The Combination of Mesenchymal Stem Cells and Bovine Colostrum in Reducing α-SMA Expression and NLR Levels in Wistar Rats After 50% Fibrotic Liver Resection

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Abstract

BACKGROUND: Liver fibrogenesis will produce α-smooth muscle actin (α-SMA) expression and a continuous inflammatory process, seen through the neutrophil lymphocyte ratio (NLR). The combination of mesenchymal stem cells (MSCs) and bovine colostrum is a novel strategy for repairing hepatic fibrosis tissue.

AIM: The aim of this study is to evaluate the combination of MSCs and bovine colostrum to reduce α-SMA expression and NLR levels in Wistar rats after 50% of fibrotic liver resection.

METHODS: Thirty-six Wistar male rats were randomly divided into five groups (sham, control, colostrum, MSCs, and combination of colostrum-MSCs). Rats were injected with CCl₄ for 8 weeks to induce liver fibrosis then underwent liver resection. NLR levels were determined using hematology analyzer, α-SMA expression of myofibroblast was analyzed by immunofluorescence staining.

RESULTS: A significant reduction in NLR levels on day 3 in the treatment Group I (1.10), Treatment II (0.83), and Treatment III (0.54) compared to the control group. A significant decrease in α-SMA in treatment Group I (0.134), Treatment II (0.68), and Treatment III (0.42) compared to the control group.

CONCLUSION: In this study, it was found that α-SMA expression and NLR levels on the 3rd day of administration were reduced in group receiving combination of MSCs and bovine colostrum in the liver of post-resection Wistar rats by 50%.

Introductions

Liver fibrosis occurs due to chronic liver damage and inflammation, from injuries of viral hepatitis, autoimmune, alcohol consumption, and non-alcoholic steatohepatitis (NASH), which characterized by deposition of the extracellular matrix with distortion of the normal liver parenchyma [1, 2, 3, 4]. The process of hepatic fibrogenesis triggers myofibroblasts to become active which then results in the expression of α-smooth muscle actin (α-SMA) as a molecular marker that continues to rise, along with the increase of extracellular matrix in the liver [4, 5]. In addition, there is a systemic increase in immune system cells, such as neutrophils and lymphocytes ratio (NLR), along with fat accumulation in liver is also one of the key contributors of liver fibrosis [6, 7, 8]. In light of the above findings, the fibrosis process will persist, resulting in reduced liver function.

Mesenchymal stem cells (MSCs) have the capacity to self-renew and differentiate into other cell types, thereby facilitating the process of cell regeneration [9, 10, 11, 12, 13]. On the other hand, bovine colostrum has antioxidant properties that can aid in tissue repair [14]. The combination of MSCs and bovine colostrum can aid in the regeneration of cells and tissues in liver fibrosis. At present, the expression of α-SMA is the most widely used marker of myofibroblasts in studies assessing the fibrosis process by taking a role in cell contraction and formation of cytoskeletal tension [15]. Judging from the functional level, myofibroblasts effectively repair defects and rebuild tissue integrity. Collagen scar formation occurs and stiffness leading to decreased tissue function and even organ failure if myofibroblast repair becomes chronic, such as during the development of fibrosis [16].

The neutrophil count was significantly lower in patients with advanced fibrosis than in patients without minimal fibrosis [17]. However, other studies

References


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have shown that NLR is significantly increased in patients with NASH and fibrosis compared with patients without NASH [18], [19]. A previous study reported that MSCs are able to accelerate the liver regeneration process [20]. MSCs will produce several paracrine factors including vascular endothelial growth factor (VEGF), hepatocyte growth factor, insulin growth factor, and interleukin 10 (IL-10) that associated with liver regeneration [11], [12], [21], [22], [23]. In addition, bovine colostrum has a total antioxidant capacity, such as superoxide dismutase, glutathione peroxidase, catalase, Vitamin E, Vitamin C, and β carotene [24]. Therefore, in this study aims to evaluate the effect of combination MSCs and bovine colostrum on the fibrotic liver mouse model.

Materials and Methods

Research design

This study employed a posttest-only control group design to evaluate the efficacy of the combination of MSCs and bovine colostrum in reducing-SMA expression and NLR levels in Wistar rats following a 50% of fibrotic liver resection. The research was conducted for 8 weeks during December 2020–February 2021 at the Stem Cell and Cancer Research Laboratory of Sultan Agung University and the Animal Health Laboratory, Semarang.

Study subjects

The subjects of this study were male rats of Wistar strain, aged 3 months and had body weight around 200–250 g. The number of samples for each study group was six rats. The research subjects were randomly divided into six groups; therefore, the total sample was 36 rats. The research subjects were adapted for 1 week and were given standard feed.

Treatment

Subjects were randomly divided into groups and then received intraperitoneal CCl4 injection 2 µL/g twice a week for 7 weeks, and performed 50% of liver resection. Subjects were divided into control Group I (NaCl 0.9% oral), control Group II (NaCl 0.9% injection), treatment Group I (bovine colostrum 15 µL/g/day for 7 days), treatment Group II (1,000,000 doses of MSCs), treatment Group III (1,000,000 doses of MSCs and bovine colostrum 15 µL/g/day for 7 days), and treatment Group IV (Sham). Then, the validation by histological examination was performed by making preparations of Hematoxylin and Eosin (H&E). Blood was collected to assess the levels of hepatic biochemistry on day 3 and day 10 of intervention. A laparotomy was performed after 10 days, liver collection and histological check of the liver samples were also taken.

Variable measurement

Analysis of α-SMA expression was using IHC technique with anti-α-SMA antibody. The result of the examination was the intensity of expression, marked in brown, seen with a 40× magnification microscope in five fields of view with 5–10 expression compartments for each field of view. Neutrophil lymphocyte ratio (NLR) was measured by hematology analyzer and be calculated in quantity/liter units.

Isolation of Umbilical cord (UC)-MSCs

UC derived MSCs (UC-MSCs) were obtained from pregnant single Wistar rats under deep anesthesia and transplanted into an ALF rat model. Briefly, the umbilical cord was cut into pieces after the blood vessels were removed and then transferred to a T25 culture flask containing complete Dulbecco’s Modified Eagle’s medium (DMEM) (Sigma-Aldrich, Louis St, MO) mixed with 10% of fetal bovine serum (FBS) (GibcoTM Invitrogen, NY, USA), and 100 IU/mL penicillin/streptomycin (Sigma-Aldrich). These cells were incubated in a 5% CO2, 37°C incubator, and the medium was changed every 3 days. After cells reached 80% confluence, the MSC-like cells were passaged with trypsin. Cells from the forth passage were used for experiments. This study was approved by the Institutional Review Board of the Ethics Committee of the Medical Department, Sultan Agung Islamic University, Semarang, Indonesia.

Induction of post-hepatectomy liver failure (PHLF) with liver fibrosis animals and experimental design

Thirty-six Wistar male rats were randomly divided into Sham group (n = 6) and the model groups (n = 30). The induction LF was performed by injecting intraperitoneally the carbon tetrachloride (CCl4) (Sigma-Aldrich, USA) with 1 ml/kg twice per week for 8 weeks. After 6 weeks, four rats in the model group were sacrificed at random and liver tissue was obtained to verify LF. 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 under aseptic condition. Each group consisted of six Wistar rats which administered into the Sham group (negative control was fed with water); NaCl PO group (was fed with NaCl 15µL/g five times a week orally); NaCl injection (was injected 15µL/g five times a week intraparenchymal); Colostrum (Colostrum milk powder (Good Health, NZ) 15 µL/g 5 times a week orally); MSC (doses 1 x 10^6 cells dissolved in 500 µL in NaCl through...
intraparenchymal injection), and MTC (MSC doses 5 x 105 cells dissolved in 500 µL NaCl 5 times a week through intraparenchymal injection + Colostrum doses 15µL/g 5 times a week orally).

**Flow cytometric immunophenotyping of UC-MSCs**

The immunophenotypes of MSCs were analyzed at fourth passage. MSCs were stained with antibodies conjugated: fluorescein isothiocyanate-conjugated CD90, Allophycocyanin-conjugated CD73, Peridinin Chlorophyll Protein Complex-conjugated CD105, and phycoerythrin (PE)-conjugated Lin monoclonal antibodies for 30 min at 4°C in the dark. The fluorescence intensity of the cells was evaluated through 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, mol/L/0.1 µM dexamethasone, 50 µmol/L ascorbate-2-phosphate (all from Sigma-Aldrich, Louis St, MO), at 37°C, and 5% CO2. The fixed cells were stained with 0.2% Alizarin Red solution (Sigma Aldrich) to represent calcium deposition (cells used were from the fourth passage).

**Statistical analysis**

Test for normality, validity, and reliability was using ANOVA. The degree of significance limit was p ≤ 0.05 with a 95% confidence interval. Data analysis was performed using a SPSS 21.

**Ethical approval**

Animal ethics was obtained from submission to the Ethics Committee for Health Research, Faculty of Medicine, Diponegoro University No. 09/EC/H/FK-UNDIP/II/2021. Treatment of animals is adjusted to the standards of animal maintenance and research report was adjusted according to NC3R ARRIVE Guidelines for Animal Research.

**Results**

**NLR Day 3**

On day 3, the control group that received injections and oral NaCl 0.9% had mean NLR values of 2.54 and 2.27, respectively. The number of NLRs in the group that received colostrum and MSC alone decreased significantly to 1.10 and 0.83, respectively (Figure 1).

![Figure 1](https://oamjms.eu/index.php/mjms/index)

**NLR levels on day 10**

The NLR profile on day 10 has a similar profile to day 3. The control group that received injections and oral NaCl 0.9% had mean NLR values of 1.49 and 1.05, respectively. The number of NLRs in the group that received colostrum and MSC alone decreased significantly to 0.76 and 0.64, respectively (Figure 2).

**α-SMA expression**

The control group which was given 0.9% NaCl orally and by injection had a mean α-SMA expression
value of 0.268 and 0.272, respectively. The combination group experienced a significantly greater decrease in $\alpha$-SMA expression up to 0.42 compared to the colostrum and MSC single-therapy group (Figure 3a and b). These results were stated to be significant with a decrease in the graphic of treatment group with the combination of MSCs and bovine colostrum compared to the control group of NaCl injection and oral NaCl.

**Discussion**

Fibroproliferation disorders of the liver are surprisingly common and essentially untreatable disease that results in substantial morbidity and mortality. The previous study reported that neutrophil contributed to the proliferation of fibroblast [25]. However, the role of MSCs and colostrum on the NLR and fibroblast proliferation through $\alpha$-SMA expression remains unclear. In this study, we found that combination of MSCs and colostrum inhibit liver fibrosis through suppress level of NLR associated with decrease levels of $\alpha$-SMA.

The research data showed that all NLR levels on day 3 were significantly decrease by MSCs group treatment. This result showed that the MSCs have the role of immunosuppressants during inflammatory process in liver fibrosis. This finding supported the previous study revealed that MSCs-mediated immunosuppression mainly acts through the secretion of soluble molecules that are induces or upregulated indoleamine 2,3-dioxygenase [23], [26]. In addition, MSCs released soluble factor of anti-inflammation such as IL-10 and growth factor including transforming growth factor-6, fibroblast growth factor, platelet-derived growth factor, and VEGF [27], [28]. This obvious reduction even exceeds the combination of MSCs and bovine colostrum in the early inflammatory phase of liver fibrosis.

NLR levels on day 10 were also reduced. The graphic showed that the combination of MSCs and bovine colostrum (MSC+C) has the lowest value with 0.54. This result showed that the role of immunosuppressants in MSCs is supported by the control of bovine colostrum as an immunoregulator. It could be seen that bovine colostrum needs time to assist MSCs in supporting this role.

In the results of data regarding $\alpha$-SMA expression, there was a decrease in $\alpha$-SMA expression,
and it was seen that the combination of mesenchymal stem cells and bovine colostrum (MSC+C) had the lowest value with 0.042. This result also supported the facts that the role of immunosuppressants in mesenchymal stem cells is assisted by bovine colostrum function as an immunoregulator.

Conclusion

In this study, α-SMA expression was reduced in the group receiving combination of mesenchymal stem cells and bovine colostrum in the liver of Wistar rats after 50% of liver resection. NLR levels were also reduced on day 3 and day 10 of intervention in the group receiving combination of mesenchymal stem cells and bovine colostrum in the liver of Wistar rats after 50% of liver resection.

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Author Contributions

MMH, YWP, and AP were responsible for experimental design and developed methodology. MMH and NDA carried out the experiments. MMH, AP, and YP interpreted the results, performed data analysis, and prepared the figures and tables. AP and NDA wrote, reviewed, and revised the manuscript. AP provided administrative, technical, and material support. AP and YP supervised the study.

References


