lack of expressions of CD79a, CD11b, CD14, CD34, CD45, and CD19 (Ly et al., 2014). These cells have low immunogenicity, self-renewal, and multidirectional differentiation properties (Zhang et al., 2018). Previous studies have shown that MSCs have immunomodulatory properties by secreting soluble cytokines to inhibit inflammatory cells and prevent excessive inflammatory damage to the liver tissue in drug-induced liver failure animal model (Hu, Wu, and Li, 2020). Bone marrow-derived mesenchymal stem cells (BM-MSCs) reduced the expression of TGF β lead to inhibition of the TGF β signalling pathway in liver fibrosis formation (Jang et al., 2014). A previous study reported that the post hepatectomy LF animal model, which received MSC treatment is successfully survived with lower ALT and AST levels (Ding et al., 2019)

BC is a complex mixture of protein, lipids, lactose, vitamin, and mineral. It includes immunoglobulin, multiple growth factors, and total anti-oxidants capacity affecting liver injury due to decreased TGF- β associated with fibrogenesis inhibition and SGPT level decreased (Sinn et al., 2017). Although MSCs and BC could resolve liver injury in LF, the efficacy of both modalities in decreasing the progressivity of liver damage, in this case, need further investigation. Therefore, in this study, we compare the effectiveness of MSCs with BC combination compared to MSCs alone in

decreasing the progressivity of liver destruction in PHLF by analyzing the regulation of TGF β and SGPT level.

2 MATERIALS AND METHODS

2.1 Isolation of UC-MSCs

The umbilical cord (UC) derived MSCs (UC-MSCs) were obtained from pregnant single Sprague-Dawley (SD) rats under deep anaesthesia and transplanted into an ALF rat model. The umbilical cord was cut into pieces after the blood vessels were removed. It was then transferred to a T25 culture flask containing complete Dulbecco's Modified Eagle's medium (DMEM) (Catalog #2192773 Sigma-Aldrich, Louis St, MO) enriched with 10% Fetal Bovine Serum (FBS) (Catalog #42A1190K GibcoTM Invitrogen, NY, USA) and 100 IU/mL penicillin/streptomycin (Catalog # 15070063 Sigma-Aldrich, USA). These cells were incubated in a 5% CO₂, 37°C incubator, and the medium was changed every three days. After the cells reached 80% confluency, the MSC-like cells were passaged with trypsin. The cells from the 4th passage were used for experiments. The Institutional Review Board approved this study of the Medical Department's Ethics Committee with number 254/VIII/2020/ commission bioethics, Sultan Agung Islamic University, in Semarang, Indonesia.

2.2 PHLF Animal Models Induction of PHLF by Made Remnant Liver Fibrosis Animals and Experimental Design

Eighteen Sprague-Dawley (SD) male rats were randomly divided into three groups (n= 18). LF induction was performed by injecting intraperitoneal the carbon tetrachloride (CCl₄) (Catalog #56235 Sigma-Aldrich, USA) with 1 ml/kg twice per week for eight weeks. After six weeks, three rats in the model group were sacrificed randomly, and the liver tissue was obtained to verify LF with Sirius red. All of the PHLF with LF rats undergoing liver resection 50% of the liver in the median and right lateral lobes. The surgical procedure was performed within the sterile condition under intravenous anaesthesia using xylazine + ketamine (5 mg/kg + 100 mg/kg intramuscularly) (Constandinou et al., 2005).

The Group of NaCl 0.9 % and parenchymal injection NaCl 0,9% 500 μ L, group MSC with parenchymal injection with doses 1 x 10⁶ cells

dissolved in 500 μ L of NaCl, and group combination: MSCs parenchymal injection 1×10^6 cells dissolved in 500 μ L of NaCl and oral administration of BC doses 15 μ L/g per oral, daily with milk powder Good Health.

2.3 Flow Cytometry Immunophenotyping of UC-MSCs

The immunophenotypes of MSCs were analyzed in the fourth passage. MSCs were stained using conjugated antibodies: fluorescein isothiocyanate (FITC)-conjugated CD90, Allophycocyanin (APC)-conjugated CD73, Peridinin Chlorophyll Protein Complex (PerCP)-conjugated CD105 and phycoerythrin (PE)-conjugated Lin monoclonal antibodies for 30 min at 4°C in the darkroom. The cell's fluorescence intensity was evaluated through flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA).

2.4 In Vitro Differentiation

MSCs differentiation potential was determined to characterize the isolated cells. These cells were cultured in DMEM medium supplemented with 10% FBS, ten mmol/L β -glycerophosphate, 10^7 mol/L/ 0.1 μ M dexamethasone, 50 μ mol/L ascorbate-2-phosphate (Catalog #SLBL4673V Sigma-Aldrich, Louis St, MO), at 37°C and 5% CO₂. The fixed cells were stained with 0.2 % Alizarin Red solution (Catalog #MKBS9114V Sigma-Aldrich) to represent calcium deposition (the cells were used from the fourth passage).

2.5 Elisa TGF- β

The rat's blood was harvested via peri-orbital venous plexus bleeding under general anaesthesia on day 3rd, 7th day after treatment, and the serum was collected by centrifugation at 4°C. The TGF- β levels were measured by ELISA kits, based on the manufacturer's instructions (*Abbkine*) and according to a standard curve constructed for each assay. The colourimetric absorbance was recorded at a wavelength of 450 nm.

2.6 SGPT Levels

The levels of SGPT were measured on the 3rd, 7th days after treatment to determine liver function. Blood samples were collected from the peri-orbital vein under anaesthesia, using xylazine + ketamine (5 mg/kg + 100 mg/kg intramuscularly) (Alfasan, Netherlands). The serum level of SGPT was

measured using automatic analyzers (BT 3000 PLUS, Italy).

2.7 Statistical Analysis

All data were presented as mean \pm standard deviation with differences between groups analyzed by a one-way ANOVA and least significant difference comparison *post hoc* LSD test. Analysis using a $p < 0.05$ significant statistical value.

3 RESULTS

3.1 Characteristics of UC-MSCs and Differentiation Test

The isolated cells showed peculiar fibroblast-like (spindle shape) morphology. To determine and verify the MSCs marker, we assessed MSC's marker expression using flow cytometry after the fourth passages (Figure 1). The results showed that the isolated cells expressed an MSC-specific marker including positive expression of CD105 (96.7%), CD73 (99.2%), and CD90 (96.7%) and lack of Lin (0.03%). In line with the flow cytometry analysis, we also examined the osteogenic capabilities of MSCs. We found the differentiation of MSC into osteogenic has occurred, indicated by calcium deposits as red appearance using the Alizarin red dye staining method (Figure 2). It is according to the International Society of Cellular Therapy (ISCT).

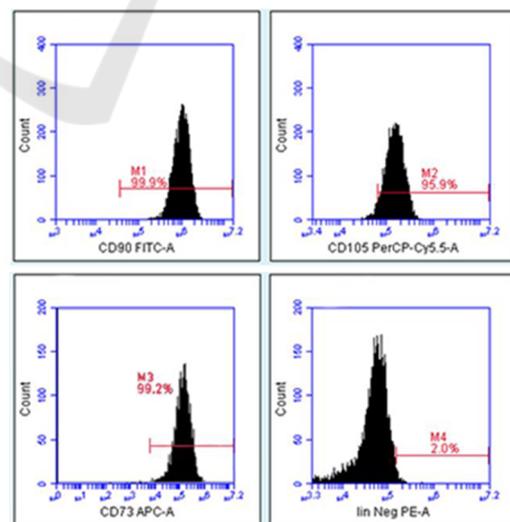


Figure 1: MSCs characterization. The graph displayed the expression MSC, positive markers (CD105, CD73, and CD90), and lack of the negative marker Lin (Lin) expression.

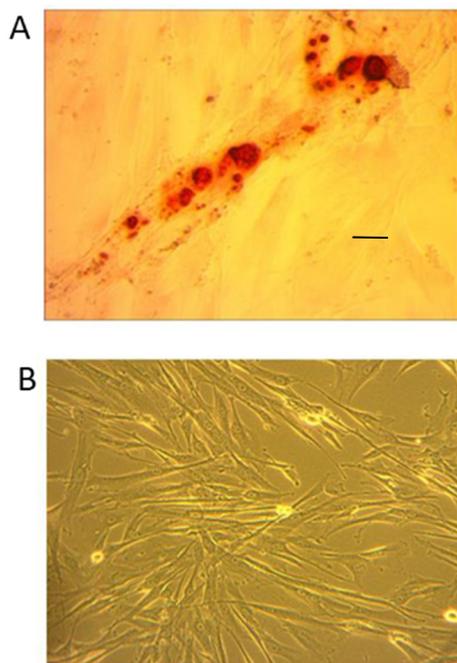


Figure 2. The morphology and differentiation of MSCs. (A)MSC Differentiation. Alzarin red dye showed a red colour appearance in the MSC differentiation test (magnification x40, scale bar 50 μ m). (B) Morphological characterization. MSCs appearance; homogeneous, spindle-shaped, fibroblast-like cells (magnification x10, scale bar 200 μ m).

3.2 MSCs Suppress TGF- β and SGPT Levels in PHLF Animal Model

The TGF- β is one of the primary growth factors with pleiotropic capability particularly associated with fibrosis formation due to its ability to changes activate hepatic stellate cell (HSC) into myofibroblast (MF) in producing collagen type III. To determine the role of MCS in decreasing TGF- β level in PHLF, we analyzed using ELISA. In this study we found, there is a significant decreased ($p < 0,05$) of TGF- β level on day 7th in MSC treatment group (day 3rd 29,66 pg/mL $\pm 0,76$; day 7th 34,11 pg/mL $\pm 4,18$, respectively) and Combination group (day 3rd 36,54 pg/mL $\pm 3,72$; day 7th 34,5 pg/mL ± 2.94) compared with the control group NaCl Injection (day 3rd 36.33 pg/ml ± 3.04 , day 7th 56.7pg/ml ± 6.22) (Figure 3).

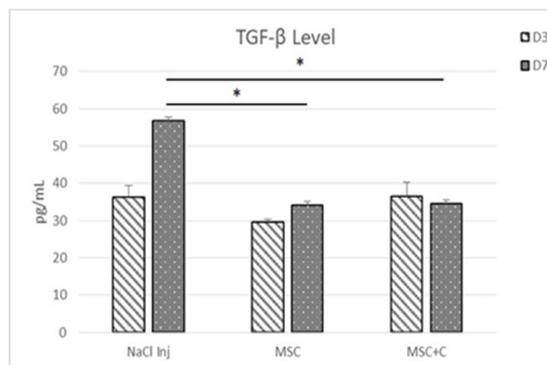


Figure 3. The level of TGF- β at 3rd, and 7th day in the NaCl, MSC, and combination group. *The level of TGF- β day MSC (34,11pg/mL $\pm 4,18$) and combination group, (34,5 pg/mL ± 2.94) on day 7th was decreased significantly compared to NaCl group (56.7pg/ml ± 6.22) $p < 0,05$. C: Colostrum

The increase of SGPT level indicated that there was a hepatocellular injury, including on PHLF. We examined the level of SGPT after MSC treatment compared to a combination of both treatments to regenerate the damaged liver tissue in PHLF. In this study, we found a significant decrease ($p: 0,024$) of SGPT level in the MSC group only on day 3rd (day 3rd 93,6 U/L $\pm 23,8$, day 7th 58,8 U/L ± 2.68). We did not found a significant decrease in the combination group on day 3rd and 7th (day 3rd 131,4 U/L $\pm 41,2$, day 7th 52,8 U/L $\pm 10,57$) compared with NaCl group (day 3rd 163,2U/L $\pm 73,62$, day 7th 66U/L ± 17.01) (Figure 4).

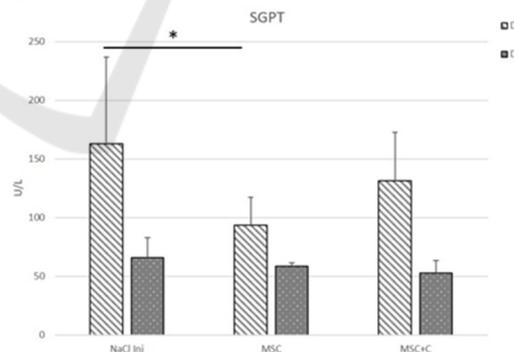


Figure 4. SGPT level on 3rd, and 7th day in NaCl, MSC and combine group. Data are presented as the mean \pm standard deviation. *The level of SGPT decreased significantly in MSCs group only on day 3rd (93,6 U/L $\pm 23,8$) compared to NaCl group (163,2U/L $\pm 73,62$) $p < 0,05$. C: Colostrum

4 DISCUSSION

The aims of this study were evaluated the effectiveness of MSCs and combination of MSCs and BC in the regeneration of liver tissues on PHLF by analyzing the regulation of TGF β and SGPT level. The ability of MSCs decrease fibrosis formation by controlling the prolonged release of TGF- β produced by MF as associate cell product fibrosis deposits. BC containing anti-oxidant also can improve LF and restore the damaged structure and function of liver tissue. As the potent mediators, the TGF- β initiates consistently with the activation and differentiation of HSCs to be active MFs resulting in collagen type III deposition. We injected CCl₄ as a hepatotoxic chemical to induce LF and performed liver resection 50 % to induce PHLF by major resection liver to make a small liver remnant (Golriz et al., 2015)

We present here that MSCs and combination group can suppress the release of TGF- β in PHLF model animals. We believe this is a novel discovery to demonstrate the mechanism of how the PHLF with fully developed TGF- β -dependent fibrosis can be disrupted by a combination of BC and MSCs application. We found that MSC has two mechanisms of action. First, MSCs can decrease TGF- β levels by depolarizing macrophage type I (M1) into macrophage type II (M2) (Darlan et al., 2020). Second, we suggest that MSCs affect immunomodulatory mechanisms by releasing IL-10. The receptor of Kupffer-IL-10 binding might activate Janus tyrosine kinase 1 (JAK1) and tyrosine kinase-2, leading to activation of signal transducer and transcription 3 (STAT3), then translocated into the nucleus to binds the promoters of target genes, the suppressor of cytokine signalling 3 (SOCS3) correlated with the decreased expression of tumour necrosis factor (TNF)- α , IL-1 β , and TGF- β (Sziksz et al., 2015). Our findings were in line with a previous study that showed MSCs could prevent peritoneal fibrosis by releasing IL-10 (Muhar et al., 2018). Our finding MF cell produced TGF β for autocrine to stay active production collagen type III. MSC Group and combine group has released immunomodulatory properties, leading to the decreasing of TFG β longer day 7th in PHLF

In line with the decrease of TGF β level, we also found SGPT level decreasing just in the MSCs group only on day 3rd. Phase inflammation on PHLF disrupts hepatocyte cells because injury from liver resection and stagnant portal venous blood stimulates inflammation with collagen deposition on the portal venous release by MF (Golriz et al., 2015, Ray et al., 2015). MSC has an effect immunomodulator and

enhances regeneration hepatocyte by release secretome. Our previous study releases that intraparenchymal of MSCs administration in the damaged liver tissue leads to the MSC migration to the injured areas for repairing and restoring the damaged liver structure and its function. BC previous study can decrease SGPT level by release anti-oxidants in acute liver injury (Sinn et al., 2017).

We assume the combination group can not affect inflammatory phase PHLF because BC has a product growth factor pro-inflammation and disrupts immunomodulator from MSC (Quiles et al., 2006). In repairing and restoring damaged tissue, MSCs should previously control inflammation by releasing IL-10 to inhibit the prolonged release of TGF, leading to healing process acceleration (Putra et al., 2019). Under prolonged controlled inflammation, MSC induces decreased remnant liver destruction and restored liver function (Fiore, 2018). Taken together, the single MSCs treatment more effective than the combination of MSCs and BC to regenerating liver tissues on PHLF.

5 CONCLUSIONS

We conclude that MSC and BC's combination is not better than MSCs alone in decreasing TGF β and SGPT. We suggest further research about the regeneration of the PHLF by examining marker regeneration and portal flow using MSC to explain the liver's healing process.

Conflict of Interest

The authors declare that they have no conflict of interests.

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