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Human mesenchymal stem cell secretome increases hepatocyte growth factor expression and promotes liver regeneration in Wistar rats with cholestasis

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ABSTRACT

Globally, the annual mortality rate due to liver cirrhosis is 2.2%, which ranks 12th as the cause of death in the United States. Human mesenchymal stem cell secretome (Hu-MSC-S) is known to reduce liver injury and inflammation. Thus, the present study aims to determine the effect of Hu-MSC-S on hepatocyte growth factor (HGF) expression and liver regeneration in cholestatic rats after choledochal duct ligation receiving standard ursodeoxycholic acid (UDCA) therapy. A randomized experimental study was conducted on 24 male Wistar rats. All rats were randomly assigned into four groups such as control, UDCA, Hu-MSC-S, and UDCA+Hu-MSC-S. The expression of HGF and mitotic index as markers of liver regeneration were evaluated using histopathologic examination. Both HGF expression and the mitotic index were significantly higher in the UDCA and Hu-MSC-S groups ($P < 0.05$) compared to the control group. The combined administration of UDCA and Hu-MSC-S resulted in significantly higher HGF expression and mitotic index than the individual intervention ($P < 0.05$). The additional administration of Hu-MSC-S increased HGF expression and mitotic index in rat model of hepatic cholestasis. In conclusion, HuMSC-S administration could be considered as a therapy in patients who received standard UDCA therapy for liver regeneration.

INTRODUCTION

Cholestasis is an impairment of bile flow, causes an accumulation of bile in the blood or hepatocytes, and potentially leads to cirrhosis [1]. The annual mortality rate due to liver cirrhosis is reported at 2.2% in the world, which ranks 12th as the cause of death in the United States [2]. The primary therapy for liver cirrhosis is liver transplantation which requires time, high costs, and a long process of searching for a matched donor. The risk of disease progression increases while waiting for a transplant, in these circumstances, ursodeoxycholic acid (UDCA) is given to prevent it. However, UDCA cannot induce liver cell regeneration, as it inhibits the process of apoptosis [3]. The connection between UDCA and liver cell regeneration has been inferred indirectly from research on microRNAs which play a significant role in modulating cell proliferation [4]. Thus, searching for alternative therapy is necessary immediately.

Stem cell therapy is considered a regenerative therapy. Stem cells are unspecialized cells of the body, capable of differentiating into any cell and regenerating [5]. It plays a role in liver differentiation by producing new liver tissue to replace damaged ones [6]. Three main functions of stem cell therapy are the replacement of tissue via multipotent differentiation, immunomodulatory and anti-inflammatory effects, and secretion of

molecules (secretome) that assist in tissue repair [7]. Recent evidence suggests that most of the therapeutic effect on stem cells comes from a secretome, a substance secreted by stem cells. Secretomes are easy to produce and low-cost compared to stem cells and can be produced more than stem cells [8].

Human mesenchymal stem cell secretome (Hu-MSC-S) is known to reduce liver injury and inflammation and promote liver regeneration. Hu-MSC-S secretion modulates innate and adaptive immune effector cells leading to an immunosuppressive environment and anti-inflammatory effects [9]. Mesenchymal stem cells (MSCs) are multipotent and can differentiate into several cell types, making MSCs able to function as a cell reservoir for regenerative medicine [10]. A study showed that the addition of HuMSC-S to UDCA lowered caspase-3 levels and apoptotic cell count in rats with hepatic cholestasis after choledochal duct ligation [11].

Tissue injury, including biliary obstruction, will recruit proinflammatory cytokines to the injured hepatic or portal area and lead to the activation of hepatic cells. Damage to hepatocytes causes the release of reactive oxygen species (ROS) and fibrogenic mediators, induces hepatic stellate cell activation, and stimulates myofibroblast fibrogenic action, which causes tissue inflammation, fibrogenesis, and liver cirrhosis [12-14]. Based on the explanation above, the present study aimed to determine the effect of Hu-MSC-S on HGF expression and liver regeneration in rat models with cholestasis after choledochal duct ligation receiving standard UDCA therapy.

MATERIALS AND METHODS

This study was approved by the Health Research Ethics Committee of the Faculty of Medicine at Diponegoro University, Indonesia (No. 147/EC/H/FK-UNDIP/XII/2022). This randomized experimental research with a "post-test only control group" design was conducted from April to December 2022 at Gajah Mada University Experimental Animal Laboratory.

UDCA and Hu-MSC-S preparation

The UDCA (Dexa Medica, Tangerang, Indonesia) dose for this study was calculated based on the range of doses used in humans, and conversion was carried out from human dosage (250 mg in 70 kg human) to experimental animals using the Laurence-Bacharach formula [15], resulting in a dose of 4.5 mg for 200 g bw of rats [16].

The dose of Hu-MSC-S was based on the effective dose range in regeneration from prior studies, namely 0.2 ml/kg in rats intraperitoneally [17]. Hu-MSC-S was produced at Gajah Mada University Experimental Animal Laboratory, Yogyakarta, Indonesia, consisting of TGF-β, PDGF, and FGF. It was processed through 4 phases and cultured, was derived from the human umbilical cord, reaching 60% harvested using the warm trypsinization, method of treating the cells with 0.25% trypsin EDTA (Merck KgaA, Darmstadt, Germany) under warm conditions at a temperature of 36.5-37°C for 3-4 min. After trypsin neutralization, the cell suspension was centrifuged at 3000 rpm for 10 minutes. The supernatant was removed, and the cell precipitate was washed with PBS 3 times. The cell precipitate was then resuspended in a new medium with a concentration of 10.000 cells every milliliter. Stem cells are modified into embryoid bodies and grown in culture media with complete media until a junction between the embryoid bodies is formed. MSC-CM production was carried out by washing the embryoid body culture with sterile phosphate-buffered saline (PBS) and filling the embryoid body culture plate

with 10 mL of complete medium without serum. After 48 hours, MSC-CM was stored at −20 °C until use.

Animal models and surgical procedures

Twenty-four male Wistar rats aged two months weighing 200-250 g were used as experimental models. All rats that met the inclusion criteria were then adapted and given food and drink *ad libitum* for five days. Blood samples were obtained to measure the alanine transaminase (ALT) and aspartate aminotransferase (AST) serum levels before the procedure using kinetic methods for transaminase assay. Hepatic cholestasis was induced after choledochal duct ligation. Briefly, the surgical procedure begins with the administration of 0.5 cc ketamine hydrochloride anesthesia intramuscular and cefotaxime 18 mg intravenous as a prophylactic antibiotic. After disinfection of the surgical area with shaving and 0.5% chlorhexidine, a 2 cm incision was made in the midline until it reached the peritoneum and liver. For convenience, a stainless steel Colibri retractor was inserted into the peritoneal cavity to widen the surgical field. Repositioning was performed for the organs to visualize the common bile duct. The connective tissue around the peritoneum and liver was removed and the peritoneal cavity was opened. Exploration was performed to expose the portal vein, hepatic artery, and choledochal duct using micro-serrated forceps with a 0.5 mm curved tip. Ligation was performed by tying two surgical knots using a 4-0 silk suture around the bile duct above the duodenum for maximum obstruction. Suturing with 5.0 silk suture was done to close the incision wound. After 2 weeks of ligation, all rats were randomly divided into four groups such as Control, UDCA, Hu-MSC-S, and UDCA+Hu-MSC-S. The control group was the cholestasis group because this study aimed to know the difference effects of therapy with UDCA, Hu-MSC-S, and a combination of UDCA and Hu-MSC-S in cholestatic rats. The UDCA was given orally, and Hu-MSC-S was given intraperitoneally. Blood samples from the tail were obtained for ALT and AST level analysis to measure liver damage. UDCA (4.5 mg) and Hu-MSC-S (0.2 ml/kg) were given one weekly dose for four weeks. All experimental animals were cared for and managed according to animal maintenance standards [18]. The experiments were conducted following the institutional guidelines and the study has been approved by Health Research Ethical Committee, Faculty of Medicine, Universitas Diponegoro (Protocol Number: 147/EC/H/FK-UNDIP/XII/2022). The Wistar rats were managed following animal welfare regulations.

Measurement of liver enzymes

ALT and AST serum levels were analyzed using kinetic methods with DiaSts Kit (Diagnostic System GmbH, Alte Strasse 9, 65558 Holzheim, Germany) to measure liver damage.

Measurement of mitotic index

Liver biopsies were done twice, after ligation and after secretome injection. Liver biopsies after ligation aim to assess the histopathological features and liver biopsies after secretome injection aim to determine the degree of liver regeneration. The degree of liver regeneration was assessed through the mitotic index. The observation was carried out using a microscope at 100x magnification with H&E stain (Abcam, Cambidge, UK). The mitotic rate was determined by randomly evaluating at least 100

hepatocytes and presented as a percentage. Each pathologic slide was measured by two independent pathologists with the help of ImageJ software [19], and an inter-rater agreement test was done through an intra-class correlation coefficient [20].

Measurement of HGF expression

HGF levels were assessed through immunohistochemistry using the hot-spot method. 50uL of standard solution or sample is added to the appropriate tube and 50uL of antibody cocktail is added. Incubation was carried out at room temperature for 1 h at 400 rpm. Aspiration and washing of each tube were carried out 3 times. 100uL TMB Development Solution was added to each tube and incubated for 10 minutes at 400 rpm. The final step is the addition of 100 uL stop solution and readings are carried out at a wavelength of 450 nm. Assessment of hepatocyte growth factor expression was carried out using a digital microscope Carl Zeiss-Axioskop 40 (Zeiss, Oberkochen, Germany) at 400x magnification using a 40x objective lens and a 10x eyepiece. The assessment was carried out on ten visual fields.

Statistical analysis

The normality distribution of HGF expression data and the degree of liver regeneration were analyzed using the Shapiro-Wilk test. Parametric analysis was carried out on data that was normally distributed, while on data that were not normally distributed the Kruskal-Wallis test was carried out followed by the Mann-Whitney test to determine group differences. P value < 0.05 with a 95% confidence interval indicates a significant difference The conformity test used the Interclass Correlation Coefficient (ICC) test, and the kappa (κ) value of > 0.75 can be considered suitable.

RESULTS

Effect of HuMSC-S on baseline characteristics

The body weight of all rats was measured on the sixth day of acclimatization, which was also the first day of the treatment procedure. The analysis result showed a normal and homogenous distribution of the body weight of rats, p>0.05 (Table 1).

Table 1. Baseline characteristics.

Group	Mean ± SD	Median (min – max)	P**	Levene Statistic
Control	191.57 ± 3.36	191 (188 – 197)	0.212*	0.735***
UDCA	191.71 ± 3.25	191 (188 – 197)	0.610*	
Hu-MSC-S	186.57 ± 3.41	187 (182 – 191)	0.760*	
UDCA+Hu-MSC-S	189.86 ± 2.41	190 (186 – 193)	0.976*	

Control group (Cholestasis), UDCA group (UDCA+Cholestasis), Hu-MSC-S group (HU-MSC-S+Cholestasis), the combination of UDCA and HuMSC-S group (UDCA + Hu-MSC-S+Cholestasis). *Normal (p > 0.05) **Shapiro-wilk, and ***Homogeneity (p > 0.05)

Effect of HuMSC-S on liver enzymes

Liver enzymes were measured before and after ligation to assess liver damage. Table 2 showed a significant difference between liver enzymes in serum before and after ligation in all groups, with data distributed normally and homogeneously (p>0.05). These result showed that there was an increase in liver enzymes in serum, which indicates liver damage.

Table 2. Descriptive analysis and normality test of liver enzyme levels.

Level	Pre-Ligation (Mean ± SD)	Post-Ligation (Mean ± SD)	*P*†
ALT	18.48 ± 0.49	37.71 ± 1.19	<0.001*
AST	35.52 ± 0.52	77.09 ± 1.23	<0.001†*

*Reached statistical significance ($P < 0.05$); †Paired T-test

Effect of HuMSC-S on HGF expression

HGF is a growth factor that is mainly secreted by mesenchymal cells. HGF has trophic effects on various cell types, such as epithelial, endothelial, and stromal cells. HGF induces mitotic, growth, migration, and antiapoptotic activities. Also, HGF is known to have an important role in liver regeneration after hepatectomy. HGF induces migration and fusion of Hu-MSC-S which will differentiate into hepatocyte-like cells and play a role in increasing the survival rate of recipients.

The highest HGF expression was in the UDCA+Hu-MSC-S group (4.42 ± 0.16), and the lowest HGF expression was in the control group (0.45 ± 0.10) (Figure 1A and Figure 2). Based on the results of the Mann Whitney test, significant increase in HGF expression was found in UDCA, Hu-MSC-S, and UDCA+Hu-MSC-S groups compared to control (Figure 1A and Figure 2).

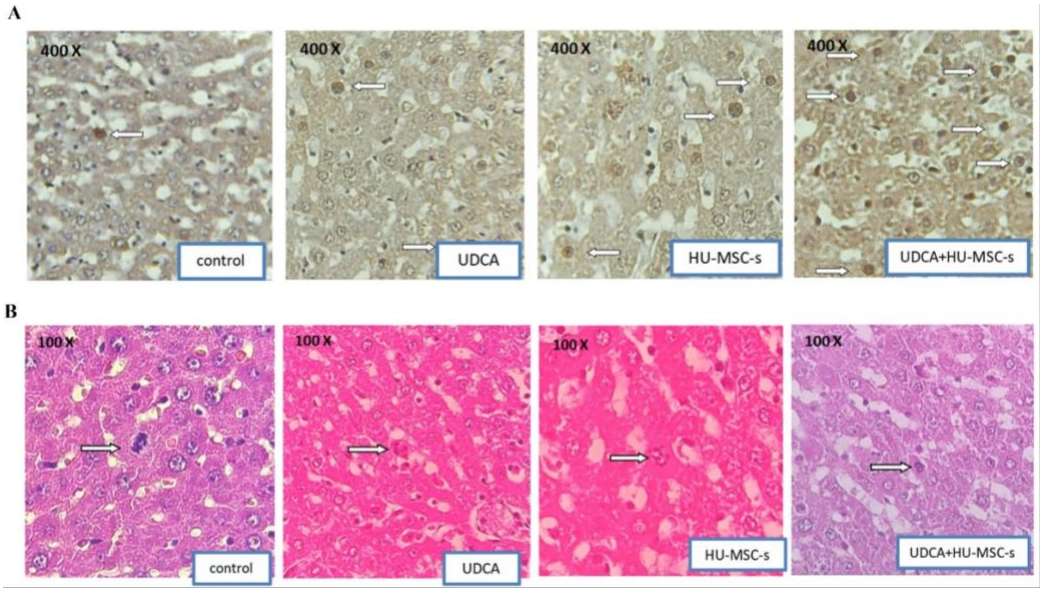

Figure 1. Immunohistochemistry of HGF expression (A) and microscopic examination of the mitotic index with hematoxylin-eosin (HE) staining (B). The arrow show HGF expression (A) and mitotic index (B). Control group (Cholestasis), UDCA group (UDCA+Cholestasis), Hu-MSC-S group (HU-MSC-S+Cholestasis), the combination of UDCA and HuMSC-S group (UDCA + Hu-MSC-S+Cholestasis).

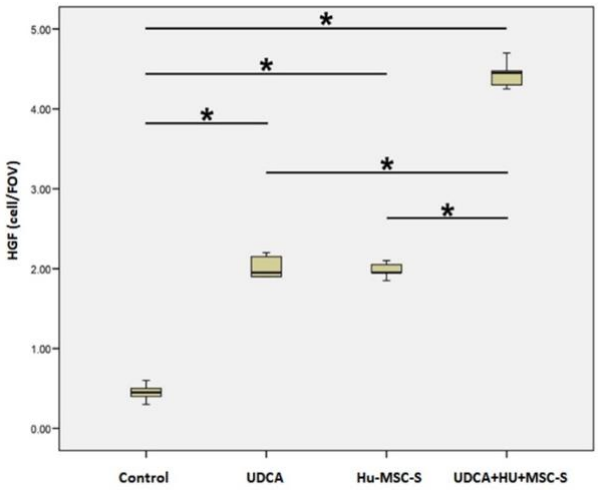

Figure 2. The boxplot graphic of HGF expression in each group. Control group (Cholestasis), UDCA group (UDCA+Cholestasis), Hu-MSC-S group (HU-MSC-S+Cholestasis), the combination of UDCA and HuMSC-S group (UDCA + Hu-MSC-S+Cholestasis). A statistically significant difference was also found when comparing each group. Asterisk (*) means it reached statistical significance (P < 0.05).

Effect of HuMSC-S on mitotic index

The secretome and its components are known to have a role in liver cell regeneration through immune system modulation mechanisms, anti-apoptotic activity, pro-angiogenic activity, antioxidants, or the induction of cell proliferation. In this study, determining the degree of liver regeneration was performed by measuring the mitotic index (the ratio between the number of cells in mitosis and the total number of cells). Data analysis of the mitotic index was not normally distributed, hence the difference on mitotic index in each group was analyzed using Kruskal Wallis test. There was a significant difference in mitotic index between the study groups. The highest mitotic index was determined in UDCA+Hu-MSC-S group (0.96 ± 0.09) and the lowest mitotic index was in control group (0.13 ± 0.04) (Figure 1B and Figure 3). The Mann Whitney test showed a significant difference in mitotic index in the UDCA, Hu-MSC-S, and UDCA+Hu-MSC-S group compared to control (Figure 1B and Figure 3).

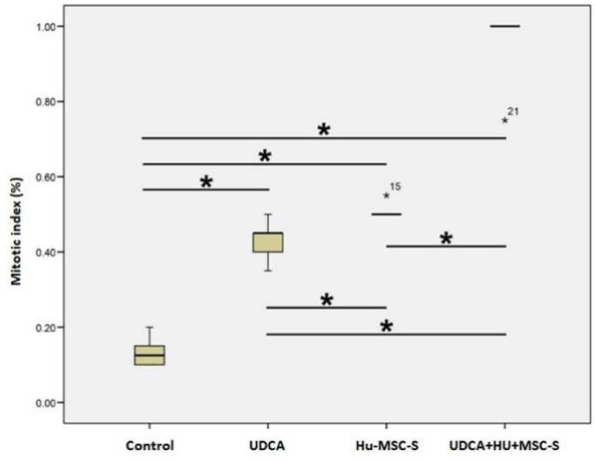

Figure 3. The boxplot graphic of the mitotic index in each group. Control group (Cholestasis), UDCA group (UDCA+Cholestasis), Hu-MSC-S group (HU-MSC-S+Cholestasis), the combination of UDCA and HuMSC-S group (UDCA + Hu-MSC-S+Cholestasis). Asterisk (*) means it reached statistical significance (P < 0.05).

DISCUSSION

This study aimed to prove the effect and feasibility of UDCA and Hu-MSC-S administration on tissue repairment in the cholestatic liver in Wistar rats. All of the subjects were still alive until the end of the study. The effect was assessed based on the expression of HGF and the mitotic index after choledochal duct ligation-induced cholestasis in liver. The result showed that UDCA group, Hu-MSC-S group, and UDCA+Hu-MSC-S group significantly expressed more HGF than the control group. The expression of HGF in UDCA+Hu-MSC-S group was significantly higher compared to individual intervention (UDCA group and Hu-MSC-S group). The UDCA group showed higher HGF expression than the Hu-MSC-S group, but it was not statistically significant. These findings indicate that administering a combination of UDCA and Hu-MSC-S can lead to the repairment of liver tissue through HGF expression compared to administering only UDCA or Hu-MSC-S alone.

Previous studies have shown that circulating stem cells migrate to injured livers and contribute to liver regeneration. MSCs are known to differentiate into cells resembling hepatocytes, thereby triggering the expression of HGF. Hu-MSC-S has been found to express HGF at both RNA and protein levels [16]. One study on the effect of MSC administration on the regeneration of small-for-size liver grafts reported that HGF-expressing MSCs play a role in regulating liver tissue regeneration [21]. HGF expressed by MSCs not only protects small tissue grafts from ischemic injury/reperfusion but also accelerates liver regeneration [22].

Administration of Hu-MSC-S alone resulted in lower HGF expression levels than when co-administered with UDCA, which may be due to the regenerative potential of MSCs that largely depends on their ability to migrate to the site of injury after administration. Homing of MSCs was found to be inefficient, and only a small percentage of cells reached the target tissue. Combined with the hepatoprotective effect of UDCA, the co-administration of UDCA and Hu-MSC-S can produce a better level of tissue preservation [23].

The secretome and its components play a role in liver cell regeneration through immune system modulation mechanisms, inflammation, anti-apoptotic activity, pro-angiogenic activity, antioxidants, or induction of cell proliferation [24]. This study assessed the liver regeneration degree through the microscopically observed mitotic index. The results showed that UDCA, Hu-MSC-S, and UDCA+Hu-MSC-S had a significantly higher mitotic index than the control group. We found that the mitotic index in the UDCA group was lower than the Hu-MSC-S group and the UDCA+Hu-MSC-S groups. Furthermore, the mitotic index in the Hu-MSC-S group was significantly lower than in the UDCA+Hu-MSC-S group. These results indicate that all intervention groups have an association with the occurrence of liver regeneration.

Previous studies stated that HGF is an angiogenic growth factor regulating endothelial cells. HGF plays a role in inducing mitotic activity, growth, migration, and anti-apoptosis [6, 8]. Previous research stated that HGF was not only protected against injury but also accelerated organ regeneration in 30% of models during the early post-transplant period [25]. Our study also showed a significantly positive correlation between HGF expression and the mitotic index.

CONCLUSION

The present study showed that additional concurrent administration of Hu-MSC-S significantly increased HGF expression in rats with hepatic cholestasis after

choledochal duct ligation that received standard UDCA therapy. In addition, it was found that administering UDCA in combination with Hu-MSC could significantly increase the mitotic index in rats with hepatic fibrosis after choledochal duct ligation. HGF expression and mitotic index have a significant positive correlation. Altogether, HuMSC-s administration could be considered as a therapy in patients who received standard UDCA therapy. However, further research should determine the effective therapeutic dose for promoting liver regeneration.

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AUTHORS CONTRIBUTION

MD: conceptualization; formal analysis; investigation; project administration; software; writing – original draft. NS: methodology; project administration; resources; validation; visualization; writing – review & editing. PB: investigation; supervision; validation; writing – review & editing. NM: investigation; resources; supervision; validation; writing – review & editing. EP: investigation; supervision; validation; writing – review & editing. IR: methodology, project administration; resources; supervision; validation; writing – review & editing. All authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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