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Research Article

Fabrication a Natural 3D-scaffold by Mixing Collagen and Decellularized Mouse Liver Extracellular Matrix for Tissue Engineering

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Abstract

Objective: Liver transplantation is the traditional method for patients who suffer from liver failure. Due to the lack of donor organs, bioengineered liver produced from whole liver decellularized scaffold can be a potential applicable method. The aim of this study was to fabricate and characterize a natural 3D-scaffold by mixing collagen scaffold and decellularized mouse liver extracellular matrix (ECM) for tissue engineering.

Methods and Materials: After washing and removing the blood from the livers completely, they were shaken at room tempreture at 200 rpm on an orbital shaker in deionized water (DW) for 30 min and then shaked in 1% SLES at 200 rpm for about 16-18 h. Thereafter, they were washed in 1% triton and followed by DW for several times. The livers were lyophilized and mixed with collagen. All the scaffolds were evaluated by scanning electron microscope and H&E staining. Scaffold porosity was also determined and cell viability was checked by MTT assay.

Results: The data showed that since SLES led to losing nuclear material, it prevented the degradation of the liver's ECM ultrastructure. DNA and cell debris clearance were verified. Although cells survived on the decellularized liver scaffold, their growth rate was slower than when mixed with collagen.

Conclusion: Combining collagen with decellularized liver ECM provides a biologically relevant microenvironment that closely mimics native tissue chemistry and protect cell survival.

Keywords: Collagen, Liver, Decellularization, Tissue Engineering

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1. Introduction

Liver dysfunction is the cause of many diseases and sometimes leads to death, and it is predicted that this condition will become the 14th leading cause of death by 2030 [1].

One of the great problems of medicine is the provision of definitive treatment for the reconstruction of damaged or defective tissues. The most common method of treating such cases is the organ transplantation [2]. However, the shortage of a donated organ is the main limiting factor [3]. In addition, Moral and technical obstacles in pharmacology and toxicology, limits the animal-based models [4–7], and new alternative strategies are urgently needed to overcome.

Scaffold design and fabrication are one of the key components of biomaterial research in tissue engineering and reconstructive surgery. Engineering of a biomimicry scaffold with appropriate three-dimensional (3D) porous structure that promotes cell adhesion, proliferation, differentiation, and new extracellular matrix (ECM) production is one of the goals in regenerative medicine [8–10].

Despite these requirements, for various biological and biophysical reasons in the field of modeling, design and construction of scaffolds for tissue engineering, there is always a big challenge. However, in the previous four decades, great progress has been achieved in scaffolding applications in the field of biomedical, and various efforts have been done to build scaffolds that can be employed in tissue engineering [9].

There are several methods for creating scaffolding in tissue engineering. Tissue decellularization, electrodynamic process (EHD), electrospinning, hydrogel, induction phase separation (TIPS), and 3D printing are a few of these methods. The raw ingredients, the intended tissue scale, and the scaffold's mechanical, chemical, and physical characteristics all influence the technique that is employed [11–13].

Techniques such as decellularization and re-cellularization are employed to create the ECM needed for tissue engineering [14]. Organs such the heart, kidney, testis, skeletal muscle, lung, liver, gastrointestinal tract, testis and ovary, have so far shown effectiveness with decellularization approaches [15–24]. Decellularized tissues retain the intricate architecture and biochemical signals of the native tissue, which are essential for cell behavior. By removing cells, the ECM retains its native composition (collagen, glycosaminoglycans, growth factors, and other proteins), providing a biologically rich environment that promotes tissue regeneration.

The secretion of tissue-dwelling cells in response to the environmental changes is known as the ECM. Chemistry and topography of the ECM show impacts on cell migration, proliferation, and differentiation as well as modulation of the host's innate immune response [3, 25]. Consequently, each tissue's unique role determines the composition and organization of the ECM [26].

The liver ECM also provides a framework for liver cells to attach and migrate, as well as control cell differentiation, repair, and proliferation [27, 28]. It also provides an appropriate environment for preserving the phenotypic and function of hepatocytes and sinusoidal endothelial cells, which is required

for liver regeneration following injury. Despite of all advantages, using decellularized ECM have some disadvantages such as pathogen transfer, uncontrolled degradation rate, and cell penetration into the scaffolds after cell loading [29]. Collagen is one of the most often used natural polymers in tissue engineering. It is the primary structural protein of many hard and soft tissues in the human and animals, and it is essential for the structural and biological integration of the ECM as well as the tissue's mechanical support. Low immunological reaction, porosity, good permeability, environmental compatibility, and biodegradability have made it one of the most widely used scaffolds in tissue engineering [30]. The goal of this research is to create and analyze a natural 3D scaffold by combining a collagen scaffold with a decellularized mouse liver for tissue engineering with potential applications in regeneration medicine.

2. Materials and Method

2.1. Decellularization

The normal male mice (8-12 weeks old) were sacrificed, then, their livers were removed and washed with PBS and deionized water (DW) for several times until all of the blood was gone. The livers were then incubated in DW for 16–18 hours at room temperature while being shaken at 200 rpm on a magnetic stirrer. The livers were then incubated for 16–18 hours in 1% Sodium Lauryl Ester Sulfate (SLES, Kimia Sanaat Ataman Co., Tehran, Iran) at room temperature while being shaken at 200 rpm on a magnetic stirrer. The samples were put in a 1% Triton X100 solution for 30 minutes. The specimens were then rinsed several times in PBS to eliminate any cell remains or chemical reagents. Decellularized tissues were treated in 10% neutral-buffered formalin and 2.5% glutaraldehyde, stored at -80˚C, and freeze-dried until use.

2.2. Analysis of Residual DNA Content

Following the manufacturer's instructions, a QIAamp® DNA Blood and Tissue Mini Kit (Qiagen GmbH, Hilden, Germany) was used to perform the DNA quantification analysis. The NanoDrop® ND-1000 (Nanodrop Technologies Inc., Wilmington, DE, USA) was used to measure the DNA concentration (ng/μL) spectrophotometrically (wavelength = 260 nm). Subsequently, the livers were infiltrated with 0.2 mg/ml of deoxyribonuclease (DNase 1) from Sigma-Aldrich, Germany, and incubated for 24 hours at 37 degrees. Finally, they were washed with PBS followed by DW.

2.3. Isolating Collagen Type I

For isolating collagen type I, first the rat tail tendons were separated and then treated with 0.02 M acetic acid, as was previously established [41]. In order to create the acquire collagen powder, we first

lyophilized the collagen solution overnight using a freeze-drying machine (Christ Alpha 2–4 LD-plus, Osterode am Harz, Germany). The collagen powder was then dissolved in 0.5 M acetic acid (1% w/v) on ice at a concentration of 5 mg/mL. A 96-well plate culture dish (Jetbiofil, China) was filled with 250 mL of the mixture.

To create a collagen sponge, the collagen-filled plates were frozen-dried after being stored at -80˚C for the whole night.

2.4. Fabricating of the Collagen and Decellularized Liver Scaffold

Lyophilized decellularized liver and collagen was mixed at a ratio of 1:1 on ice and let them to jellified by incubating in 37ºC. In order to sterilize these scaffolds, they were exposed to ultraviolet light for 20 minutes. The decellularized/collagen scaffolds were compared to collagen and decellularized liver scaffolds.

2.5. Isolation and Culture of MSCs Derived from Wharton's Jelly (WJMSC)

After receiving informed consent from parents, the umbilical cords were retrieved from a new infant born via Cesarean section at Hafez Hospital. The specimen was then placed in cold phosphate buffered saline (PBS) containing 100 μg/mL penicillin and 100 U/mL streptomycin (Sigma, USA).

The umbilical cords were separated into small pieces, followed by a longitudinal cut in the umbilical vein, scraping the inner surface, and completely removing the arteries from the umbilical cord. The jelly matrix was sliced into small explants and cultured in the α-MEM medium containing 10% fetal bovine serum (FBS) (Gibco, Germany), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 1% L-glutamine, in a CO_2 incubator at 37 ˚C. After two weeks, the cells migrated from the umbilical cord matrix. The primary cell culture was grown until it reached 80% confluency before being passaged [19]. The Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1395.289) approved all methods.

2.6. Surface Marker Analyses of Mesenchymal Stem Cells

The phenotype of WJMSCs was assessed using flowcytometry. At the third passage, one million cells were harvested and suspended in a blocking solution contained 10% FBS in PBS for 20 minutes. Then, they were labeled with Fluorescein isothiocyanate (FITC) conjugated anti-human CD44,CD144 and CD90, phycoerythrin (PE) conjugated anti-human CD73, CD 106 and CD34, and perCP conjugated CD105 antibodies. The tubes were incubated in the dark for 30 minutes, rinsed with PBS, and examined by flowcytometry (FACS, Caliber^{TM}, BD Biosciences). The data were analyzed by flowJo software.

2.7. Osteogenic and Adipogenic Differentiation

To evaluate the differentiation potential of the MSCs, thy exposed to osteogenic (DMEM-LG supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/ml streptomycin, 100 nM dexamethasone, 0.2 mM L-ascorbate, and 10 mM β-glycerophosphate) and adipogenic (DMEM-LG supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/ml streptomycin, 60 μM indomethacin, 1 μM dexamethasone, 0.5 mM IBMX, and 5 μgr /mL Insulin solution) culture conditions for four and three weeks, respectively. Then, the differentiated cells were stained with Alizarin Red S (sigma, USA) and oil red (sigma, USA), for calcium and lipid depositions, respectively.

2.8. Cell Attachment Assessments

To evaluate cell attachments, the WJMSCs were loaded on different scaffolds and after 2 h, non attached cells on the culture media removed and counted. The number of unattached cells were subtracted from the initial number of cells and the percent of attached cells was calculated.

2.9. MTT Assay

The cell survival and proliferation capacity were determined using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyle tetrazolium bromide (MTT, Sigma) assay. WJMSCs were seeded at 1×10^4/well in a 96-well plate for 1, 3, and 5 days. The culture media were then changed with 1 mg/mL of MTT in Dulbecco's Modified Eagle's Medium (DMEM, Bio idea, Iran) and incubated for 3 hours at 37°C, 5% CO_2, in a dark atmosphere. The dye was then eluted using dimethyl sulfoxide, and the optical density was measured at 595 nm.

2.10. Scanning Electron Microscopy (SEM)

Decellularized/collagen scaffolds with or without cell loading was prepared for SEM and the structure compared with collagen or decellularized scaffolds alone. To do this, all scaffolds were fixed with 2.5% glutaraldehyde and lyophilized. Then, they were coated with a thin layer of gold, using Q150R- ES sputter coater (Quorum Technologies, London, UK) and imaging was done an VEGA3 microscope (TESCAN, Brno, Czech Republic) at 10 kV accelerating voltage.

2.11. Hematoxylin-Eosin (H&E) Staining

The cell-seeded and decellularized scaffolds were fixed them with 10% neutral buffered formalin (pH 7.4) and paraffin embedded sections were prepared for histological evaluations. Hematoxylin and eosin (H&E) and Hoechst (Sigma-Aldrich) were used to stain the 5–10 μm thick sections, which were then

viewed under a light microscope (Olympus BX61, Tokyo, Japan) or a fluorescent microscope (Olympus BX51, Japan) with a digital camera (Olympus DP73), respectively.

2.12. Statistical Analysis

The data was analyzed using analysis of variance (ANOVA). The data were analyzed with SPSS 15.0 for Windows. The graphs were created with GraphPad Prism program. A p-value <0.05 was judged statistically significant. All experiments were done in triplicate.

3. Results

3.1. Evaluation of Decellularized Scaffold

The liver of the mice turned from red to white and semi-transparent during the decellularization process. Throughout the procedure, the samples maintained their integrity and shape, and the scaffolds' pores were conserved following the lyophilization of the decellularized liver (Figure **1**A-D, respectively). The H&E and Hoechst staining revealed that the scaffolds were free of nucleic material.

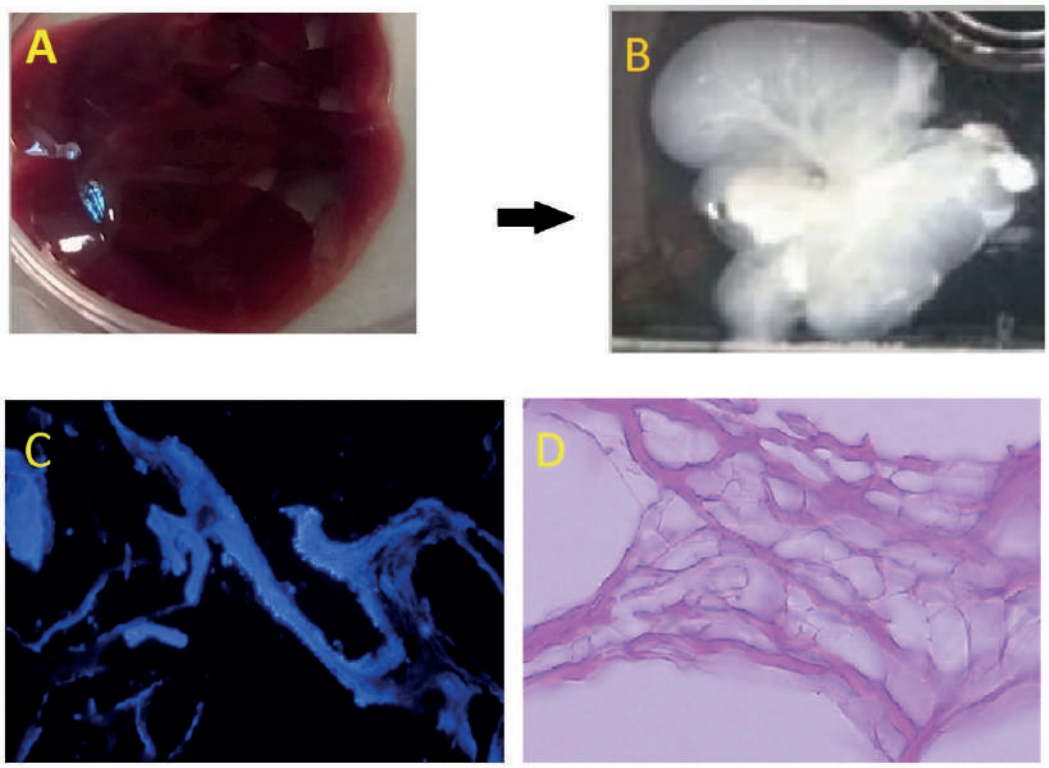

Figure 1: Macroscopic changes in the mice's decellularized liver over time by SLES-based decellularization process (A,B). A representative lyophilized decellularized scaffold with visible pores, (C) Hoechst, and (D) hematoxylin and eosin staining of the decellularized liver revealed it was free of nucleic materials.

3.2. Histological Staining and SEM Assessments

SEM assessment showed that the integrity of the microarchitecture was preserved well and the cells were removed with high efficiency after decellularization. The SEM image of the decellularized liver scaffolds represented a complex network of fibers with porous structures once filled with cells. Moreover, the investigation of the shape and structure of the decellularized liver and the collagen scaffolds by light microscope showed a complex network of fibers with good integrity (Figure **2**).

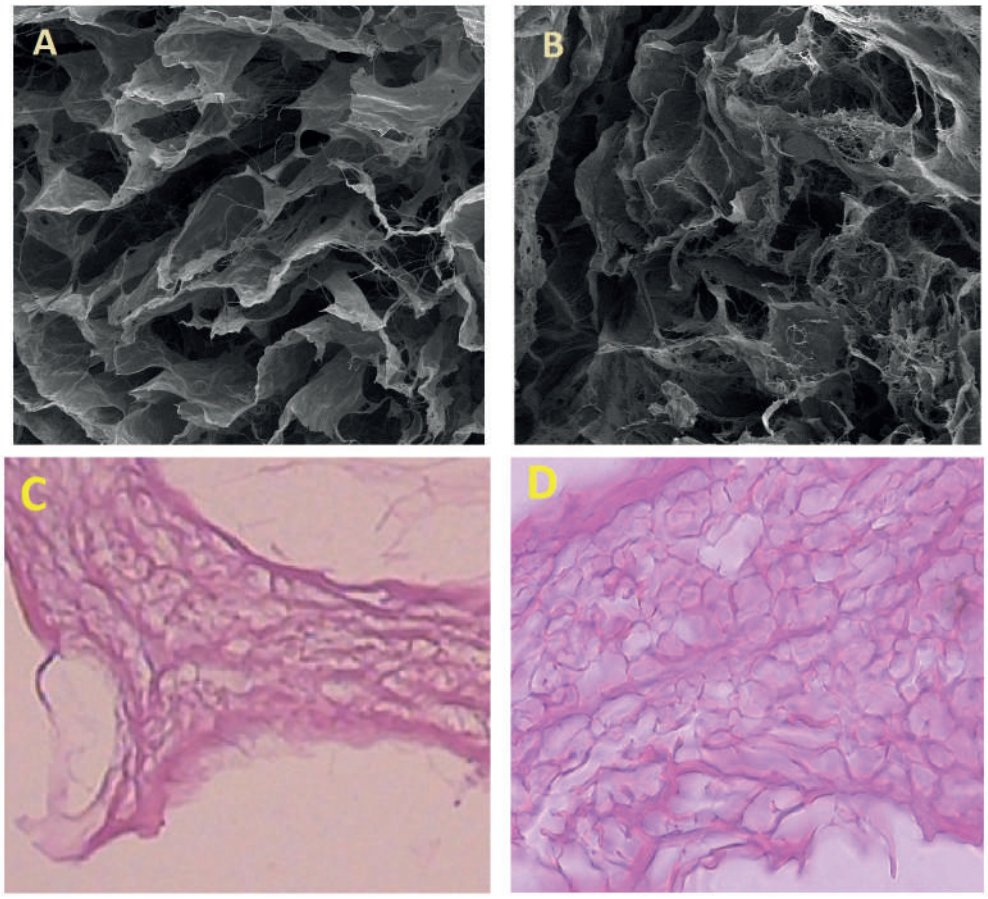

Figure 2: SEM and light microscopy images of decellularized liver and collagen scaffolds. (A) SEM microphotograph of a 3D collagen scaffold with interconnected pores, (B) SEM image of decellularized liver scaffolds, (C) H&E staining of the collagen scaffold and (D) light microscopy of the decellularized liver scaffolds, reveals a complex network of fibers and bundles with well-defined integrity.

3.3. Wharton's Jelly Characterization

The flow cytometry showed that the cells could highly express CD44, CD73, CD90. They could also express CD106 and CD106 moduratly. They failed to express CD144 (endothelial cell marker) and CD34 (hematopoietic marker). They showed the potential to differentiated into adipocytes and osteocytes as well (Figure **3**).

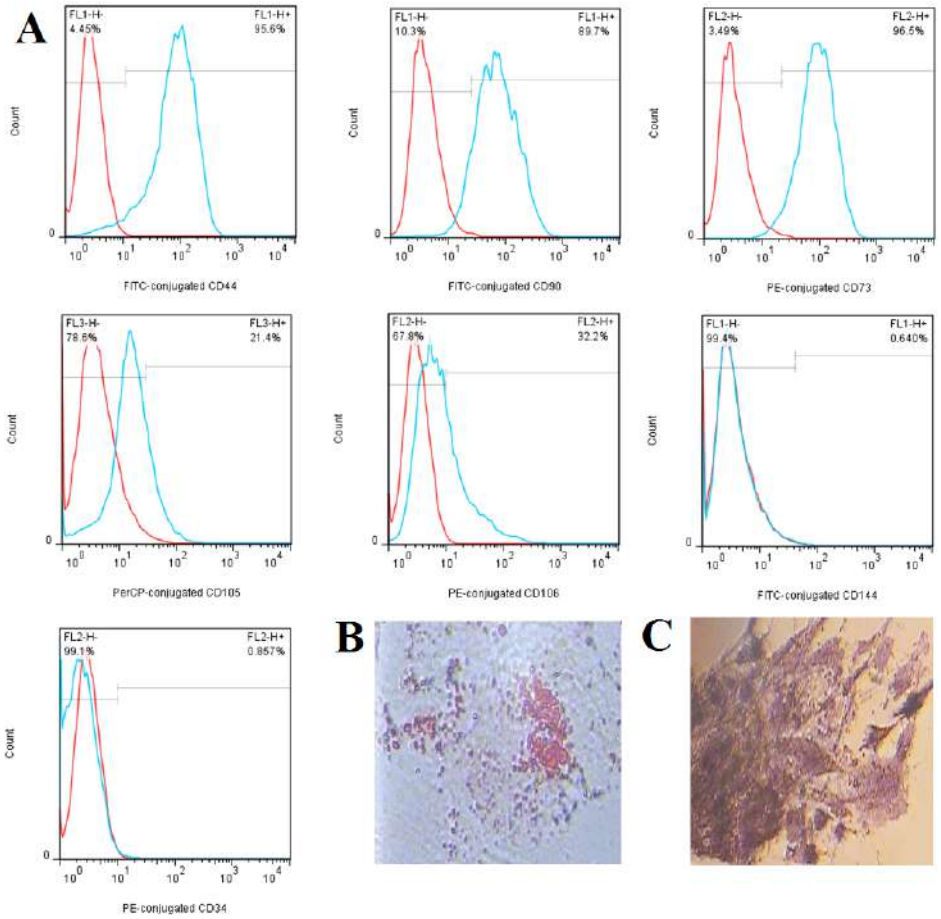

Figure 3: The flow cytometry showed the WJMSC could express CD44, CD90, CD73, CD 105 and CD106, but could not express CD144 and CD34 (A). They also couuld differentate into adipocytes (B) and osteocyte (C).

3.4. Assessment of Attachment

According to the cell attachment experiment, 90.83%±3.253% of the cells was attached to the collagen scaffolds, while 89.08%±3.95% of the cells was attached to the decellularized liver scaffold. The two scaffold types' adhesion properties were statistically comparable (Figure **4**A).

3.5. MTT Assay

The vitality and proliferation of WJMSCs on decellularized liver, collagen scaffolds and decellularized/collagen scaffolds were evaluated and the results were compared with 2D culture conditions (WJ) in order to determine the cytocompatibility of decellularized scaffolds. Compared to the other groups in the 3D cultures on the same day, the cell viability in the 2D culture condition was considerably higher on the first day ($P < 0.05$). When compared to the equivalent cultures on the first day, the number of viable cells on the collagen scaffolds increase significantly on the third day ($P = 0.014$).

The greatest number of viable cells appeared on the decellularized liver scaffold (DCL) on the fifth day ($P<0.05$). Additionally, compared to the cells cultivated on the decellularized scaffold, the number of viable cells on the mixing collagen scaffold with DCL scaffold increased significantly on the third and fifth day ($P = 0.027$). The MTT assay demonstrated the viability of the WJMSCs seeded on the decellularized scaffolds was statistical equal to the number of live cells on decellularize/collagen scaffolds (Figure **4**B).

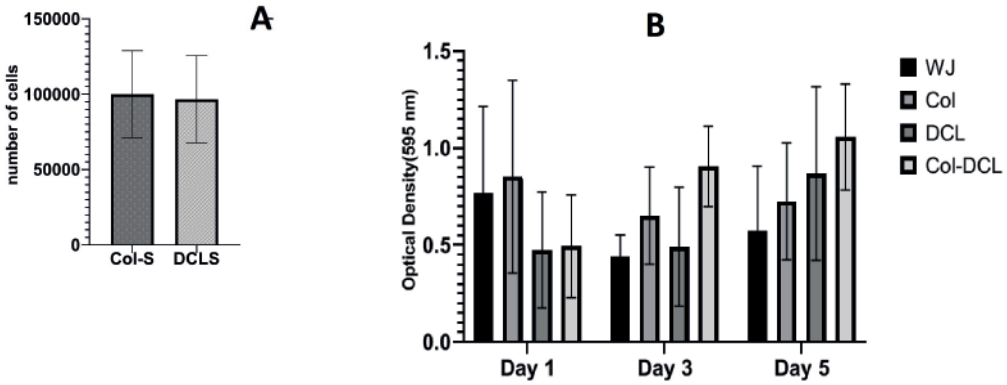

Figure 4: The adhesion of cells to decellularized liver and collagen scaffolds was evaluated. (A) There was no significant difference in cell adhesion between the two scaffolds. (B) The MTT test showed that the statistically more viable cells on decellularized / collagen scaffolds was detected.

3.6. Cell Morphology Assessments

H&E staining was used to examine the cell morphology on collagen and the decellularized liver scaffold. WJMSC cells were evenly distributed and adhered to collagen strands in the decellularized liver scaffolds as well as collagen in this staining. But the collagen scaffold appeared to have a significantly larger cell count (data was not displayed). Furthermore, the pictures showed that the cells appropriately grew on both scaffolds (Figure **5**).

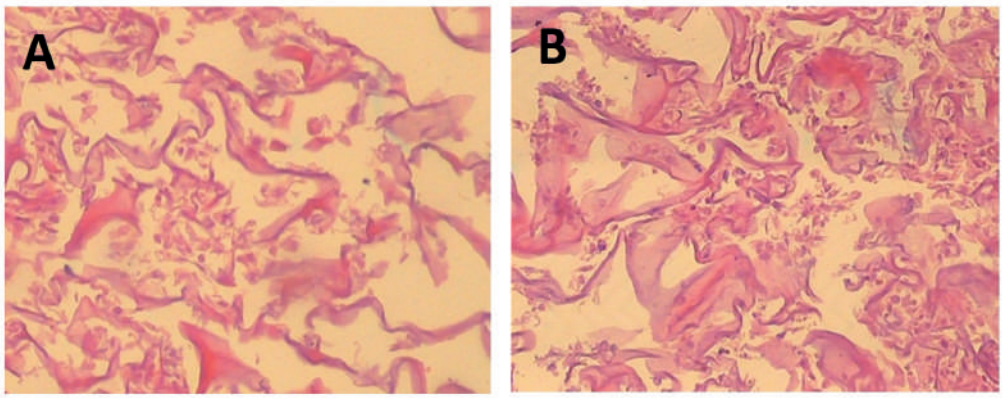

Figure 5: The cells within the collagenous component of the scaffolds have a fibroblast-like structure, which could represent WJMSC. The microphotograph (A) reveal cell distribution on collagen scaffolds and (B) decellularized liver scaffold.

4. Discussion

In regenerative medicine, scaffolds play a pivotal role by providing the necessary structural support that enables cells to grow, proliferate, and differentiate into functional tissues. These scaffolds are often used in conjunction with stem cells or other types of cells to promote tissue regeneration and repair, especially in cases where damaged tissues or organs need to be replaced or repaired. Research suggests that the cells in 3D culture environments exhibited better physiological functions such as cell survival, morphology, proliferation, differentiation, response to stimuli, migration, angiogenesis, drug metabolism, gene expression, protein synthesis, and cell function [31]. Furthermore, the use of natural ECM-based scaffolds—those derived from the body's own ECM is particularly significant because they better mimic the native tissue environment, offering a more faithful reproduction of the biochemical, physical, and mechanical properties of tissues. These scaffolds typically retain bioactive molecules like collagen, elastin, fibronectin, and glycosaminoglycans (e.g., hyaluronic acid), which are crucial for cell recognition, attachment, and function [32].

The liver ECM provides a framework for cell activity, tissue development, and repair [33, 34]. The ECM framework influences liver-specific gene expression and cellular responses through its chemical composition, biophysical properties, and interactions with cultured liver cells. Peripheral signals generate an ideal environment for liver cells to maintain their phenotypic and function [35, 36].

One of the objectives of the current study was to develop a standard methodology to maintain the architecture of a cell-free ECM. Although sodium dodecyl sulfate (SDS) is a common agent in many decellularization procedures, its significant effects on critical ligands and ECM proteins have led to the employment of other agents or techniques. SDS can damage the ECM's biological composition, protein structure, and functional integrity even if it is an effective way to remove cellular debris. SDS is commonly used in decellularization protocols, but its harmful effects on important ligands and ECM proteins have led to the use of milder detergents like SLES and Triton X100. These detergents preserve the ECM structure and compositions [37,38].

Our data showed that decellularized liver scaffold in combination with collagen could provide an appropriate environments of cell proliferation. Our SEM, H&E, and Hoechst results verified that SLES led to clearance of nuclear material as well as the preserving liver's ECM, While, the cell embedding can be done by mixing cell and liquid scaffold in low temperature and jellified the scaffold by increase the temperature. This scaffold provides a better condition for penetrating the cells. In regenerative medicine, this is especially troublesome when the objective is to preserve the ECM's capacity to promote cell attachment, penetration, proliferation, and differentiation. Moreover, the scaffold could also be used for building liver grafts or supporting liver regeneration in vivo [39]. Another point about the decellularized scaffolds is the toxicity of the cellular debris and detergent traces inside the scaffold which may induce cell harm during culture. In our investigation, WJMSC survived on the decellularized liver scaffolds. SEM,

and light microscopy revealed that decellularized liver scaffolds were non-toxic and compatible with cell survival and function. Furthermore, it was discovered that, there was no significant difference in proliferation rate of the cells cultivated in collagen scaffolds compared to DCL scaffold. This could be because they were in a microenvironment comparable to their existing niche. However the proliferation rate was significantly higher in when the cells cultured in decellularized/collagen-scaffold compared to other groups.

Previous research suggests that the 3D scaffold of the ECM promotes the proliferation and boosted the function of many liver cell types [40, 41]. These studies were in line with our study. Evidence suggests that using ECM as a bioink in 3D printing creates a structure for hepatocytes can survive and function. Li, et al showed that decellularized liver scaffolds enhance the differentiation of WJMSCs into hepatocytes and improve their phenotype [42]. This study confirmed earlier results, showing that the combination of two scaffolds—collagen and decellularized liver scaffold has more cells than either one alone. A study found that co-culturing hepatocytes and endothelial cells can improve hepatocyte functions [43].

Scarritt et al, reported about the challenge of decellularization, particularly the potential loss of important ECM components or incomplete removal of cellular debris during decellularization process[31]. This fabrication method that we used in this study could be adapted to other tissues (e.g., skin, cartilage, heart).

5. Conclusion

Combining collagen with decellularized liver ECM to fabricate a 3D scaffold for tissue engineering provides a biologically relevant microenvironment that closely mimics native tissue chemistry. The decellularized liver ECM contributes essential biochemical cues, while collagen provides structural support. This approach can be considered for liver tissue engineering or other applications where ECM composition is critical.

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