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Maintenance of hiPSC-derived Hepatocytes in a Perfusion Bioreactor Integrated with Stem Cell Hepatic Intuitive Apparatus

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Abstract. In clinical terms, end-stage liver disease is a group of liver diseases that includes advanced liver disease, liver failure, and decompensated cirrhosis. Liver transplantation has been the most effective treatment for cirrhosis. The limited number of available and suitable living liver donors is a significant limitation in liver transplantation. Acute or chronic rejection could be the cause of liver transplant failure. To overcome rejection, usage of the long-term immunosuppressive drug is a standard post-transplant regimen. However, this therapy can increase the risk of severe viral or fungal infection and malignancy. Various attempts were made to address the liver transplant shortage. One of them is liver tissue engineering. This research was conducted with an artificial liver prototype of Stem Cell Hepatic Intuitive Apparatus (SHiNTA) with a perfusion bioreactor, whose manufacturing process is simple in the form of a liver microstructure consisting of differentiated hepatocytes from an hiPSCs from a modification of the Blackford protocol in a liver biologic scaffold. Liver biologic scaffolds were made from pieces of rabbit liver stored in the Stem Cell and Tissue Engineering (SCTE) laboratory, Fakultas Kedokteran Universitas Indonesia, by decellularization. This study aimed to develop the SHiNTA BALs to sustain the viability of hiPSC-derived hepatocytes in the artificial liver prototype. SHiNTA artificial liver prototype with a

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perfusion bioreactor connected to a perfusion pump with a specific perfusion rate of 2mL/min showed a higher cell count and confluence, with evenly distributed in the extracellular matrix than SHiNTA blood bag with orbital shaker group up to day 7. Furthermore, the SHiNTA artificial liver with perfusion bioreactor showed a positive signal of cell maturation in the scaffold (ASGPR, HNF4-α, and CEBP-α) through immunofluorescence.

Keywords: Bioreactor; hiPSCs; Histology; SHiNTA; Tissue engineering

1. Introduction

End-stage liver disease is a term used to describe a collection of liver conditions, including severe liver disease, liver failure, and decompensated cirrhosis. In the USA, there are currently 11,514 people on the liver transplant waiting list, and approximately 70.19 percent of them received a transplant in 2017 (Kim *et al.,* 2019). According to WHO data from 2012, the mortality rate for liver cirrhosis in Indonesia is 52.7 (for males) and 16.6 (for women) per 100,000 deaths (Kim *et al.,* 2016). Cirrhosis has been most successfully treated through liver transplantation. A key barrier to liver transplantation is the death of appropriate and available living liver donors. The failure of a liver transplant may result from acute or chronic rejection. To prevent rejection, the standard post-transplant procedure involves the use of long-term immunosuppressive drugs. However, this therapy has been associated with an increased risk of severe viral or fungal infections and malignancies. Various approaches have been utilized to address the shortage of liver transplants. The transplantation of hepatocyte cells as a therapeutic approach is limited by the low engraftment rate of the cells, which in turn requires a large number of hepatocyte cells (Alwahsh, Rashidi, and Hay, 2018). The present study reports that the engrafted cells fail to differentiate into functional liver cells, leading to the requirement of liver transplantation (Hamooda, 2016). Plasmapheresis, hemodiafiltration, and artificial liver (AL) have been proposed as potential alternatives to liver transplantation in the field of liver tissue engineering (Lee, Kim, and Choi, 2015).

The field of liver tissue engineering primarily focuses on four primary endeavors (Hosseini *et al.,* 2019): 1. The development of complete, functional, and implantable liver constructs; 2. The creation of bioartificial liver (BAL) systems to support the lives of patients awaiting liver transplantation and in vitro hepatocyte-based models; 3. The establishment of culture models for drug metabolism and toxicity screening in drug discovery; 4. The provision of a foundation for researchers studying liver regeneration, disease, pathophysiology, and pharmacology. Like other types of tissue engineering, liver tissue engineering requires three main components that are interconnected and have an impact on one another: cells, scaffolds, and signaling molecules, like growth factors and other active biomolecules. Complex tissue architecture is created when cells derived from primary or stem cells are combined with three-dimensional scaffolds. Scaffolds and signaling molecules work together to provide structural, biochemical, and biomechanical support for cell and tissue development (Sibuea, 2021). Recent progress in the development of implantable engineered hepatic tissues has demonstrated promising potential as a feasible alternative to overcome the constraints associated with existing cell-based strategies. The limited engraftment of cells and their short-term survival post-implantation are significant challenges that remain to be addressed. Various methodologies have been utilized to generate hepatic micro-tissues, including cell encapsulation, 3D printing, microfluidic systems, and decellularization/recellularization techniques (Heydari *et al.,* 2020; Forbes and Newsome, 2016).

The AL technique integrates blood dialysis treatments with extracorporeal perfusion system equipment, utilizing non-biological hemofiltration to effectively cleanse the blood of individuals suffering from acute and chronic liver failures. The primary function of AL is to facilitate liver transplantation by eliminating metabolic waste and blood toxins while also preventing potential damage to the brain and kidneys. The device's efficacy is limited to providing temporary relief and stabilization of the patient's symptoms for a brief duration. Therefore, liver transplantation continues to be imperative. A BAL, specifically the hollow fiber bioreactor that involves the adherence of hepatocytes within a cartridge, was applied to hollow fiber membranes that serve as a scaffold for cell attachment and compartmentalization. The methodology involves the integration of a synthetic liver with operational hepatocytes to reconstruct or restore the metabolic function that has been compromised due to liver cell loss. This enables partial replacement of the patient's hepatic function during the period of awaiting liver transplantation (Vacanti and Kulig, 2014; Zhang *et al.,* 2014).

Bioreactors are utilized in tissue engineering for three primary purposes: 1.) Replicate the in vivo state of cells in vitro, thereby facilitating the comprehension of normal cellular and molecular physiology; 2.) Expand cells for potential clinical applications, such as gene and cell therapies, or simulate a pathological state to study pathophysiology; 3.) Employed to establish novel therapeutic targets and evaluate potential new treatments in a more realistic setting than traditional in vitro culture. Success in this area would also reduce the burden of using animals in pharmacological testing (Selden and Fuller, 2018). Contemporary bioreactors typically comprise tri-dimensional cell constructs composed of a solitary phenotype, co-cultures of diverse phenotypes such as epithelial and endothelial cells, or epithelial and fibroblastic cells. Alternatively, multiple cell types are combined to simulate the in vivo microenvironment. Mass transfer enhancement can be achieved by introducing dynamic characteristics to bioreactors, specifically through the utilization of convection. This fluid flow mechanism significantly facilitates mass transfer (Charmet *et al.,* 2020). Various types of bioreactors, such as spinner flasks, rocking bioreactors, and waveform bioreactors, have the capacity to achieve a dynamic state through mixing. Nevertheless, these do not constitute imitations of any physiological system within the human body (Egger *et al.,* 2017; Birla, 2014). In contrast, perfusion bioreactors provide a more accurate simulation of the in vivo environment. Micro bioreactors that have achieved success utilize perfusion systems, which may involve either a basic downward or crossflow approach or the provision of a microgravity setting. The former mass transfer method is surpassed by the latter, which utilizes rotating wall cell culture systems and fluidized bed bioreactors. However, optimizing the flow for improved tissue-specific expression through optimal perfusion is imperative. Excessive perfusion can negatively impact cell proliferation, survival, and function by potentially removing crucial paracrine factors necessary for cell survival (Irsyad *et al.,* 2022; Nadhif *et al.,* 2020).

Tissue engineering techniques, such as liver regeneration using cell-based artificial liver prototypes, are still being developed to address the high demand for liver transplantation. The need for artificial liver prototypes that are microstructurally and functionally similar to the liver in vivo presents challenges in tissue engineering and method refinement. The extracellular matrix (ECM) microstructure of artificial liver prototypes made from the natural or synthetic matrix is insufficient for cell proliferation and differentiation. The decellularized liver scaffold retains the original organ's ECM, resulting in a scaffold with an ideal microstructure for liver cell proliferation and differentiation (Antarianto *et al.,* 2022; Dewi, Antarianto, and Pawitan, 2021). Therefore, artificial liver prototype research is still a developing area of research, one of which is Stem

Cell Hepatic Intuitive Apparatus (SHiNTA). This study is developed from previous artificial liver prototype studies (Sibuea *et al.,* 2020). The novelty of this study is the use of hepatocytes that are differentiated from human induced pluripotent stem cells (hiPSCs) by using a native liver scaffold with a decellularization method. The hepatocytes that differentiate from hiPSCs are able to mimic the pattern and development of hepatocytes through stages of definitive endoderm differentiation, hepatic specification and hepatic organ-like maturation (Takeishi *et al.*, 2020). These methods are made by the Stem Cell and Tissue Engineering (SCTE) laboratory in IMERI FKUI with multiple syringe injection methods for the more straightforward manufacturing process as a central component of SHiNTA (Antarianto *et al.,* 2022; 2019). Another component of SHiNTA is using a bioreactor made from silicon and a perfusion pump machine that flows the medium at a specific rate to achieve a higher hepatocyte maturation and function. The bioreactor's overall design combines with specific resulted surface roughness produce a distinct perfusion flow can be used to modulate the perfusion (Qosim *et al.,* 2018; Whulanza *et al.,* 2016). These BAL can be used to overcome the problems and weaknesses of existing studies in the form of price and availability constraints. This research aimed to develop the SHiNTA BAL for sustaining the viability of hiPSCs-derived hepatocytes in the artificial liver prototype.

2. Materials and Methods

2.1. hiPSCs Culture

In vitronectin-coated (A14700, Gibco™, USA) 12-well plates, the hiPSCs cell line from human bone marrow mesenchymal stem cells was purchased from EBiSC (A14700, Gibco™, USA) was thawed and cultured with the complete Essential 8 medium culture (A14700, Gibco™, USA). Half of the medium was removed every two days and replaced with an equal volume of fresh medium. Colony morphology and confluency were evaluated daily through microscopic observation and documentation. The passage was completed when confluency in each well reached 25–50%. The iPSC colony was washed by using phosphate-buffered saline (PBS) (10010023, Gibco™, USA), then broken up and separated from the vitronectin in the 12-well plates using Versene solution (15040066, Gibco™, USA) and incubated for 4-5 minutes at 37°C and 5% CO_2. A cell scraper was used to scrap the hiPSCs from the vitronectin-coated 12-well plates completely. To halt the dissociative effect, the iPSC suspension was collected in a 5 mL complete medium with 10 mM ROCK inhibitor (Y-27632, STEMCELL Technologies, Canada). The split ratio ranged from 1:2 to 1:4.

2.2. Liver scaffold production and hepatic differentiation to generate artificial liver prototype

Based on previous research, the researchers utilized decellularization methods to create a native liver scaffold from the liver of New Zealand White Rabbits through multiple syringe injections (Antarianto *et al.,* 2019). Five liver lobules were cut to 1.5 cm x 1.5 cm with a thickness of 0.7–1 cm. The liver cubes were placed in a petri dish and immersed in 0.001 M Ethylene Glycol Tetraacetic Acid (EGTA) (E3889-100G, Merck, Germany) for 30 minutes. A 1 mL syringe was secured with a fixation device and a toothpick attached to a red wire on top of the Styrofoam. Aquadest (B000002784, OneMed, Indonesia) was the first administered to liver cubes using the fixated syringe technique. The sodium dodecyl sulphate (SDS) (BIO-2050-100g, 1st BASE, Singapore) with graded concentrations of 0.1%, 0.25%, 0.5%, 0.75%, and 1% was administered 25 times at the same location until translucent. Distilled water was injected into the liver biological scaffolds to wash, then placed into tubes filled with NaCl 0.9% (GKL9230500149A1, Widatra Bhakti, Indonesia) solution and stored in the freezer at −80°C.

The biological scaffolds of the liver were taken out from the freezer and thawed in a biosafety cabinet (BSC) prior to usage. Using sterile surgical scissors, the scaffold was then cut into three pieces and placed on a 12-well plate. Before recellularization, the BSC was sterilized with ultraviolet (UV) light for 1 hour.

The recellularization stage was completed by injecting $1x10^6$ - $2x10^6$ cells/mL from total harvested hiPSCs into five pieces of the liver biological scaffolds with a 1 mL syringe. Based on a modified protocol, the liver scaffold was cultured for 21 days in a static 12-well plate culture with medium changes (Blackford *et al.,* 2018). Hepatocyte differentiation induction medium included: complete Essential 8 medium (A1517001, Gibco™, USA) for days 1-2, RPMI-1640 (RPMI-XRXA, Capricorn Scientific, Germany) medium with human serum albumin (HSA) 10% (13533-692-71, Grifols, USA), Antibiotic & Antimycotic 1% (15240062, Gibco™, USA), Glutamax 1% (35050061, Gibco™, USA) for day 4-8, and HepatoZYME-SFM (17705021, Gibco™, USA) medium, fetal bovine serum (FBS) 10% (SH30071.02, Cytiva, USA), Antibiotic & Antimycotic 1% (15240062, Gibco™, USA), Glutamax 1% (35050061, Gibco™, USA) for day 9-21. Small molecules and growth factors for hepatocyte differentiation included 1.5 µM CHIR99021 (72054, STEMCELL Technologies, USA) (day 1), 5 ng/mL bone morphogenetic protein 4 (BMP4) (PHC9534, Gibco™, USA) (day 1-2), 5 µM LY29004 (PHZ1144, Gibco™, USA) (day 1), 40 ng/mL fibroblast growth factor 2 (FGF2) (78046, STEMCELL Technologies, USA) (day 1-3), 50 ng/ mL and 25 ng/ mL Activin A (PHC9564, Gibco™, USA) (day 1-4 for 50 ng/ mL and day 5-8 for 25 ng/ mL), 5 ng/ mL Oncostatin M (OSM) (PHC5015, Gibco™, USA) (day 9-21), and 25 ng/ mL hepatocyte growth factor (HGF) (78019.1, STEMCELL Technologies, USA) (day 9-21). An inverted microscope was used to observe the scaffold microscopically during the differentiation process. On day 21, after the differentiation process was completed, the liver tissue engineering construct samples were collected to initiate the perfusion process.

2.3. SHiNTA artificial liver system prototyping

This research was consisted of four groups: Group 1 was SHiNTA BALs perfusion with the bioreactor for seven days (SH-H7), Group 2 was SHiNTA BALs inside the blood bag on top of orbital shaker for seven days (BL-H7), Group 3 was decellularized native liver scaffold only as a negative control (K-), and Group 4 was artificial liver prototype as a positive control (K+).

The SHiNTA BALs perfusion with the bioreactor group (SH-H7 group) was placed in a bioreactor made of polydimethylsiloxane according to previous studies (Irsyad *et al.,* 2022; Sagita *et al.,* 2018; Whulanza *et al.,* 2016). A closed perfusion circuit was created with the installation of 3 mm silicon tubes in the bioreactor, a blood bag contained with complete hepatocyte medium, and a perfusion pump (Perista Pump SJ-1211, Atto, Japan), as shown in the illustration in Figure 1. Complete hepatocyte medium consists of Williams's E medium (12551032, Gibco™, USA) with platelet-rich plasma (PRP) 10% provided from Palang Merah Indonesia (PMI), dexamethasone 1% (GKL8619906143A1, Phapros, Indonesia), Insulin-Transferrin-Selenium (ITS) 1% (41400045, Gibco™, USA), Antibiotic & Antimycotic 1% (15240062, Gibco™, USA), Glutamax 1% (35050061, Gibco™, USA), and heparin 1% (GKL1731539143A1, Fahrenheit, USA) (Sibuea *et al.,* 2020). Perfusion pumps were run for seven days with a perfusion rate of 2 mL/min. Samples were collected on day 7 after the perfusion process was completed for histological analyses.

To conduct the blood bag with orbital shaker method, the BL-H7 group was introduced into a blood bag containing a complete hepatocyte medium. The blood bag was then positioned on an orbital shaker (PSU-10i, Biosan, Latvia) at 100 rpm for a duration of seven days. Samples were obtained on the seventh day following the completion of the perfusion process for histological analyses.

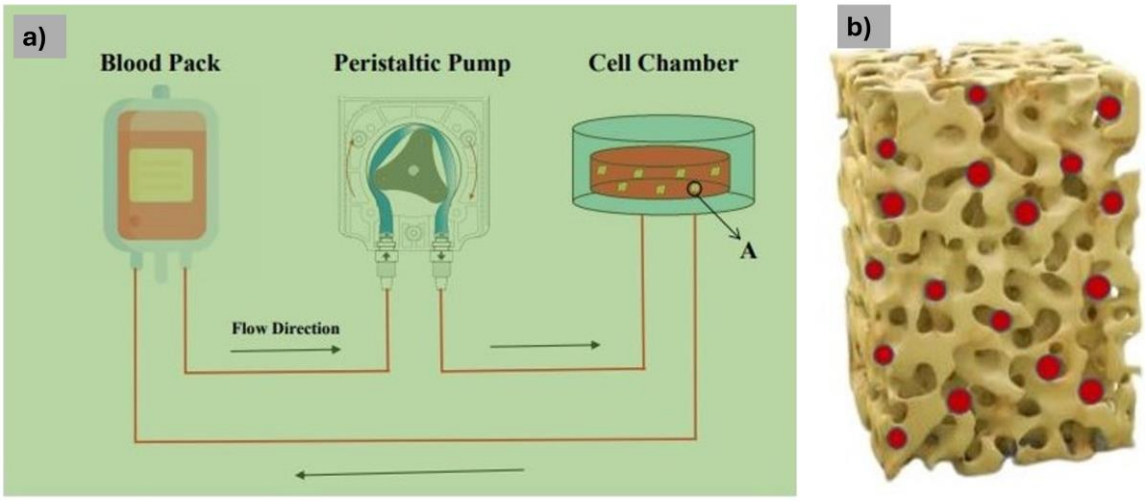

Figure 1 (a) An illustration of a closed-circuit SHiNTA with a bioreactor inside an incubator. Arrow: Flow direction of complete hepatocyte medium (b) An illustration of cell distribution inside a liver scaffold

2.4. Cell counting and viability analysis

The preparations of cell viability in liver tissue engineering construct groups were made with a working solution of Live/Dead Viability/Cytotoxicity Kit (L3224, Invitrogen, USA) by combining Ethidium Homodimer-1 with Dulbecco's phosphate-buffered saline (DPBS) (14190144, Gibco™, USA) and Calcein AM (4:2). Samples were fixated with methanol for 30 minutes, then added with 100-150 µl working solution, and incubated in a moist chamber at dark room for 30 minutes. After incubation, samples were added with DPBS. The viability and cell counting results were examined using Biotek Cytation1 Image Reader (Agilent, USA) at Integrity Laboratory and Research Center (ILRC), Universitas Indonesia.

2.5. Histological analysis for cell adhesion to extracellular matrix in scaffold and measurement of collagen area fraction

Haematoxylin and eosin (HE) staining was carried out by deparaffinization with xylol (1.08661.2500, Merck, Germany) and rehydration in decreasing graded alcohol concentrations (1.00983.2500, Merck, Germany) (100%, 96%, 80%, and 70 %). After being treated in a hematoxylin solution (3801562, Leica, USA) for 10-15 minutes, the samples were washed under running water and then incubated in eosin (3801602, Leica, USA) for 5-10 seconds. The samples were dehydrated with increasing alcohol concentrations. (70%, 80%, 96%, and 100%) and cleared with xylol. After clearing, the samples were covered with a cover slip and enclosed with Entelan (1.07960, Merck, Germany).

Masson's Trichrome (MT) staining was carried out by deparaffinized with xylol and rehydration in decreasing graded alcohol concentrations (100%, 96%, 80%, and 70%). The samples were fixated in Bouin's fixative solution (HT10132, Merck, Germany) for one hour at 56°C, then washed in running water, submerged in Weigert hematoxylin solution (115973, Merck, Germany) (30 minutes), Biebrich scarlet acid fuchsin (HT151, Merck, Germany) (five minutes), phosphomolybdic acid (HT153, Merck, Germany) (five minutes), and aniline blue (B8563, Merck, Germany) (30 minutes). Tissue samples were dehydrated with increasing alcohol concentrations (70%, 80%, 96%, and 100%), followed by xylol clearance. . After clearing, the samples were covered with a cover slip and enclosed with Entelan. To calculate the fraction of collagen area relative to scaffold area on MT staining, ImageJ software was used to evaluate the micro photo findings from the Optilab, with replication of scaffold area photo taken three times per group sample (n=3).

2.6. Immunofluorescence analysis of artificial liver prototype

The immunofluorescence technique was conducted to investigate the expression of hepatocyte transcription factors in the liver tissue engineering construct groups. The samples were deparaffinized using xylol and rehydrated through a series of decreasing graded alcohol concentrations (100%, 96%, 80%, and 70%). The samples were then incubated with primary antibodies, including anti-HNF4-α (ab92378 Abcam, UK) at a 1:100 dilution, anti-CEBP-α (ab40761 Abcam, UK) at a 1:250 dilution, and BD Pharmigen™ PE Mouse anti-ASGPR1 (Clone 8D7, BD Biosciences, USA) at a 1:100 dilution. The incubation procedure for anti-HNF4-α and anti-CEBP-α was carried out in a moist chamber at a temperature of 4°C for the night. The incubation process for anti-ASGPR1 was carried out in a moist chamber and dark room at room temperature for two hours in the absence of secondary antibody incubation. The experimental procedure involved a washing step with 0.1% PBST, followed by incubation of Goat anti-Rabbit IgG (H+L) Cross-Adsorbed secondary antibody Alexa Fluor™ 488 (A-11008, Invitrogen, USA) in 1:200 dilution for anti-HNF4-α and anti-CEBP-α. The secondary antibody was incubated for one hour at room temperature in a dark room. Following the second antibody incubation, the samples were washed with 0.1% PBST. Subsequently, the samples were incubated with 4',6-diamidino-2-phenylindole (DAPI) (ab228549, Abcam, UK) staining for five minutes at room temperature in a dark room. Following the immunofluorescence staining procedure, the samples were subjected to analysis utilizing a ZOE™ Fluorescent Cell Imager (Bio-Rad, USA) at the Gerontology laboratory IMERI FKUI.

2.7. Data analysis

Data analysis in this study was carried out by statistical tests using GraphPad PRISM Version 9 software. A statistical analysis was performed using quantitative histological analysis using ImageJ software (collagen area). The normality test was carried out using the Shapiro-Wilk test, and the homogeneity test was carried out using the Levene test. The P value <.05 indicates a significant difference between the two groups.

3. Results

3.1. SHiNTA bioreactor perfusion

The configuration of the SHiNTA BALs with perfusion (SH-H7) group study comprises a blood bag containing complete hepatocyte medium attached to a perfusion pump, demonstrating the capability for perfusion. The perfusion did not cause the sample in the bioreactor chamber to rupture or be forced into tiny tubes during the seven days of perfusion with electrical power application. The 2 mL/min perfusion rate allows for appropriate perfusion while subjecting the sample to the least degree of shear stress throughout its stay in the bioreactor chamber. In contrast, the orbital shaker methods used in the SHiNTA blood bag (BL-H7) group trials showed that the samples did not rupture throughout the seven days. Each group's artificial liver prototype can be used for histopathological and molecular examination.

3.2. SHiNTA histological staining

Based on observation made on hematoxylin-eosin (HE) staining, the basophilic nuclei of the hiPSCs derived-hepatocytes linked to the scaffold were visible in the histology of the BL-H7 group and the SH-H7 group, as illustrated in Figures 2a and 2b. The SH-H7 group and the BL-H7 group showed a variety of cell morphology, ranging from flattened spindle shape to ovoid shape with heterogeneous size. The connected cells were more extensive and more evenly dispersed across the ECM, with the nucleus and cytoplasm being more apparent in the SH-H7 group than in the BL-H7 group. The histologic characteristic is visible

in Figure 2c K- group of decellularized liver scaffolds is a pink-stained ECM. Homogenous cell morphology was found in the K+ group of liver tissue engineering construct in 21 days, as shown in Figure 2d. From the examination results with HE staining, cell adhesion to ECM in the scaffold was observed in both SH-H7 and BL-H7 groups with different profiles.

The scaffolds were still present in both cultures, and the SH-H7 group's HE staining revealed more cells than the BL-H7 group. Additionally, the SH-H7 group showed more cells adhered to the liver biological scaffold than the BL-H7 group.

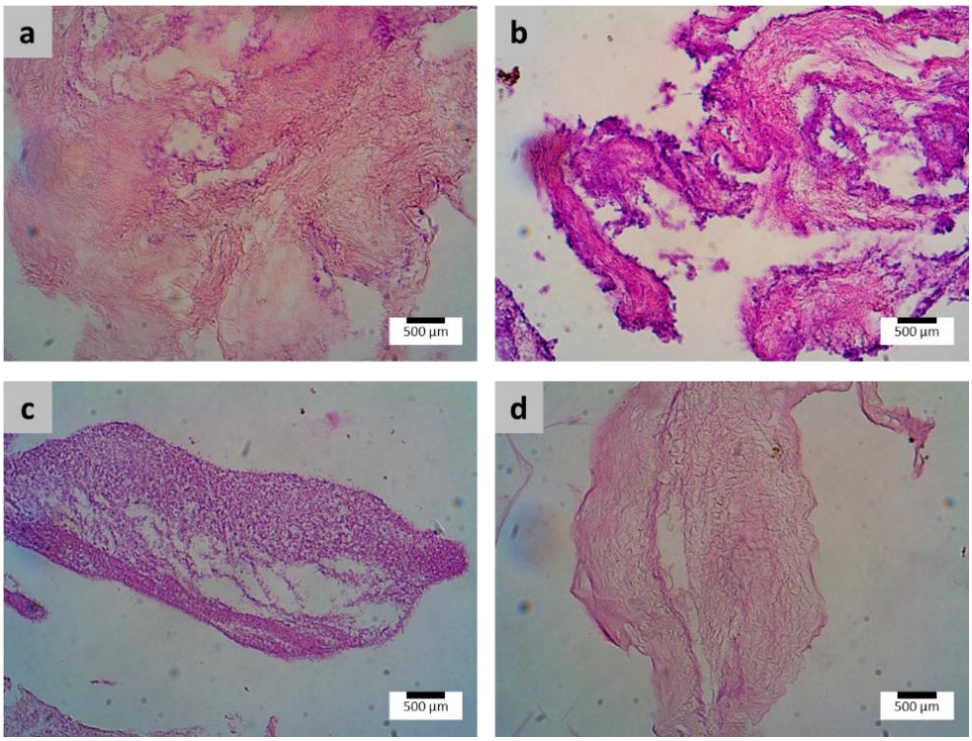

Figure 2 Histological features of SHiNTA using HE staining (Mag. 40x): (a) SHiNTA BALs perfusion with the bioreactor for seven days (SH-H7), (b) SHiNTA BALs inside the blood bag on top of orbital shaker for seven days (BL-H7), (c) artificial liver prototype as a positive control (K+), and (d) decellularized native liver scaffold only as a negative control (K-)

According to findings from Figure 3a's Masson's Trichrome (MT) staining, cells in the SH-H7 group had eosinophilic cytoplasm and blue-black nuclei. They could be visible in the pores of the scaffold or on the collagen fibers' surface. As illustrated in Figure 3b, the cells in the BL-H7 group could also be observed on the surface of the collagen fibers or by filling the holes of the scaffold. The ECM in the negative control (K-) group of decellularized liver scaffolds (Figure 3d) was made up of blue-colored collagen fibers with voids between them (which appeared as hollow white spaces between fibers). In K+ group of liver tissue engineering construct in 21 days showed the cells on the surface of collagen fibers of ECM (Figure 3c).

In the study, the mean percentage of collagen area relative to scaffold area in the SH-H7 group, as measured by ImageJ in Figure 3e, was found to be 22.33%, with a standard error of the mean of 2.02%. The mean percentage of collagen area in the BL-H7 group was 32.85% ± 1.43%. The mean collagen area percentage in the K- group was 36.67% ± 1.14%. In the K+ group, the mean collagen area percentage was 30.52% ± 1.43%. The collagen area results showed a statistically significant difference between the groups, especially between the SH-H7 group and the BL-H7 group ($P = 0.001 < 0.5$), the K- group ($P = 0.008 < 0.5$), and the K+ group ($P = 0.33 < 0.5$).

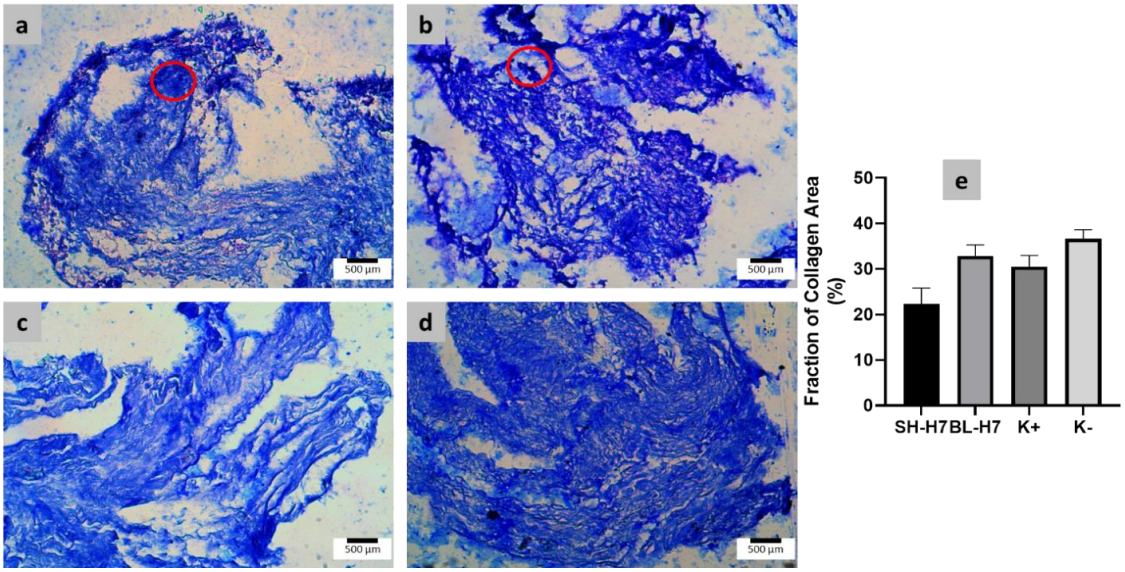

Figure 3 Histological features of SHiNTA in MT staining (Mag. 40x): SH-H7 (a), BL-H7 (b), K+ (c), and K- (d). (e) Quantification of fraction collagen area using ImageJ measurement for SH-H7 group, BL-H7 group, K+ group, and K- group (%) (n=3). Red circle: Cells

3.3. The microenvironment in the SHiNTA liver bioreactor improved cellular viability

According to findings in this study, cells in the SH-H7 group had a total cell number of 55, with the confluence of samples being 32%, as shown in Figure 4a. In comparison, in Figure 4b, the cells in the BL-H7 group had a total cell number of 44, with the confluence of samples being 18%. The ECM in the negative control (K-) group of decellularized liver scaffolds had a total cell number of 29, with a confluence of 10.8%.

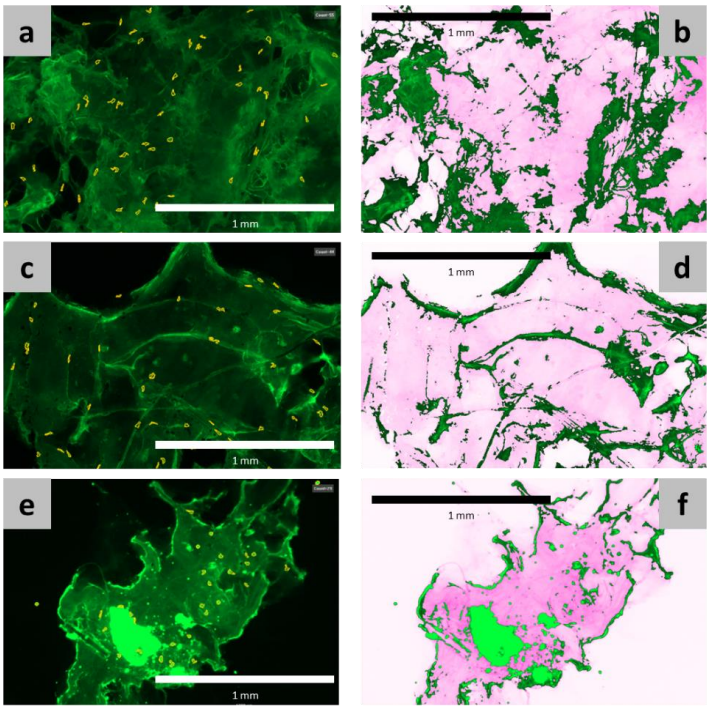

Figure 4 Cell counting microscopic figures of cell viability LIVE/DEAD Viability/Cytotoxicity Kit *For Mammalian Cells* (Mag. 4x): (a-b) SH-H7, (c-d) BL-H7, and (e-f) K-. (a,c,e) are images from the confocal fluorescent microscope with yellow fluorescent color indicated dead cells (calcein stained), green fluorescent color indicating viable cells. (b,d,f) are extrapolated images to visualized the clusters of viable cells showed in green

fluorescent color. The pink background color in b,d and f are from brightfield images of native liver scaffold.

3.4. Determination of hepatocyte maturation in SHiNTA artificial liver prototype

Histological analysis to determine hepatocyte maturation in SHiNTA was performed using three hepatocyte transcription factors such as ASGPR1, HNF4-α, and CEBP-α, in regulating the expression of genes in mature hepatocytes. The immunofluorescence analysis of ASGPR1 in the SH-H7 and BL-H7 groups (as depicted in Figures 5a and 5b) revealed the existence of cells that were positively stained for ASGPR1 and were observed to have adhered to the surface of the scaffold. The immunofluorescence findings of HNF4-α and CEBP-α in the SH-H7 and BL-H7 groups (as depicted in Figures 5e, 5f, 5i, and 5j, respectively) revealed the existence of cells that were positive for HNF4-α and CEBP-α, which were adhered to a section of the scaffold.

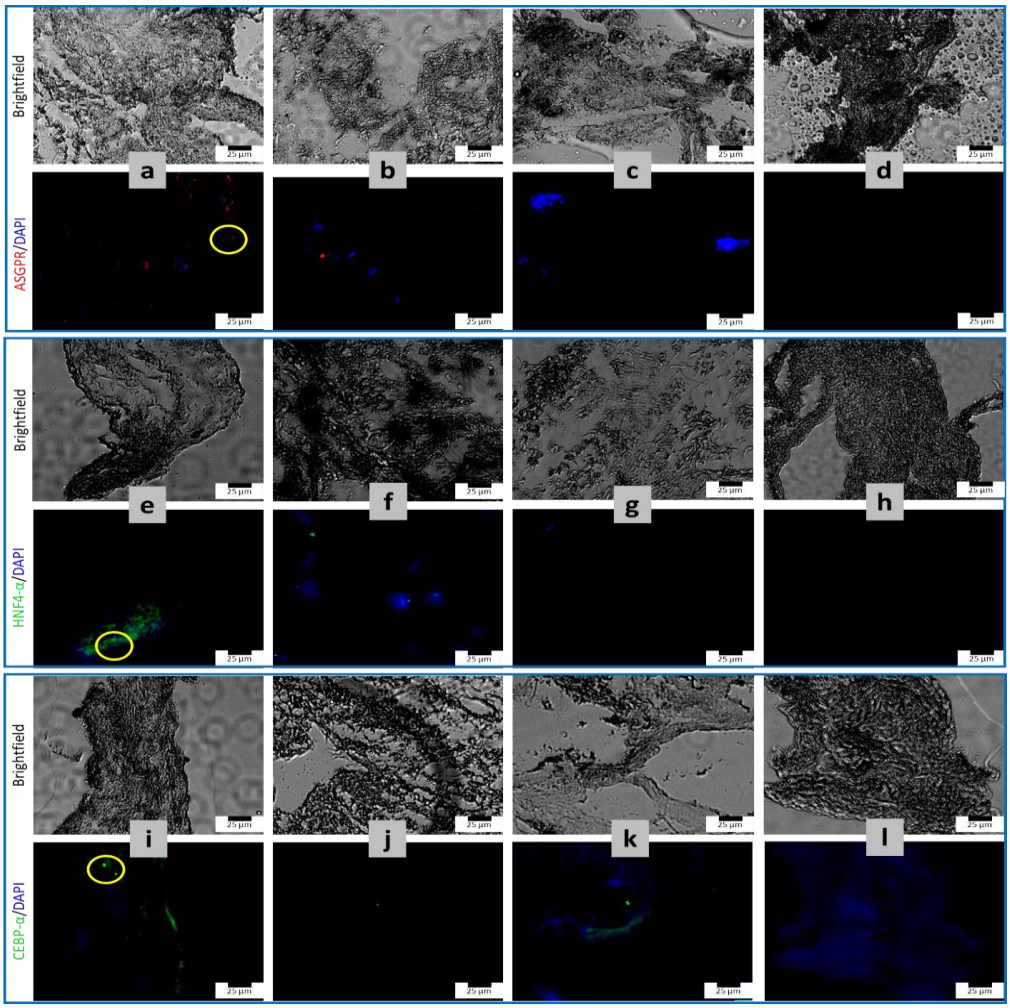

Figure 5 Immunofluorescence staining in SHiNTA samples with 3 different staining techniques (Mag: 40X): ASGPR (SH-H7 (a), BL-H7 (b), K+ (c), and K- (d)), HNF4-α (SH-H7 (e), BL-H7 (f), K+ (g), and K- (h)), and CEBP-α (SH-H7 (i), BL-H7 (j), K+ (k), and K- (l))

4. Discussion

The preservation of cell morphologies and their development into various tissues is influenced by tissue architecture and composition, in combination with the extracellular matrices. To achieve relevant perfusion *in vitro*, bioreactors must enquire controllable flow rate to the cultivated cells (Nadhif *et al.,* 2017; Whulanza *et al.,* 2014). Bioreactor designs

consider physical and technical requirements specific to the targeted organ to create optimal conditions for organogenesis (Utomo *et al.,* 2021). Theoretically, the human body possesses the optimal conditions for bioreactor functionality. However, it is imperative to create a conducive milieu that facilitates the in vivo maturation of a neo-organ, enabling it to regain its intended function eventually. Prior to *in vivo* maturation, additional procedures must be carried out, some of which may pose a potential risk to human life if performed *in vivo* (Hillebrandt *et al.,* 2019; Chen and Hu, 2006; Pei *et al.,* 2002). The findings of this investigation suggest that applying a perfusion rate of 2 mL/min over seven days within the bioreactor chamber utilizing a perfusion chamber did not result in the rupture of the artificial liver prototype. As mentioned above, the approach ensures adequate perfusion of the artificial liver prototype while minimizing the impact of shear stress, thereby facilitating the even distribution of cells within the extracellular matrix during its residence in the bioreactor chamber. The customary constituents of perfusion bioreactors encompass reservoirs for medium, tubes, and chambers for culture. The dimensions of these entities are custom fitted to individual systems, and the associated volume of the medium must be adjusted accordingly. Achieving a consistent flow in a perfusion bioreactor typically necessitates a substantial volume. However, this volume must be tailored to the requisite cells on the scaffolds (Yamada *et al.,* 2022; Mirdamadi *et al.,* 2020).

The results of the HE staining indicated a higher cell count in the SH-H7 group than in the BL-H7 group. Additionally, the liver biological scaffold remained structurally sound throughout the experiment. Furthermore, cellular adhesion to the scaffold was observed in the SH-H7 and BL-H7 groups. The present investigation involved a reduced quantity of hiPSCs that were introduced onto the scaffold compared to prior research endeavors. The present study involved the collection of hiPSCs from a culture that had achieved over 80% confluence, resulting in a higher number of viable cells. Before seeding, the confluence of hiPSCs cultures in this investigation was approximately 50% of the value previously documented, and the viability of these cells was diminished. However, it is commonly believed that hiPSCs can proliferate indefinitely and differentiate into hepatocytes. Research conducted by Minami *et al.* (2019) has shown that when these cells are harvested at a confluence level below 80%, there is a decrease in the number of viable cells. This phenomenon, in turn, reduces the quantity of cells that can be seeded onto the liver's biological scaffold, especially when dealing with hepatocyte-differentiated hiPSCs.

This study indicates that the collagen area observed in the artificial liver prototype indicates the interaction between cells and the ECM. The MT staining analysis findings suggest a different ratio of collagen within the ECM between the SH-H7 and BL-H7 groups. The SH-H7 group demonstrated a reduced collagen area compared to the BL-H7 group. A decrease in the collagen area signifies an increase in the number or size of cells occupying a more significant proportion of the collagen area. The observed outcome may be attributed to the dispersion of cells that adhere to the scaffold during the perfusion of the bioreactor chamber. The divergent cellular behavior observed in the SH-H7 group instead of the BL-H7 group could be attributed to dissimilarities in the collagen area. Furthermore, the cellular distribution is impacted by the scaffold's pore size and geometry (Schmid *et al.,* 2018).

The cell counting and viability analysis results indicate that the SH-H7 group had a total cell count of 55, with a corresponding cell confluence of 32%. In comparison, the BL-H7 group exhibited a total cell count of 44 and a cell confluence of 18%. The findings presented in this study are consistent with the conclusions drawn in prior research (Sassi *et al.,* 2021). Previous studies have demonstrated that the arrangement of cells in liver tissue engineering plays a significant role in modulating intercellular communication and

interactions with the extracellular environment during bioreactor-based culture. The physical characteristic of multicellular organisms is the mode of cell interactions. The physical characteristics mentioned significantly impact various cellular processes such as cell density, ligand-receptor interactions, signal gradient processing, intracellular signal transduction, and the microenvironment (Muncie *et al.,* 2018; Wang *et al.,* 2016).

The findings of this investigation indicate that the differentiation of hepatocytes from the SH-H7 group occurred at a more advanced stage than the BL-H7 group, as evidenced by the positive signals for ASGPR, HNF4-α, and CEBP-α through immunofluorescence. The research findings demonstrate a favorable result, suggesting an enhanced level of hepatocyte maturation in terms of transcriptional activity within the SH-H7 subgroup of SHiNTA compared to the BL-H7 subgroup of SHiNTA. Previous studies have elucidated the role of HNF4-α and CEBP-α in the hepatocyte differentiation process. The transcription factor HNF4-α plays a crucial role in differentiating mature hepatocytes. It is an essential element in constructing a transcription factor network that regulates hepatic mRNA expression. Maintaining transcriptional regulation of gene expression during hepatocyte maturation relies on HNF4-α, which serves as the transcription factor network in the liver that becomes increasingly intricate (Walesky and Apte, 2015). CEBP-α is a member of the CEBP transcription factor family, which exhibits specificity towards the liver and is linked to hepatocyte maturation. CEBPA regulates the genes encoding albumin and alpha-fetoprotein (AFP) (Reebye *et al.,* 2016). The ASGR1 hepatic surface marker has been extensively recognized for its usefulness in the identification of circulating hepatocellular carcinoma cells, the purification of hepatocyte-like cells (HLCs) that originate from hiPSCs, and the demonstration of the effectiveness of differentiating HLCs from hiPSCs. The significance of ASGR1 as a marker for identifying hepatocyte identity is widely acknowledged. However, further research is required to comprehensively examine the transcriptional level of the specific group of cells that express ASGR1 in hepatocyte-like cells HLCs derived from hiPSCs (Peters *et al.,* 2016).

The limitations of this study include the absence of a scanning electron microscope (SEM) to determine the spatial distribution of cells in the artificial liver prototype, as well as the lack of immunohistochemistry (IHC) analysis for each group of artificial liver prototypes. This study is also limited to the use of a single flow rate, referring to previous studies. Nonetheless, it may be of interest to discover the effect of different flow rates to the cell and ECM development in the future. This SHiNTA BALs system, in contrast to the limited availability of viable hepatocyte differentiated iPSCs, requires a significant number of cells in the biological liver scaffold in order to achieve optimal results and perfusion in the bioreactor chamber. To further establish the formation and preservation of the hepatic system, as well as the applicability and functionality of the 3D scaffold systems in vitro, additional long-term data and profiling of genes associated with hepatic maturation and functionality are necessary.

5. Conclusions

The SHiNTA study using a bioreactor combined with a perfusion pump in a closed circuit with the speed of 2 F/min has the potential to run on electricity for seven days non-stop without rupturing the samples. HE staining, MT staining, and viability of cells in the ECM revealed more cells are viable with more reduction of ECM collagen area in SHiNTA with bioreactor group than in the blood bag group. These results were confirmed by the positive signals of HNF4-α and CEBP-α immunofluorescence in SHiNTA with the bioreactor group. This study has the potential for further research for safety and effectiveness, especially with animal models of cirrhosis.

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