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Judul	:	Effect of mesenchymal stem cell conditioned medium on hepatocyte matrix implant to alleviate liver cirrhosis in rats
Nama Media	:	REGENERATIVE MEDICINE
Penerbit	:	Taylor & Francis Group
Volume/Tahun	:	Vol 20, Issue 9, 2025
URL Repository	:	https://doi.org/10.1080/17460751.2025.2574178

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Regenerative Medicine
September 2025 Vol. 20 No. 9
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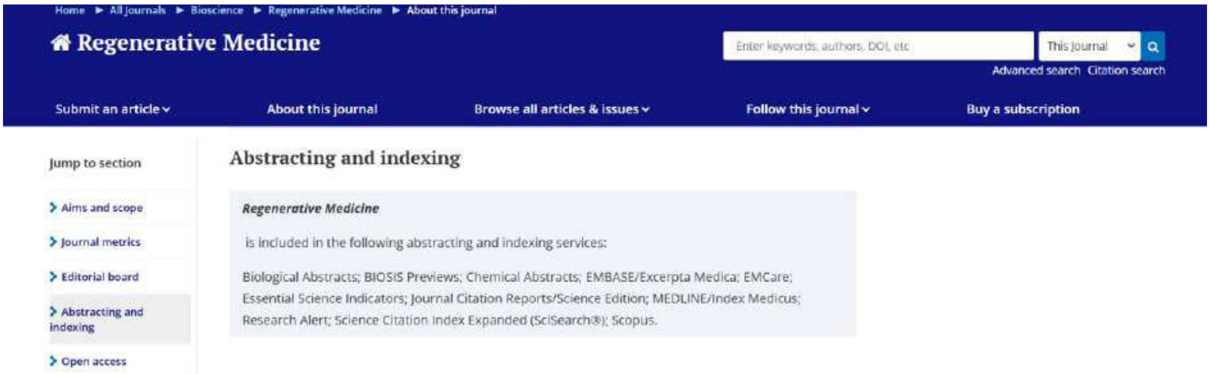

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COUNTRY	SUBJECT AREA AND CATEGORY	PUBLISHER	SJR 2024
United Kingdom Universities and research institutions in United Kingdom Media Ranking in United Kingdom	Engineering └ Biomedical Engineering Medicine └ Embryology	Taylor and Francis Ltd.	0.509 Q2 H-INDEX 77

PUBLICATION TYPE	ISSN	COVERAGE
Journals	17460751, 1746076X	2006-2025

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Regenerative Medicine

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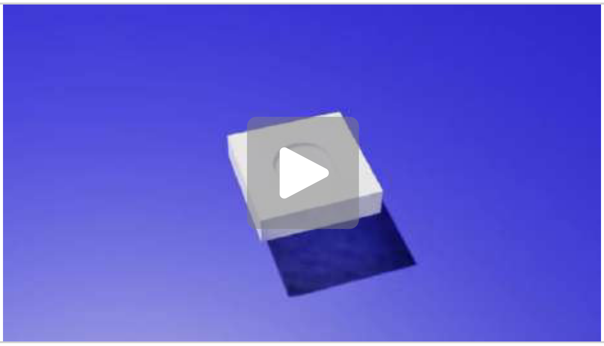

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Effect of mesenchymal stem cell conditioned medium on hepatocyte matrix implant to alleviate liver cirrhosis in rats

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To cite this article: Siufui Hendrawan, Jennifer Lheman, Fitriya Nur Annisa Dewi, Nuraeni, Permanawati & Hans Ulrich Baer (14 Oct 2025): Effect of mesenchymal stem cell conditioned medium on hepatocyte matrix implant to alleviate liver cirrhosis in rats, Regenerative Medicine, DOI: 10.1080/17460751.2025.2574178

To link to this article: https://doi.org/10.1080/17460751.2025.2574178

Published online: 14 Oct 2025.

REGENERATIVE MEDICINE
https://doi.org/10.1080/17460751.2025.2574178

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ORIGINAL RESEARCH

Effect of mesenchymal stem cell conditioned medium on hepatocyte matrix implant to alleviate liver cirrhosis in rats

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ABSTRACT

Background: Hepatocyte transplantation has gained importance as an alternative treatment to orthotopic liver transplantation for end-stage liver disease. This study explores the modification of the hepatocyte matrix implant (HMI) procedure by replacing islet cells with conditioned medium (CM) derived from human umbilical cord-MSC (hUC-MSC) supplementation to alleviate liver cirrhosis in a rat model.
Methods: The male Sprague Dawley rats were induced liver cirrhosis using thioacetamide for 11 weeks, and implanted with matrices on the small bowel mesentery, according to the groups. Four groups were assessed: blank matrix (cell-free), hepatocytes seeded-matrix (Hep), hepatocytes+islets co-seeded-matrix (Hep:Islet), and hepatocytes seeded-matrix supplemented with CM (Hep+CM).
Results: *In vitro*, the Hep+CM group showed significantly higher hepatocyte proliferation than the Hep:Islet group, though albumin production was similar. The *in vivo* study further confirmed that the implanted hepatocytes remained viable and were able to produce albumin for at least 4 months post-implantation. Liver function parameters were shown to be improved in Hep:Islet and Hep+CM groups. Notably, collagen deposition in the liver was lower in Hep:Islet and Hep+CM groups compared to other groups.
Conclusion: These findings suggest that CM can effectively replace islet in supporting hepatocyte proliferation and function, enhancing the therapeutic potential of HMI procedure. No writing assistance was utilized in the production of this manuscript or liver cirrhosis treatment.

PLAIN LANGUAGE SUMMARY

Liver cirrhosis is a severe liver disease that often leads to life-threatening complications, with the only curative option for advance cirrhosis cases is through liver transplantation. However, due to the shortage of donor organs, alternative therapies are urgently needed. In our previous study, we developed a method called hepatocytes matrix implant. In this approach, liver cells were placed together with pancreatic cells on a polymer scaffold as cell carrier, and implanted into the small bowel mesentery to temporarily support liver function as a bridging therapy. In the present study, we explored a modified approach by replacing the pancreatic cells with conditioned medium derived from mesenchymal stem cells (CM MSC, which are known to exert regenerative properties. Through this innovation, additional pancreatic resection is no longer needed, preventing the risks of complication. Our findings show that liver cells stimulated with CM MSC showed equal or even better results compared to those stimulated by pancreatic cells. In animal studies, these implanted cells survived for several months, improving liver function. These findings suggest that CM-MSC could become a simpler and more promising alternative to pancreatic cells in supporting hepatocytes transplantation, potentially offering a new therapeutic option for liver cirrhosis patients.

ARTICLE HISTORY
Received 5 August 2025
Accepted 9 October 2025

KEYWORDS
Liver cirrhosis; hepatocytes matrix implant; islet cells; conditioned medium; mesenchymal stem cell

1. Introduction

Liver cirrhosis is a debilitating disease, often associated with complications such as ascites, hepatic encephalopathy, hepatorenal syndrome, and hepatocellular carcinoma, which could lead to decreased quality of life and increased mortality rates [1]. It is one of the leading cause of death in global, contributing to 2.4% of all deaths in 2019 [2–4]. The major underlying cause of cirrhosis include hepatitis B, hepatitis C, and alcohol abuse [5,6]. Cirrhosis is a subsequent result of chronic liver injury, where the regenerative nodules enclosed by fibrous bands replace the normal liver structure, and in the course of time will result in liver failure [7–9]. Unfortunately, patients suffering from conditions leading to liver failures have limited treatment options. While current treatments mainly aim to delay the progression of fibrosis and latter-stage therapies focus on managing specific complications, these approaches are still incapable to restore complete functionality of the

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Article highlights

- Collagen coated poly-L-lactide acid (PLLA) matrix may serve as a suitable three-dimensional platform for hepatocyte transplantation
- The implanted hepatocyte-loaded matrix improves liver functions of cirrhotic rats
- Conditioned medium (CM) from human umbilical cord mesenchymal stem cell (hUC-MSC) may replace the role of islet cells in stimulating hepatocytes proliferation and function
- The implantable hepatocyte-loaded matrix stimulated with CM hUC-MSC could serve as a bridging strategy for orthotopic liver transplantation.

damaged tissue [8]. The only curative option for most patients with end-stage liver cirrhosis is orthotopic liver transplantation (OLT); however, the global shortage of donor organs presents a significant barrier [10–12]. Additionally, the OLT may not be suitable for patients with end-stage cirrhosis [13].

Cell-based therapy, such as hepatocyte transplantation, has gained importance as an alternative or bridging treatment to OLT. In these cell-based therapies, tissues from the patient are extracted, processed into viable single cells, and cultivated to obtain a large viable cell population. These cells are then directly transplanted to facilitate the innate regeneration of failing organs or damaged tissues [14]. However, challenges still persist, *e.g.* difficulties in hepatocytes cultivation and limited long-term survival of the transplanted cells, presenting major obstacles for broader application of this cellular approach [14–17]. A recent study explored a novel engineered cell-scaffold approach using hepatocyte matrix implantation, showing promises as a supportive therapy for OLT [18]. In this method, hepatocytes were co-cultured with pancreatic islet cells and seeded on 3D collagen-coated PLLA matrices. It has been found that proliferation and survival of hepatocytes can be stimulated by co-cultivation with cells of the islets of Langerhans [17,19]. On the downside, production of the autologous liver implant (*i.e.* a carrier matrix loaded with viable hepatocytes) requires not only an extraction of liver tissue from a patient but also a separate extraction of the patient's pancreatic tissue through an open or laparoscopic surgery. During the pancreatic tissue removal, there is a considerable risk of an acute inflammation (pancreatitis) or pancreatic duct damage, which can lead to a dangerous or even life-threatening pancreatic juice fistula formation [20–22]. Therefore, alternative methods are being explored to stimulate the hepatocyte growth – ideally without the need of co-cultivation with pancreatic cells, which can provide a safer and cost-efficient preparation for hepatocyte implant.

Stem cell-based therapy has gained attention for its ability to support regeneration in degenerative diseases [23]. Mesenchymal stem cells (MSC) especially from umbilical cord, exhibit high self-renewal ability, low immunogenicity, and can be obtained through noninvasive procedures and easily cultured, which make them potentially superior to the MSC from other sources [24]. The MSC secretome can either be provided as a conditioned media (CM)-MSC or purified MSC-derived extracellular vesicles (EVs). CM-MSC is particularly notable for its bioactive factor contents, including hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF). These factors are well-known for their angiogenic, antiapoptotic, and mitogenic properties, all of which aid liver regeneration and inhibit hepatocyte apoptosis [25–30]. Therefore, we aim to provide a new way to stimulate cell growth and proliferation of hepatocytes without needing to co-cultivate them with cells of islets of Langerhans. Here, we reported the study on the potential of CM derived from hUC-MSCs to stimulate hepatocyte function, serving as a replacement for islets, in hepatocyte matrix implant to alleviate liver cirrhosis in rat model. Rats were commonly used and served as a good model for liver cirrhosis study [31].

2. Materials and methods

2.1. 3D collagen coated PLLA matrices production

The Poly-L-lactic acid (PLLA) polymer was purchased from Lactel® (Evonik, USA). Chloroform and sodium chloride were purchased from Fujifilm Wako, Japan; bovine collagen type I solution was purchased from Koken, Japan. PLLA scaffold was fabricated according to the previously described method [32,33]. In brief, the salt leaching method was conducted to form the porous in the scaffold. Then, the scaffold was cut into 1 cm in diameter and coated with 0.5% (w/v) bovine collagen type I solution, followed by plasma treatment with oxygen and low-temperature sterilization process using 30% H_2O_2 (Merck, Germany), in a custom-built plasma machine treatment (Diener Electronic GmbH, Germany).

2.2. Isolation of primary rat cells for matrix embedding

All procedures involving the care and use of animals for primary cell isolation has been approved by the Institutional Animal Care and Use Committee (IACUC) of Faculty of Medicine, Tarumanagara University, Jakarta, Indonesia; with ethical approval number 012–013.KEPH/UPPM/FK/III/2021.

2.2.1. Isolation of primary rat hepatocytes

Primary hepatocytes were isolated from the liver of male Sprague Dawley (SD) donor rats (age 9–10 weeks, $n = 5$) through a 2-stage perfusion technique that has been described previously with some modification [17]. The isolated primary hepatocytes were cultured in selective media Hepatocytes Basal Medium (HBM) (Lonza, USA), supplemented with HCM SingleQuots kit (Lonza, USA), 20% Fetal Bovine Serum (FBS) (Gibco, UK), and 1% Antibiotic/Antimycotic (Ab/Am) (Sigma, USA). Culture media was replaced every 3 days until the cells reached 90% of confluency. The confluent hepatocytes were dissociated enzymatically using TrypZean solution (Sigma, USA), then counted using Trypan Blue (Sigma, USA) exclusion method.

2.2.2. Isolation of primary rat islet cells

The islet cells were obtained from male SD donor rats (age 9–10 weeks, $n = 5$). The isolation process was carried by the 1-stage perfusion technique using ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (Sigma, USA) buffer solution. The pancreas tissue was dissected, then processed mechanically and enzymatically according to previously described methods [34]. Islet cells were then counted using the Trypan Blue exclusion method and then mixed with previously cultivated hepatocyte cells according to a predetermined ratio of 50:1.

2.3. MSCs isolation and conditioned medium (CM) production

The human umbilical cord was obtained from a healthy donor post-cesarian surgery with a parental consent (Ethical approval number PPZ20192062, by the Universitas Tarumanagara Human Research Ethics Committee, Tarumanagara University). The procedure was done according to a previous study [35]. In brief, the umbilical cord was washed with phosphate buffer saline (PBS) (Sigma, USA) and EGTA buffer solution, cut into smaller pieces, and cultured in a culture flask to obtain mesenchymal stem cells (MSCs). MSCs were cultured until confluent and passaged until reaching passage 5–6. Afterward, pre-conditioning was applied to the MSCs in the form of hypoxic condition for 72 hours. The culture medium was then collected and filtered to obtain the conditioned medium (CM). The CM was measured for hepatocytes growth factor (HGF) (Elabscience, USA) and vascular endothelial growth factor (VEGF) (R&D Systems, USA) using enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's protocol.

2.4. In vitro assays

To assess the optimum culture duration of cells in PLLA matrix, viability assay was performed in designated time points. Hepatocytes were seeded onto a sterile matrix with total cells of 5×10^5 cells/100 µL/matrix, then cultured in a standard medium. The standard medium contained William E Medium (Sigma, USA) supplemented with 10% of fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotic/antimycotic (Ab/Am) (Sigma, USA). The cell-seeded matrices were incubated at 37°C, 5% CO_2, for a total of 48 hours, 72 hours, and 1 week, with the culture media replaced at the first 24 hours post-seeding and every 48 hours thereafter. Cell viability and adhesion were assayed using CCK-8 Assay (Sigma, USA). For CCK-8 assay, the culture medium was replaced with CCK-8 diluted in fresh standard culture medium (1:10). Then, the samples were incubated for 4 hours at 37°C, 5% CO_2. The absorbance was measured at 450 nm using a microplate reader (Multiskan EX, Thermo Scientific, USA). Results were expressed as percent adhesion fold change relative to 48 hours.

Further *in vitro* assay was proceeded to compare cell adhesion into matrix between Hep, Hep:Islet, and Hep+CM groups. In the Hep group, the matrices ($n = 4$) were seeded with hepatocytes (total cells of 5×10^5 cells/100 µL/matrix), then added with 1 mL of standard medium. In the Hep+CM group, equal total number of cells (hepatocytes only) were seeded onto dry matrices ($n = 4$), and then added with standard medium supplemented with 1% of CM. While in the Hep:Islet group, a mixture of hepatocytes:islet cells suspension (50:1) with total cells of 5×10^5 cells/100 µL/matrix were seeded onto dry matrices ($n = 4$), followed by adding 1 mL of standard medium. The Hep group served as a control group in this assay. CCK-8 assay was performed at 72 hours after seeding, which is the optimum duration obtained from the previous study, with the culture media replaced at the first 24 hours post-seeding. CCK-8 assay is based on the reduction of the tetrazolium salt WST-8 into a yellow-colored formazan product, which exclusively occurs in viable cells. Hence, the formazan generated in this assay was proportional to the overall population of viable cells, without distinguishing different cell types. Results were expressed as the percent adhesion fold change relative to the Hep group (cells adhering to the matrix in Hep group, 100%).

The function of hepatocytes in producing albumin was measured through ELISA (Elabscience, USA), by comparing Hep, Hep:Islet, and Hep+CM groups.

2.5. Animal experiment

All procedures involving the care and use of animals for the *in vivo* experimentation was approved by the IACUC of Faculty of Medicine, Tarumanagara University, Jakarta, Indonesia with ethical approval number 015.KEPH/UPPM/FK/IX/2022. The sample size in this study was determined using Resource Equation method [36]. Rats were maintained under controlled condition (temperature, humidity) and lighting condition (12 hours light/dark cycle), with free access to water and laboratory standard feed *ad libitum*, and structural environmental enrichment.

2.5.1. Cirrhosis induction

Male Sprague Dawley (SD) rats were used in this study ($n = 30$). Rats were obtained (age 6–7 weeks) from the Experimental Animal Housing Unit (UKH), Center of Tropical Biopharmaceutical Studies, IPB University, Bogor, Indonesia. Rats were acclimatized for 1 week prior to the experiment. Thioacetamide (TAA; Supelco, Merck, USA), a hepatotoxic agent was used to induce cirrhosis condition. Rats (body weight (BW): 180–200 gram) were injected with TAA intraperitoneally (dosage 200 mg/kg BW), 2 times per week for 11 weeks [37,38]. Prior to each injection process, TAA was freshly prepared and dissolved in 1 mL of sterile 0.9% salt solution (NaCl). Blood from rat tail vein was collected from each rat (restraint) before and after cirrhosis induction process for liver function analysis.

2.5.2. Matrix implantation procedure

The TAA-induced rats were divided randomly into 4 groups: cell-free group was implanted with blank matrices (without cells) ($n = 6$), hepatocytes (Hep) group was implanted with hepatocytes seeded matrices ($n = 8$), Hep:Islet group was implanted with hepatocytes and islet cells seeded matrices ($n = 8$), and Hep+CM group was implanted with hepatocytes seeded matrices supplemented with CM ($n = 8$). Animals in each group were divided into 2 endpoints, half of them were euthanized at 2 months following implantation for histopathological analysis, and the remaining rats were euthanized at 4 months post-implantation.

A B

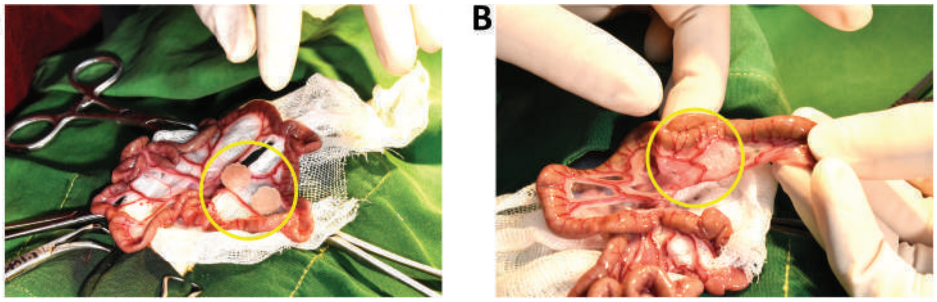

Figure 1. Representative images of matrix implantation procedure. Yellow circles indicated the matrix implants. (A) Matrices were placed between the serosal surface of the mesentery loops of the small bowel. (B) Matrices were fixed with 6–0 nylon non-absorbable suture.

Each rat received 4 pieces of blank- or cell-seeded matrices according to the corresponding group. Each matrix was seeded with total cells of 7.5×10^5, prepared accordingly to each assigned group, and cultured for 72 hours prior implantation. Briefly, rats were anesthetized with Ketamine 10% (40–80 mg/kg BW) and Xylazine 2% (5–10 mg/kg BW). Then, under an aseptic condition, a laparotomy procedure was performed with a 2 cm incision along the midline abdomen. The matrices were placed between the serosal surface of two adjacent mesenteric loops of the small bowel and fixed with interrupted sutures using 6–0 non-absorbable nylon suture (Ethilon®, Ethicon, Raritan, NJ) (Figure 1). Afterward, 3–0 absorbable coated vicryl (Ethicon, Raritan, NJ) running sutures was used to close both the muscle and abdominal skin, the surgical wound was finally secured with Hypafix®. Post-surgery, rats were given antibiotic (Ampicillin 20 mg/kg BW, 2 times/day) and analgesic (Ketoprofen 5 mg/kg BW, 1 time/day) for 5 days, then were closely monitored until endpoint.

At each endpoint, the animals were anesthetized (Ketamine 10% (40–80 mg/kg BW) and Xylazine 2% (5–10 mg/kg BW) and blood was collected from the tail vein to obtain serum for liver function parameter analysis, *i.e.* alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin, and albumin. Subsequently, euthanasia was performed by intracardiac injection of sodium pentobarbital (200 mg/kg BW). At necropsy, liver and small bowel mesentery as the implantation site were collected, and preserved in 4% paraformaldehyde solution for histopathological analysis.

2.6. Histopathological analysis

The liver specimens were stained with Hematoxylin and Eosin (HE) and Sirius Red (SR). The fibrosis stage and collagen percentage were determined through SR staining according to Standish *et al.* (2006) [39]. The implantation sites were stained with HE and immunohistochemistry (IHC) against albumin. An albumin recombinant rabbit monoclonal antibody (Invitrogen, Massachusetts, USA) was used in IHC staining. The number of hepatocytes on the implantation site (IHC-stained) was counted from 10 field of view. This procedure was performed by the Pathology and Anatomy Laboratory, Primate Research Center, IPB University, Bogor, West Java, Indonesia.

2.7. Statistical analysis

All results were expressed as means ± SD. Shapiro–wilk test was used to determine the normal distribution of the data. For multiple comparisons, if the data were normally distributed, one-way analysis of variance (ANOVA) was used, otherwise, the Kruskal–Wallis test was performed. For determining significance between two independent groups, Student's *t*-test was used for normally distributed data, otherwise the Mann–Whitney test was used. The values were considered statistically significant at $p < 0.05$ and highly significant at $p < 0.01$.

3. Results

3.1. CM enhances hepatocytes proliferation and function in vitro

In our previous study, we found that 3D PLLA matrix could serve as a carrier for different cell types, including hepatocytes and fibroblasts [33,40]. This 3D biomaterial provides structural support and enables effective nutrient transfer within the matrix system, which aims to deliver viable and functional cells during transplantation. *In vitro* finding showed that hepatocytes cultured in the PLLA matrix had the highest viability at 72 hours of culture ($p < 0.01$ compared to 48 hours) and retained its viability until 1 week of culture (Figure 2(A)). Hence, further experiments employed 72 hours of cell culture into 3D PLLA matrix prior to implantation.

Islet cell has been previously known to enhance hepatocytes viability and function when co-cultured [19]. Concurrently, CM is also known to contain several growth factors, such as HGF and VEGF, important factors in facilitating liver regeneration [25,26]. In this study, we utilized CM derived from hUC-MSC, which contained a particularly high concentration of HGF and VEGF, that is 1595 ± 252 pg/ml and 3312 ± 793 pg/ml, respectively (Figure 2(B)). To evaluate whether the CM exerts a stimulatory effect on hepatocytes compared with islets, primary hepatocytes and islets were isolated. Isolated hepatocytes were cultured in a hepatocyte-selective medium to limit the growth of other cell types and verified by their characteristic polygonal structure [41], while islets were identified by cell clusters displaying honeycomb-like structure under light microscopy (Figure 2(C)) [42]. Then, we compared hepatocytes proliferation and albumin secretion between Hep, Hep:Islet, and Hep+CM groups in 3D platform at 72 hours post-seeding.

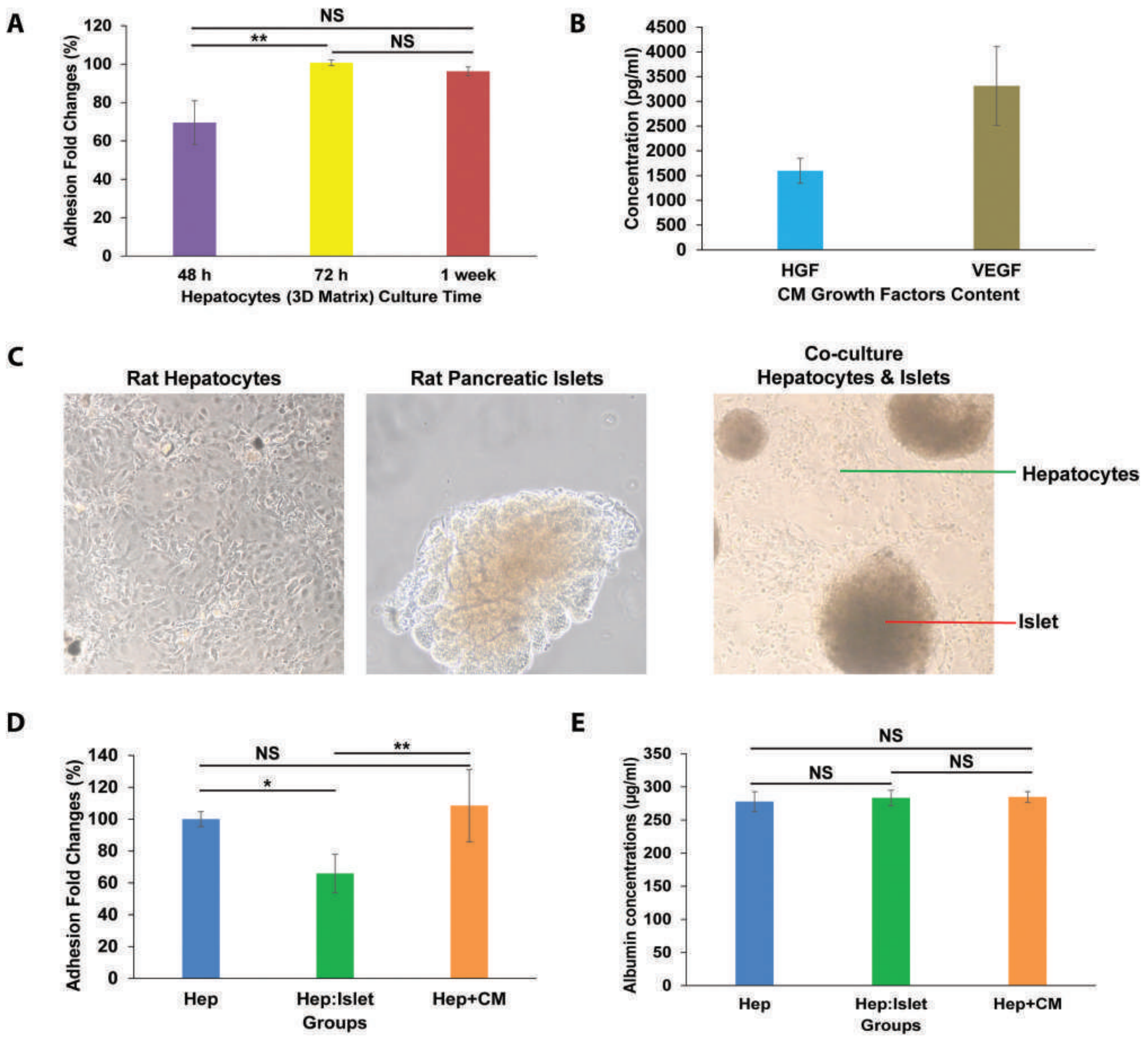

Figure 2. Effect of CM on hepatocytes adhesion and function in 3D platform. (A) Hepatocytes viability in the collagen-coated PLLA scaffolds under designated culture duration. (B) Growth factor concentration in the CM. (C) Representative images of hepatocytes, islet, and hepatocytes : islet co-culture. (D) Percent adhesion fold change normalized to Hep group. (E) Comparison of albumin concentration secreted by hepatocytes in the collagen-coated PLLA scaffolds after 72 hours. (A, D) Samples were analyzed with CCK-8 assay. (B, E) Samples were analyzed with ELISA. Data showed means ± SD ($n = 4$/group). *$p < 0.05$, **$p < 0.01$, NS: not significant.

Hepatocytes proliferation was significantly enhanced ($p < 0.01$) in the CM-treated group (Hep+CM) (108.4 ± 22.7%) compared to the islet co-seeded group (65.9 ± 12.1%), which result was normalized to the Hep group. Although intriguingly, the Hep:Islet proliferation is significantly lower than Hep group (100 ± 4.6%) (Figure 2(D)). In terms of albumin secretion, no significant differences were observed among the groups. The islet co-culture (Hep:Islet) and CM-treated (Hep+CM) groups, however, showed slightly higher albumin concentrations compared to the hepatocytes only (Hep) group (Figure 2(E)).

3.2. CM-enhanced hepatocyte matrix implant improves liver function parameters

To assess the effect of hepatocytes matrix implant in alleviating liver cirrhosis, liver function parameters such as ALT, AST, total bilirubin, and albumin were analyzed. The data were collected at designated time points and compared between groups of cirrhosis-induced rats at 4-month post implantation. The liver enzymes ALT and AST were significantly elevated in all groups after 11 weeks of TAA induction (Figure 3), indicating the occurrence of liver injury and potential liver cirrhosis. Compared to prior implantation, the ALT and AST levels were gradually reduced across all groups at 2- and 4-month post implantation; with Hep+CM group showing the lowest concentration of ALT and AST (Figure 3 (A,B)). Total bilirubin levels were also significantly decreased in all cell-seeded groups (Hep, Hep:Islet, Hep+CM) at both 2- and 4-month post-implantation. Moreover, the Hep+CM group had the lowest concentration of total bilirubin, significantly lower compared to the cell-free group (Figure 3 (C)). This observation was further supported by the albumin levels, which showed significant increment ($p < 0.05$) at 2-

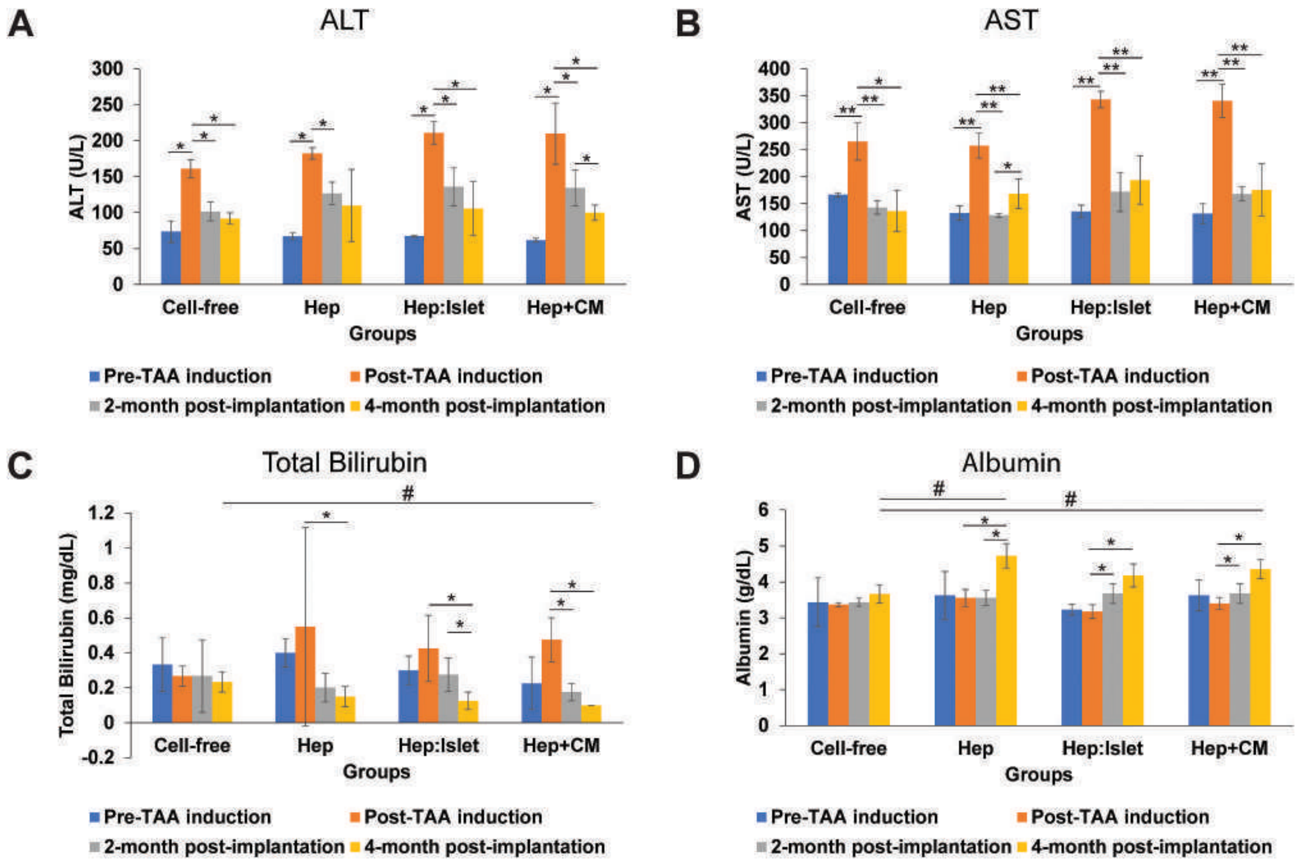

Figure 3. Serum chemistry profile of liver function parameters in cirrhosis-induced rats at pre- and post-TAA, 2- and 4-month post-implantation. (A) ALT, (B) AST, (C) total bilirubin, (D) albumin. Data were expressed as means ± SD ($n = 3$ for cell-free group, $n = 4$ for other groups). $^{*}p < 0.05$ compared within group, $^{**}p < 0.01$ compared within group, $^{\#}p < 0.05$ compared between groups at the same time point. Hep: hepatocytes, CM: conditioned medium, TAA: thioacetamide, ALT: alanine transaminase, AST: aspartate transaminase.

and 4-month post-implantation, particularly in the Hep:Islet and Hep+CM groups (Figure 3(D)).

3.3. CM-enhanced hepatocyte matrix implant reduce collagen deposition in liver tissue

Cirrhosis condition is characterized by the replacement of normal liver structure by fibrous tissue. Therefore, histological assessment of fibrosis progression is pivotal in determining the disease outcome. The fibrosis stages were assessed using Sirius Red staining of liver sections from all groups, scored based on the Ishak scoring system [39]. At 2-month post implantation, the results showed similar fibrosis stage of Ishak score 5, indicating incomplete cirrhosis in all groups; except for Hep:Islet group with the lowest collagen deposition and Ishak score 4, which indicated fibrous expansion of portal areas with marked bridging. Even though the Hep and Hep +CM groups had the same Ishak score, the collagen deposition on Hep+CM group was lower compared to the Hep group.

At 4-month post implantation, the cell-free group exhibited the highest stage of fibrosis (Ishak score: 6, cirrhosis) and collagen deposition area. Both the Hep group and Hep+CM group showed similar fibrosis stage (Ishak score: 5), with the lowest fibrosis state was Hep:Islet group (Ishak score: 4) (Table 1). The collagen deposition result also aligned with the fibrosis stage, showing Hep:Islet group with the lowest collagen deposition among other groups. Similar with 2-month post implantation, although the Hep and Hep+CM groups had the same fibrosis stage, the collagen deposition of Hep+CM was lower compared to the Hep group.

Microscopic evaluation and fibrosis assessment of liver specimen were presented in Figure 4 (at 2-and 4-month post-implantation). Liver obtained from all groups in these

Table 1. Liver fibrosis scoring and collagen deposition percentage of the cirrhosis-induced rats.

Groups	Cell-free ($n = 3$/time point)		Hepatocytes ($n = 4$/time point)		Hep:Islet ($n = 4$/time point)		Hep+CM ($n = 4$/time point)	
Time point	Ishak Stage	% Collagen	Ishak Stage	% Collagen	Ishak Stage	% Collagen	Ishak Stage	% Collagen
2-month	5	11.48	5	11.7	4	8.35	5	9.97
4-month	6	11.5	5	11.1	4	7.45*	5	9.28

Hep: hepatocytes, CM: conditioned medium. Values were expressed as means ± SD ($n = 4$/group). $^{*}p < 0.05$ compared to the cell-free group at the same time point.

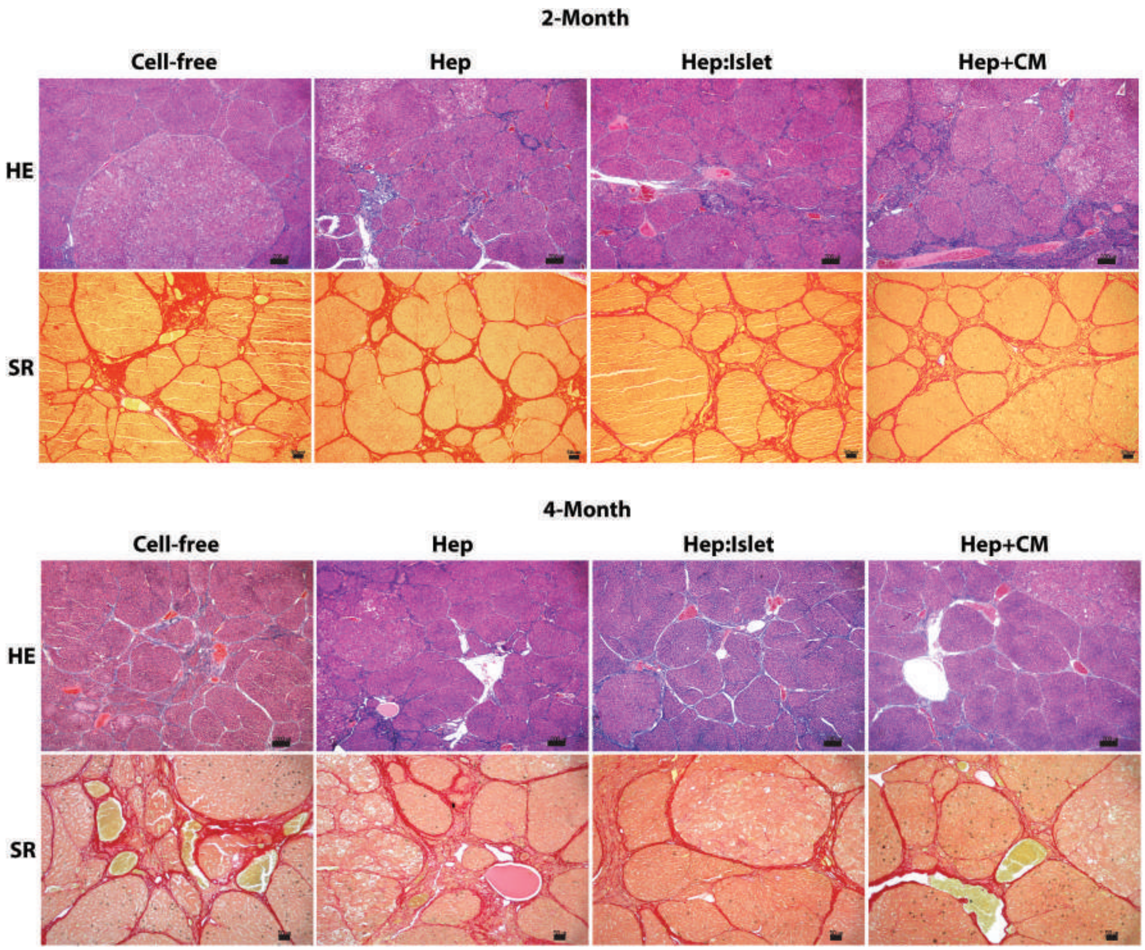

Figure 4. Histopathology analysis of cirrhosis-induced liver at 2- and 4-month post-implantation. Above panel showed liver section stained with hematoxylin-eosin (HE; magnification 40×, scale bar: 200 µm); lower panel stained with sirius red (SR; magnification 100×, scale bar: 50 µm). Hep: hepatocytes, CM: conditioned medium.

2 time points appeared congested, with the loss of normal liver architecture replaced by fibrous septae and connective tissues connected between portal and to the central vein. This was also accompanied by moderate infiltration of inflammatory cells. Regenerative nodules were also observed in multifocal areas of the parenchyma (HE staining). The degree of fibrosis and collagen deposition (SR staining) were quantified in Table 2. Collagen deposition was evident across all groups (at both time points), visualized as red strands in the SR-stained sections. Either Hep: Islet and Hep+CM groups showed milder deposition. These findings suggested that the implantation of Hep:Islet and Hep+CM is comparable and could reduce the progression of fibrosis.

3.4. Hepatocytes implant maintained viability and function up to 4-month

The small bowel mesentery (i.e., implantation site) from each rat was collected. Matrices remained on implantation sites were stained with HE staining and immunohistochemistry against albumin (Figure 5). All groups showed positive albumin expression, demonstrating the survival and functional activity of implanted hepatocytes, at least in producing albumin. The mean number of hepatocytes on Hep+CM group showing the highest hepatocytes count among groups (Table 2). Albumin expression intensity, represented by brownish coloration, was quantified following the method of Cizkova et al. [43], where lower intensity values (as the color stained darker) correspond to stronger albumin expression (Figure 5). Among the groups, the Hep+CM group exhibited the lowest albumin intensity values at both time points (Table 3). However, no statistically significant differences were observed among groups for either albumin intensity or hepatocytes count. Overall, these findings indicate that the Hep+CM group performed comparably to the Hep:Islet group in secreting albumin, suggesting that CM can effectively substitute islet in supporting hepatocytes function.

Table 2. The average number of hepatocytes per field view on the site of implantation.

Groups	2-month	4-month
Hepatocytes	131 ± 41	159 ± 26
Hep:Islet	147 ± 22	177 ± 48
Hep+CM	175 ± 49	207 ± 34

Hep: hepatocytes, CM: conditioned medium. Data were expressed as means ± SD ($n = 4$/group).

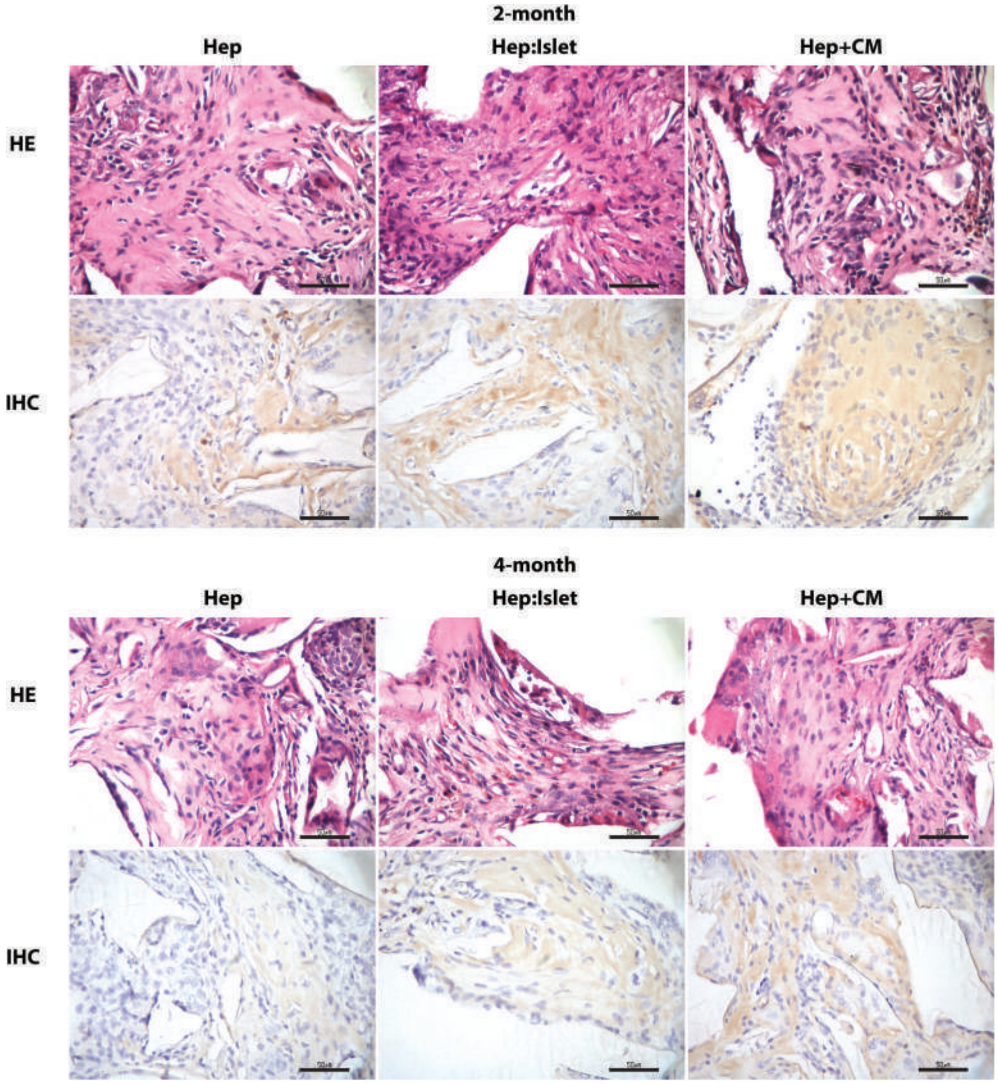

Figure 5. Small bowel mesentery (implantation site) stained with hematoxylin-eosin (HE) and immunohistochemistry (IHC) against albumin at 2- and 4-month post implantation. Magnification 100×, scale bar 50 µm. Hep: hepatocytes, CM: conditioned medium.

Table 3. Quantification of albumin intensity on the site of implantation as stained by immunohistochemistry.

Time point	2-month		4-month	
Groups	Intensity	Score*	Intensity	Score*
Hepatocytes	140.86 ± 3.25	1	141.29 ± 11.60	1
Hep:Islet	138.15 ± 6.11	1	143.42 ± 6.13	1
Hep+CM	137.78 ± 11.43	1	139.89 ± 10.95	1

Hep: hepatocytes, CM: conditioned medium. Data expressed as mean ± SD ($n = 4$/group) *Score: intensity 0–60 = 3 (strong), intensity 61–120 = 2 (moderate), intensity 121–180 = 1 (weak), intensity > 180 = 0 (negative) [43].

4. Discussion

Liver cirrhosis is a chronic condition characterized by irreversible liver damage at advanced stages [44]. Current therapies aim to delay fibrosis progression; however, it could not reverse the damage of liver tissue. Nonetheless, when liver cirrhosis reaches its final stage, liver transplantation remains the only definite treatment. Cell-based therapies, such as hepatocytes transplantation, have emerged as less invasive alternatives [45,46]. Despite their potential, these methods still face challenges such as organ donor shortage and immunological reactions. Culturing primary hepatocytes is considerably challenging; it took a great effort to optimize and modify the culture conditions that enable their growth *in vitro* [15]. The primary difficulty lies in maintaining hepatic function, as liver-specific metabolic activities deteriorate within a few days of culture [15,47]. Moreover, cell survival remains a constant challenge in hepatocytes engraftment, as well as the engraftment mechanism itself, such as migration, integration, and cell repopulation, are still largely elusive when administered intravenously [48]. Our previous study proposed the utilization of autologous hepatocytes matrix implant (HMI), which showed promising results, particularly in improving patients quality of life [18]. In this HMI procedures, autologous hepatocytes were co-seeded with islet cells in the 3D matrix to stimulate hepatocyte proliferation and survival when implanted into patients with liver cirrhosis [19]. Islets are well-known for promoting hepatocyte growth and survival when cultured together with hepatocytes [17,19]. On the other hand, obtaining pancreatic islet is risky and may lead to complications, such as pancreatitis. This study provides a new and safer way to stimulate hepatocytes growth and proliferation without the co-cultivation with cells of islets of Langerhans. In practice, this means that the production of an autologous liver implant or an autologous liver cell transplantation can be achieved by the extraction of autologous hepatocytes, cultivation, and re-implantation thereof with no additional surgical procedure for obtaining cells of islets of Langerhans (*i.e.* pancreatic cells) from the patient.

Developing a sufficient number of functional implanted hepatocytes remains a challenge [49]. To address this issue, our present study adopted various strategies, providing a carrier matrix suitable for tissue engineering and a cell suspension comprising viable hepatocytes, allowing the cells to attach and grow in more than one layer. This matrix serves as a three-dimensional scaffold that can be colonized by cells or tissue, either *in vitro* or *in vivo* [50]. Furthermore, in the context of transplantation, the matrix functions both as a scaffold for positioning the graft and as a placeholder for newly forming tissue *in vivo*. The matrix preferably has a sponge-like structure with at least partly interconnected pores of different pore sizes, in which this porous nature facilitates attachment of the cells and nutrient flow through the matrix. Our matrix consists of PLA synthetic polymers, then covered with – natural polymers, that is collagen, which provides the matrix with additional stability, and hydrophilicity to mimic the extracellular matrix, support hepatocytes proliferation, and preserve their function. Moreover, our matrix was plasma-treated prior seeded with cells to further enhance the hydrophilicity and thus the attachment rate of the hepatocytes. Then, the matrix is subjected to a hydrogen peroxide (H_2O_2) containing the environment at a temperature below 40°C. This sterilization technique has been developed without compromising their hydrophilic properties or damaging the polymeric structure. As a result, our matrix was able to provide a suitable environment for hepatocyte growth, confirmed by increasing cell viability for up to 1 week *in vitro*. Another studies also demonstrated that *in vitro* culture of hepatocytes in 3D matrix could support cell viability for 1 week [51]. Furthermore, *in vivo* findings showed that by 4 months of implantation into small bowel mesentery, hepatocytes were still viable throughout the matrix implant, which demonstrated an extended capacity of our matrix in carrying viable hepatocytes.

Following cell seeding, the hepatocytes-loaded matrix is incubated in a culture medium supplemented with CM-MSC to provide exogenous stimulation. The MSC was grown under hypoxic exposure to promote self-renewal of undifferentiated MSC and enhance their therapeutic potential through the activation of several signal transduction pathways including hypoxia inducible factor (HIF), a master transcription factor that regulates the expression of hundreds of genes to promote cellular adaptation to the hypoxic condition [52,53]. In our preliminary trial, we applied different concentrations of CM-MSC in hepatocytes culture to evaluate its effect on cell growth. While 1% of CM stimulated hepatocytes proliferation, higher concentration of 5% to 10% failed to further improve cell proliferation. Therefore, in this study, we applied low concentration of CM-MSC to stimulate hepatocytes. Our *in vitro* result demonstrated that CM-MSC significantly enhanced hepatocytes proliferation compared to Hep:Islet group in 3D matrix. This result aligns with previous reports indicating that CM-MSC in low concentration is sufficient to support proliferation and survival of hepatocytes [26,27]. Intriguingly, cell viability was lower in the Hep:Islet group compared to the Hep group. The beneficial effects of islets to support hepatocyte growth and survival are mediated exclusively through the release of soluble factors, *e.g.* insulin and glucagon, instead of direct cell-to-cell contact [54]. Hence, direct co-culture of hepatocytes and islets could induce contact inhibition and nutrient competition in a static co-culture system, especially in three-dimensional settings [55]. This might underlie the reduced cell viability in Hep:Islet group when cultured on the matrix *in vitro*.

Additionally, albumin secretion in the Hep+CM group was comparable to the Hep:Islet group, suggesting that CM-MSC effectively supports hepatocytes function and may serve as a substitute for islet cells. It is noteworthy that HGF secreted by MSC, which is abundant in the CM-MSC, also plays a crucial role in promoting hepatocytes survival [56,57]. These results are consistent with other studies showing that additional CM can enhance the viability and proliferation of hepatocytes, while having no effect on albumin production *in vitro* [58,59]. CM supplementation, however, showed positive effects on hepatocytes capacity to store glycogen and inhibited cell apoptosis [46,58].

To further determine the efficacy of Hep+CM matrix, we performed *in vivo* study using TAA-induced liver cirrhosis rat model. The dose of TAA used in this study was optimized based on literature reviews and mortality rates observed in our previous animal investigation. While liver enzymes (ALT, AST) significantly decreased in all groups, only the cell-implanted groups (Hep, Hep:Islet, Hep+CM) demonstrated a substantial decrease of total bilirubin and increase of albumin levels. This result indicates that hepatocytes matrix implant could improve the liver function of cirrhosis-induced rats. Additionally, the Hep:Islet and Hep+CM groups demonstrated equivalent stimulation of hepatocytes function. Although no significant differences were observed in the improvement serum chemistry profiles among the cell-implanted group, the addition of CM appears to support the hepatocytes engraftment as seen in the histological findings. It is also noteworthy that the cell/matrix system was implanted locally into the small bowel mesentery (in contrast to systemic cell infusion), which underscores that although implanted cells are functional and locally vascularized, systemic biochemical parameters may not show marked improvement, which was also aligned with another study [60]. Based on these results, we suggested that hepatocytes matrix implantation was an effective method to support hepatocytes growth and function *in vivo*, and the engrafted hepatocytes could temporarily substitute the loss hepatic function without the immediate need to transplant a whole liver organ. This was also confirmed by another study utilizing allogeneic hepatocytes on porous scaffolds to mimics hepatocellular function, which showed a positive result in liver function improvement [61]. Histological evaluation of liver sections showed no significant differences in fibrosis stage among groups. However, collagen percentage was significantly lower in Hep:Islet and Hep+CM group at 4-month time point. Although the Hep+CM group displayed a slightly higher collagen percentage to Hep:Islet group, this collagen deposition was still lower compared to other groups, indicating the progression of fibrosis is delayed in these two groups. Furthermore, immunohistochemistry results showed albumin and hepatocytes quantification at the implantation site in the Hep+CM group outperformed other groups, further supported the finding that CM enhances hepatocytes survival and proliferation. These results aligned with other study that CM-MSC could attenuate liver fibrosis, due to its anti-inflammatory, antioxidative, and anti-fibrotic effects [62]. MSC secreted several growth factors and cytokines which are known to play important roles in liver regeneration. HGF is a hepatocyte mitogen playing a key role in cell protection and regeneration, mainly through HGF/c-Met pathway, as well as attenuating chronic inflammation and fibrosis [56,57]. VEGF family is crucial in regulating vasculogenesis and angiogenesis phase of liver regeneration [63–65]. Fibroblast growth factors (FGFs) are also responsible for vascular angiogenesis and restoration of sinusoidal networks during liver regeneration [63]. Tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) work simultaneously to activate and prime hepatocyte proliferation [63,66,67]. Additionally, CM from MSC contains several types of extracellular vesicles (EV) which exert important roles through its paracrine mechanism, such as down-regulating the expression of fibrotic markers, inhibiting hepatocyte apoptosis, and suppressing the infiltration of inflammatory cells [68–71]. Nonetheless, this study has several limitations, including the lack of *in vitro* functional assays. Additional analyses, such as glycogen synthesis and metabolic detoxification capacity, should be addressed in future studies to provide a more comprehensive understanding of the mechanisms by which CM stimulates hepatocytes. For the *in vivo* experimentation, the limitation including the use of all male rats in this study, limiting the applicability of the findings to both sexes. Moreover, the number of hepatocytes that could be implanted in the animal model was limited, potentially contributing to the modest improvements observed in liver function. Besides the restricted capacity of each matrix to hold cell suspensions, the total number of matrices that can be implanted is also constrained by the limited anatomical space within the mesentery. However, for clinical applications in humans, the matrix dimensions and numbers can be scaled up as needed, allowing a greater number of cells to be implanted. In our previous HMI (Phase 1 clinical study) procedure involving cirrhosis patients, more than 20 pieces of matrices, loaded with hepatocytes and islets, with diameter of 2 cm were successfully implanted [18]. Recently, Phase 2 of the HMI clinical study has also been completed (unpublished data). The follow-up period in this study was also relatively short, which may not be sufficient to capture the full extent of long-term effects. Further studies with larger sample size and additional quantitative assays are needed to strengthen current findings and to determine the optimal number of cell-matrix. In addition, long-term investigations are required to evaluate the effects of matrix implantation on liver function parameters, fibrosis progression, and potential adverse events, particularly beyond 6 months.

This study showed that engrafted hepatocytes carried in biomaterial such as 3D PLLA-collagen matrix could survive at least 4-month post implantation *in vivo*. Additionally, CM from MSC enhances the survival and function of engrafted hepatocytes, suggesting that CM could replace islets in hepatocytes culture. This result is consistent with previous study, stating that CM from MSC hold a great potential in promoting the recovery of damaged hepatocytes [46]. This study provides preliminary evidence and new insights of the CM-MSC application as an alternative strategy for liver cirrhosis therapy. Although further research is necessary prior clinical application, these findings may contribute to the development of safer and more effective therapeutic approaches that could support the progression of regenerative medicine, especially for liver cirrhosis.

5. Conclusion

While liver cirrhosis remains a global health issue due to its high complications and mortality, we demonstrate the potential of utilizing hepatocyte matrix implant as a bridging therapy to liver transplantation, which can temporarily substitute the loss of liver function. Moreover, the CM has the potential to replace islet function in stimulating engrafted hepatocytes proliferation and survival. This research provides a novel strategy in combining tissue engineering and therapeutic potential of stem cells for liver cirrhosis treatment.

Author contributions

S Hendrawan: Conceptualization, Methodology, Supervision, Funding acquisition, Writing-Review&Editing; **J Lheman**: Data curation, Project administration, Formal analysis, Visualization, Writing-Original Draft; **F N A Dewi**: Conceptualization, Methodology, Validation, Writing-Review&Editing; **Nuraeni**: Investigation, Data curation, Visualization, Writing-Review&Editing; **Permanawati**: Methodology, Validation, Investigation, Writing-Review&Editing; **Hans Ulrich Baer**: Conceptualization, Methodology, Resources, Writing-Review&Editing

Disclosure statement

Hans Ulrich Baer has an interest in intellectual properties in tissue engineering. The other authors declare that they have no competing interest. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Reviewer disclosure

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

Ethical declaration

All procedures involving the care and use of animals for primary cell isolation were approved by the Institutional Animal Care and Use Committee (IACUC) of Faculty of Medicine, Tarumanagara University, Jakarta, Indonesia; with ethical approval number 012–013.KEPH/UPPM/FK/III/2021.

Funding

This work was funded by a grant from the Ministry of Education, Culture, Research, and Technology, Jakarta, Indonesia [309/E4.1/AK.04.PT/2021; 3499/LL3/KR/2021, 1045-SPK-KLPPM/UNTAR/VII/2021]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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