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Development of a Nanofibrous Scaffold Based on Bovine Tissue-derived ECM and Poly(ε-caprolactone) for Tissue Engineering Applications

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ABSTRACT

In this study, nanofibrous biohybrid scaffolds were developed by electrospinning using poly(ε-caprolactone) (PCL) and decellularized bovine tissue derived extracellular matrix (ECM). At the first part of the study, bovine ECM was decellularized by treatment with detergent for 24h and then combined with PCL. Following the evaluation of the decellularization efficiency via spectrophotometric DNA content analysis, the composite scaffolds were characterized by using SEM and FT-IR spectroscopy. Moreover, to assess the biocompatibility of the scaffolds an in-vitro cell culture based cytotoxicity test was performed. The results indicated that, DNA content of the bovine tissue was reduced by ~80% compared to the native tissue after decellularization. While FT-IR results indicated the presence of ECM in the composite scaffolds, SEM findings showed that the porous nanofibrous structure of the scaffold changed depending on the incorporated ECM amount. Cell culture based studies also revealed that, the scaffolds containing different amounts of ECM did not have any toxic effect on cell viability during 48 hours of culture period.

Keywords:
Biomaterial; Tissue engineering; Decellularization; Electrospinning.

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INTRODUCTION

The main focus of tissue engineering approach is the development of biomaterial scaffolds exhibiting similarity to the three-dimensional (3D) architecture, and its natural bioactive microenvironment of the target tissue or organ. Up till now, this goal has not been fully achieved, owing to the difficulty in completely mimicking the versatile bio-functional composition and structure of the natural extracellular matrix (ECM) [1]. A number of scaffold manufacturing techniques such as, solvent casting and electrospinning have been utilized in order to obtain 3D porous or nanofibrous scaffolds somewhat architecturally resembling the natural ECM by using natural or synthetic biocompatible polymers [2]. In particular, FDA-approved synthetic resorbable polymers such as poly(ε-caprolactone) (PCL), poly(lactic-co-glycolic acid) (PLGA), and other α-hydroxy acid polymers etc. in such forms have been widely utilized in a variety of tissue engineering applications, some of which have reached clinical applications [3]. However, in addition to being biocompatible and structurally-resembling the tissue, tissue engineering scaffolds are expected to support cell attachment and proliferation, as well as promote angiogenesis, and regeneration when transplanted. Nevertheless, the biofunctional active content of the natural ECM, required for these biological processes cannot be realistically simulated on developed scaffolds using the aforementioned polymers by common manufacturing techniques, such as electrospinning.

To overcome the limitations due to lack of bioactivity, alternative modification methods based on incorporating one or more ECM proteins such as fibronectin or laminin into such biomaterials have been evaluated. However, the natural ECM has a unique bioactive composition and structure, mainly composed of structural and functional biomolecules such as collagen, fibronectin, elastin, laminin, glycosaminoglycans and other glycosylated proteins [4]. Therefore, considering the complex bioactive compositions of the natural ECM, it does not seem possible to fully mimic the ECM with the conventional scaffold production methods. On the other hand, although the functions of the ECM are quite similar in terms of tissue types, bioactive contents show differences, which is also a limitation that cannot be overcome.

In recent years, ECM-based biomaterial fabrication technology called "decellularization" has come to the fore in order to create more realistic tissue scaffolds. This technology is based on developing biocompatible ECM-based scaffolds by removing the cells and nuclear material from natural tissues and organs by chemical, physical and/or enzymatic methods [5]. The success of the applied decellularization method depends on the preservation of the existing natural bioactive content of the ECM in the target tissue/organs during the process. Until this time, bioactive ECM-based materials have been developed via decellularization technology from a variety of tissues or organs including the bone, cartilage, small intestine submucosa, liver, heart etc. [6]. Although the decellularization approach seems to be advantageous, this technology also has obvious disadvantages. In fact, some issues such as the selection of decellularization protocol, shaping the biomaterial into the appropriate architecture, the mechanical properties of the formed scaffold and the large-scale production issues are still controversial. On the other hand, although synthetic polymers have limitations in terms of providing bioactivity, they have remarkable features in that they have adjustable mechanical properties and allow mass production. Therefore, the joint rational use of these two technologies could enable the production of bioactive superior tissue engineering scaffolds suitable for diverse regenerative applications [7].

This study aimed to bring together the natural ECM and a synthetic resorbable biopolymer forming a hybrid scaffold for prospective use in tissue engineering applications. At first, bovine liver tissue was decellularized to obtain the bioactive ECM. Decellularization efficiency and the features of the liver ECM were evaluated. Then, the PCL polymer incorporating the liver ECM was electrospun to form a nanofibrous bioactive scaffold. The chemical, morphological and in-vitro cytotoxicity evaluations of the hybrid scaffolds were carried out.

MATERIALS AND METHODS

Materials

Polycaprolactone (PCL), tetrahydrofuran (THF), dimethylformamide (DMF), sodium dodecyl sulfate (SDS) and the enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA). PBS, culture mediums and supplements was supplied from Biological Industries (Beit Ha-Emek, Israel). Bovine liver tissues were kindly supplied from the Ankara Meat and Milk Board veterinary-controlled slaughterhouse (Ankara, Turkey).

Isolation and characterization of bovine-derived liver tissue ECM

Within the scope of the study, bovine liver tissues were transferred to the laboratory in cold phosphate-buffered saline (PBS, pH: 7.4) buffer within 2-4 hours after slaughtering. Tissue samples were repeatedly washed with 0.9% saline solution and then were cut into approximately 1 mm^3 pieces before decellularization. The tissue pieces were washed with PBS for 2 hours to remove blood and waste tissues. In order to remove cells and DNA from tissues, samples were treated with 0.1% SDS solution prepared in distilled water for 20-24 hours at room temperature. At the end of the period, samples were collected and serially washed with saline solution for 2 hours. Finally, the decellularized samples were frozen at -80 °C for 24 hours and then lyophilized at -76 °C under 0.29 mbar pressure.

In order to evaluate the decellularization efficiency, samples were weighed and treated with a buffer containing 20 mg/mL proteinase K at 55°C for 48 hours [7]. Subsequently, DNA was extracted using phenol/chloroform/isoamyl alcohol method. Then, the amount of DNA reduction compared to the control was determined by taking measurements with NanoDrop ™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA).

Beside the DNA content, quantification the sulfated glycosaminoglycan (sGAG) of decellularized ECM were performed after proteinase K digestion by using the Blyscan sGAG Assay Kit (Biocolor, Newtownabbey, U.K.) [4,5]. Briefly, following digestion, tissue lysates were collected and mixed with dimethylmethylene blue dye. sGAG content of the native and decellularized tissues were determined by measurements at 656 nm (SpectraMax M5, Molecular Devices, San Jose, CA).

Preparation of bioactive ECM powder

For the purpose of bioactive composite material development, decellularized ECM in lyophilized form was digested in 1 mg/mL pepsin solution prepared in 0.01N HCl for 48 hours and then neutralized. After digestion, ECM in gel form was molded and lyophilized. The lyophilized sponges were immersed in liquid nitrogen and homogenized (Retsch MM400) for 10 minutes to obtain ECM powder with a size of approximately 50-60 μm (Fig. 1A).

Production of ECM/PCL composite material

Fibrous bioactive composite nanofibers were developed by use of the electrospinning technique with different ratios of ECM powder and PCL (Fig. 1B). For this purpose, different concentrations of decellularized ECM powder (1%, 0.1%) were added into tetrahydrofurane (THF): dimethylformamide (DMF) (1:1) solution and sonicated for 15 minutes. Then, 14% PCL (80,000) (w / v) was added to

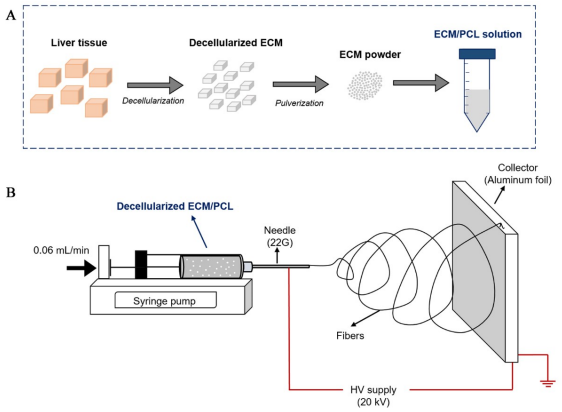

Figure 1. Schematic illustration of the experimental design. (A) Isolation of liver ECM and preparation of ECM/PCL composite solution, (B) the electrospinning process.

the solvent and vortexed for 2 hours with short breaks.

The prepared ECM/PCL solutions were loaded into injectors with a needle tip of 22G diameter and placed into the syringe pump. Then, the electrospinning was conducted at a speed of 0.06 mL/min flow rate under 20 kV and against aluminum sheets at a distance of 15 cm. In addition, in order to develop the biofilm, the prepared decellularized ECM/PCL solutions were placed in Teflon molds (100 µL/cm^2) and then incubated at 37 °C for 48 hours.

Morphological characterization of the materials

Scanning electron microscopy (SEM) analyses were carried out to evaluate the surface morphology of decellularized ECM/PCL composite scaffolds. For this purpose, the samples were coated with a thin gold layer and then analyzed with a ZEISS EVO 40 model SEM device.

FT-IR analysis

FT-IR analysis of decellularized ECM/PCL composite scaffolds prepared in different forms was carried out in order to evaluate the chemical properties of the structures. For this purpose, samples were homogenized and loaded into a Shimadzu IRAffinity model FT-IR device. Analysis was performed in the wavelength range of 600-4000 cm^{-1}.

In-vitro cytotoxicity test

Indirect in-vitro cytotoxicity tests were performed according to the International Organization for Standardization; ISO 10993-5 guidelines to evaluate the potential toxic effects of the scaffolds on human adipose-derived stem cells (hASCs) through MTT based viability assay. Briefly, commercial hASCs (TCC® PCS-500-011™) were maintained in DMEM F-12 medium containing 10% FBS, 10 U/mL penicillin and 10 µg/mL streptomycin and cultured in 24 well culture plates at a density of 4–5 x 10^4 cells/cm^2. hASCs were proliferated under standard culture conditions (5% CO_2, 37 °C and > 95% humidity) until they reached 80% confluence. In parallel with the culture study, the UV-sterilized scaffolds were incubated in culture medium for 48 hours to obtain the extraction medium (EM). Then, the cells were treated with the EM for 48h. During the study standard culture medium served as a negative control and medium containing 400 µL phenol as a positive control group. At the end of the culture period, waste medium was removed and the cell culture was washed with sterile PBS. Then, the MTT reactive was added into each well (diluted in DMEM F-12, 1:10) and incubated for 4 hours. The formed formazan crystals were monitored and solubilized by dissociation reagent. Cell viability was determined by measurements at 570 nm.

Statistical analysis

Statistical analyses were carried out in the GraphPad Prism 8 program using a one-way ANOVA test, and significant differences were identified through Tukey's post hoc analyses.

RESULTS AND DISCUSSION

In this study, different forms of composite scaffolds were developed by using decellularized bovine liver ECM and a biocompatible polymer (PCL) for use in prospective liver tissue engineering applications. The decellularization efficiency was determined by spectrophotometric analyses. The decellularized ECM powder was incorporated into electrospun nanofibrous membranes and films. Besides, the morphological and chemical features of the scaffolds, and their potential in-vitro cytotoxicity on cells was also investigated.

Chemicals to be used for decellularization vary according to the target tissue or organ type and its structural form. While acid-alkaline solutions are frequently used in decellularizing tissues in the form of thin membranes, it is seen that detergent-enzymatic approaches come to the fore in total organ decellularization [8]. In liver-specific decellularization studies, in addition to perfusion approaches, studies with sliced tissues draw attention [9, 10].

In the first stage of the study, in order to incorporate the active ECM components into the hybrid biomaterial to be developed in further steps, bovine liver tissue was decellularized and efficiency of the process was investigated. It was observed that the structural integrity of lyophilized natural liver tissue was disrupted and turned into a white fragile form with the effect of the decellularization protocol (Fig. 2A-C). As a matter of fact, SDS, which is an ionic and amp-

hipathic detergent, has a significant effect on ECM integrity while removing cells from tissues and organs. Thanks to these properties, SDS is frequently used in solid organ and complex tissue decellularization studies, such as for kidney, liver and cornea [11,12]. Indeed, in this study, it was determined that after decellularization of liver tissue pieces by treating in 0.1% SDS solution for 48 hours, the amount of DNA can be removed by 80% compared to the natural liver (Fig. 2D). Beside DNA, sGAG, which is one of the basic components of the ECM, plays a critical role in the regulation of cell behavior such as migration and differentiation. With these features, it is frequently included in decellularized ECM based studies [13]. The results demonstrated that the sGAG levels were significantly maintained in decellularized ECM following decellularization.

Scaffolds to be used in tissue engineering are expected to mimic the target tissue ECM structure and functions. Thus, a three-dimensional porous and nanofibrous scaffold mimicking the natural ECM architecture can be formed by electrospinning using natural or synthetic polymers. Poly (ε-caprolactone) (PCL) draws attention in 3D tissue scaffold production, with its suitable biodegradation and biocompatibility properties [14]. PCL in various concentrations can be solubilized in solvents, such as in chloroform, methanol, DMF, THF, or formic acid, and this polymer can be used for the production of nanofibers in the size range from micron to sub-nanometers [15]. In addition to the electrospun nanofibrous form, the sheet form of PCL can also be used in a number of tissue engineering applications [16, 17]. On the other hand, although scaffolds with appropriate architecture can be formed by using PCL or other synthetic polymers, the biofunctional active content of natural ECM cannot be mimicked [18]. Alternatively, coating or grafting of ECM proteins or attaching short peptide sequences to the biomaterial surface are other efforts in order to find an alternative solution to this limitation [19,20,21]. Considering the complex structure of ECM, it does not seem possible to combine all natural bioactive factors in bioscaffold with these traditional methods.

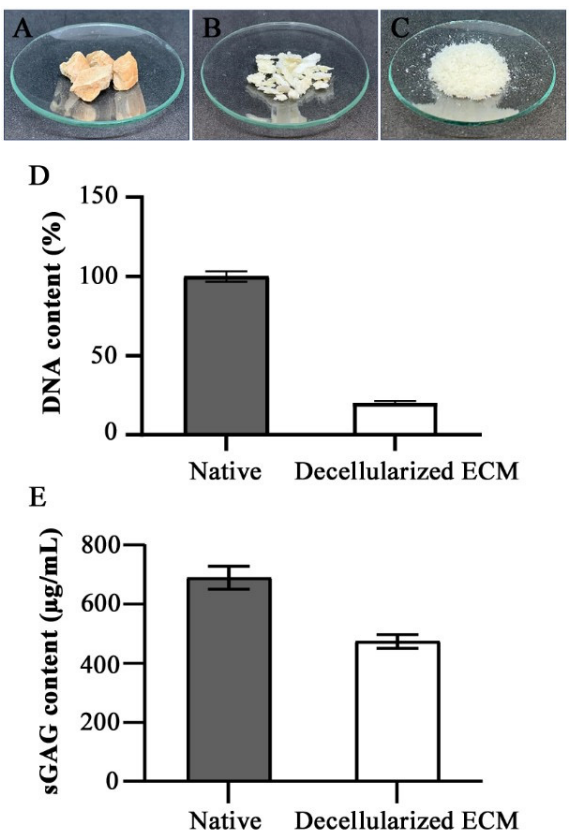

Figure 2. Schematic illustration of the experimental design. (A) Isolation of liver ECM and preparation of ECM/PCL composite solution, (B) the electrospinning process.

In our current study, liver ECM was decellularized and prepared in powder form (Fig. 2C). Then, it was used as the bioactive component for the preparation of the biocomposite PCL scaffold. SEM analysis of the electropsun constructs showed that they had a fibrous structure with varying fiber sizes. While 14% PCL-based constructs showed a homogeneous nanofiber distribution, it was observed that the homogeneous fiber formation was disrupted by the incorporated ECM in the composite group (Fig. 3A-C). Results also showed that the fiber diameters of PCL membranes without ECM were 250-750 nm (Fig. 3A). On the other hand, fibers with a size of about 3-5 µm and various bead-like structures were observed in different regions following the incorporation of ECM particles into the scaffolds. In addition, homogeneous bead-like structures were visible in the membranes containing 0.1% ECM (PCL/0.1% ECM). By the increase in ECM ratio (PCL/1% ECM), the fiber integrity deteriorated, and significant amount of particles could be observed (Fig. 3B and 3C). Similarly, PCL (PCL-F) prepared in the film form had a homogeneous surface morphology. However, it was observed that the films containing decellularized ECM had a rougher surface topography compared to that of the pure PCL films (Fig. 3D-F).

The chemical properties of the morphologically characterized biocomposite nanofiber and film constructs were evaluated by FTIR analysis. Although there are a limited number of studies on FTIR analysis of decellularized native tissues, it basically gives some clear clues regarding the basic composition of ECM. PCL basically has characteristic peaks in wavelength ranges of 1150-1250 cm^{-1} (COC, -COC-), 1250-1300 cm^{-1} (CC) 1700-1750 cm^{-1} (CH_2) and 2800-3000 cm^{-1} (CH_2) [22,23].

In addition, although the liver tissue ECM has characteristic peaks related to the protein, polysaccharide and lipid components within the structure, some of these peaks are

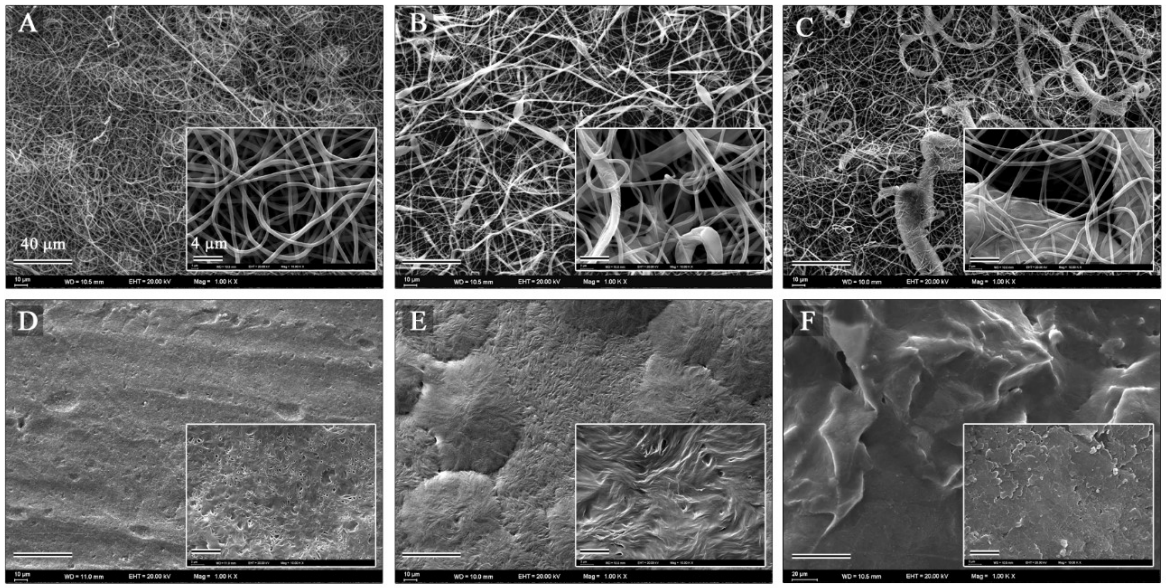

Figure 3. SEM micrographs of the composite nanofibers and films. (A) PCL-N, (B) PCL-N/0.1% ECM/PCL, (C) PCL-N/1% ECM, (D) PCL-F, (E) PCL-F/0.1% ECM/PCL, (F) PCL-F/1% ECM.

overlapping with PCL. Similar to PCL, –CH (2800-3000 cm^{-1}) tensions and bends (1300-1500 cm^{-1}) caused by the polysaccharides are also seen in the liver ECM group. It has flat peaks in the range of 3100-3600 cm^{-1} depending on the protein and water composition. On the other hand, unlike PCL, characteristic peaks of Amide I, II and III bonds are expected to be observed in the wavelength range of 1400-1700 cm^{-1} [24,25].

The findings revealed that PCL-based specimens both in the form of nanofiber and film have characteristic peaks, whereas lower intensity peaks were observed in the film membranes due to its permeability compared to fibrous membranes (Fig. 4A). In the composite scaffolds containing decellularized ECM, peaks of Amide I, II, III bonds in the range of 1500-1700 cm^{-1} were observed similar to the results of the control liver ECM (Fig. 4B-4C). These characteristic peaks, which are particularly stronger in the nanofiber formation, are the indication of the presence of ECM in the developed composite bioscaffolds.

It is critical to evaluate the biocompatibility of biomaterials to be developed for tissue engineering applications. In this framework, different biocompatibility testing approaches, such as extract dilution, direct contact, or indirect contact testings are recommended within the scope of the ISO 10993-5 guide [26,27]. The indirect in-vitro cytotoxicity test findings of the biocomposite scaffolds are presented in Fig. 5. The results showed that both the composite scaffolds, in film or nanofibrous membrane form containing different concentrations of ECM, do not have a toxic effect on the viability of cells following 48 hours of culture. The formation of MTT formazan crystals, demonstrating the metabolic activity of viable cells, was determined bothe by invert microscopy and by quantitative spectrophotometric measurements (Fig 5A-B). On the other hand, 90% of cell viability was lost in the positive control group (medium containing phenol). These findings support the potential for the use of developed biomaterials in future in-vitro and in-vivo applications.

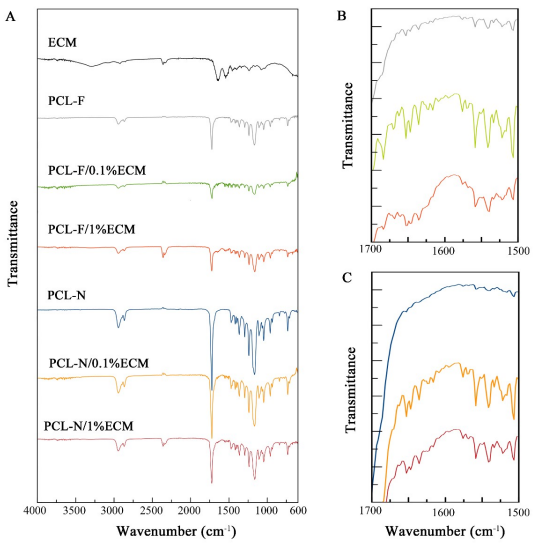

Figure 4. FTIR results of ECM/PCL composite nanofibers. (A) All groupus, (B) PCL-F, PCL-F/0.1%ECM, PCL-F/1%ECM, (C) PCL-N, PCL-N/0.1%ECM, PCL-N1%ECM.

CONCLUSION

In this study, composite scaffolds in both nanofibrous membrane and film forms could be developed using decellularized bovine ECM and synthetic PCL polymer. The presence of ECM in the biocomposite scaffolds was determined by chemical and morphological analy-

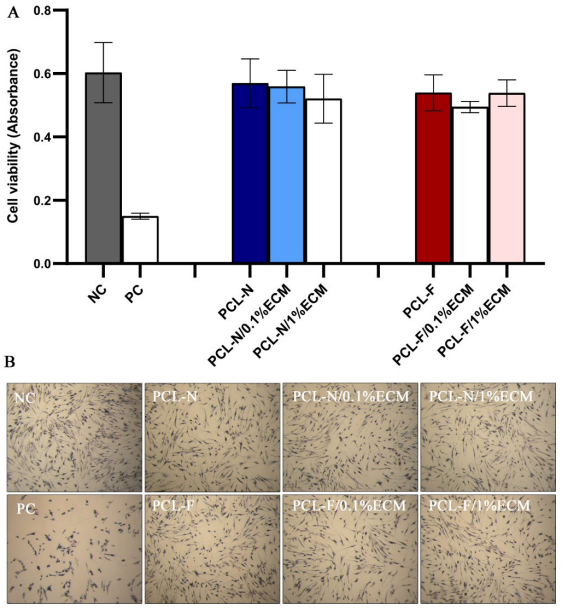

Figure 5. Indirect in-vitro cytotoxicity findings. (A) Spectrophotometric MTT findings. (B) Phase-contrast micrographs demonstrating the formation of formazan crystals on cell cultures.

ses. Subsequently, the potential cytotoxic effects of these scaffolds on human adipose mesenchymal stem cells were examined by in-vitro studies and it was found that biocompatible scaffolds could be developed. It is thought that the scaffolds in different forms containing liver ECM have the potential to be used in advanced liver tissue engineering studies.

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CONFLICT OF INTEREST

Authors approve that to the best of their knowledge, there is not any conflict of interest or common interest with an institution/organization or a person that may affect the review process of the paper.

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