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ORIGINAL RESEARCH REPORT

Evaluation of 3D PLLA scaffolds coated with nano-thick collagen as carrier for hepatocytes

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Abstract
Orthotopic liver transplantation is presently the most effectual method for the treatment of end-stage liver diseases. Though, one major issue is the restricted number of donor organs that are accessible. Hence, liver tissue engineering is under investigation with the goal of restoring liver functions. In this study, we investigated 3D porous scaffolds made of PLLA coated with a nano thick collagen layer (matrices). Primary rat dermal fibroblasts were used in a first study phase to check matrices' cytocompatibility. More than 70% of seeded cells could adhere and remain viable 24 and 48 hours after the seeding. To test the suitability of the matrices for human primary hepatocytes, HepaRG cells were seeded and analyzed for viability, adhesion rate, and functionality such as albumin secretion. About 80% of seeded HepaRG adhered to the scaffolds remaining viable up to 72 hours. Cells were homogeneously distributed in the entire scaffold with albumin secretion increasing with time. Our results indicate that PLLA collagen-coated matrices allow hepatocytes attachment and distribution throughout the 3D structure, as well as support cell functionality. Such matrices have been applied in our clinical phase II trial. Functional hepatocytes were successfully implanted in patients suffering from liver-cirrhosis with higher cell numbers and adhesions rate compared to our previous trial with the first matrix type and a general improvement in clinical condition.

KEYWORDS
3D PLLA scaffolds, hepatocyte, nano-thick collagen coating, tissue engineering

1 | INTRODUCTION

Chronic end-stage liver disease is related to high mortality rate due to loss of liver functions. This medical condition causes about 1 million deaths per year globally. The current standard treatment for end-stage liver cirrhosis and liver insufficiency is orthotopic liver transplantation.[1] Unfortunately, the demand for liver donor organs frequently, if not always, surpasses the actual number of donor organs available.[2] Living related transplantation, split liver transplantations, and new methods of non-heart-beating donors do not solve the problem; and the attempts to build extracorporeal devices to support the insufficient liver metabolism have so far failed; therefore, the demand for additional therapeutic options remains high.[3-6]

Implantation of functional cells, including hepatocytes and hepatic lineage stem or progenitor cells may provide a substitute cell-based therapy for the treatment of hepatic failure. A very topical debate remains whether hepatocytes directly infused into the veins will implant and survive at various locations in the body or need an extracellular support in order to remain in situ and to be functionally active. Many studies show the functional failure of scaffold free implantation through the bloodstream.[7,8] Therefore, the need to find a scaffold that can support adhesion and growth of cells in situ mimicking native tissue is mandatory.[9]

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Williams and co-workers found that at the beginning of tissue engineering, the selection of the support material was primarily based on regulatory approval for use in human patients.[10] Synthetic polymers (e.g., polylactide-co-glycolide, polyethylene glycol, and polycaprolactone) were initially used for engineering biological tissues.[11] Unfortunately, although these materials presented good mechanical strength were not designed to support cell attachment, growth, and functionality. Therefore, the development of functional scaffolds, also named templates, able to mimic the environment of cells or naturally occurring extracellular matrix (ECM) tissues[10] was fundamental for liver regeneration.

Various methods of producing scaffolds are known, among others 3D–Printing,[12] electrospinning,[13,14] and salt leaching techniques.[15,16] The salt leaching method has been very commonly used in the manufacture of scaffolds for tissue engineering applications,[17] which can be used to achieve tunable mechanical properties, porosity, and pore size that can be effectively controlled.[18]

In this report, we conducted in-vitro studies based on previous clinical studies' results. In a clinical phase I trial, we implanted up to 30 million freshly isolated autologous human hepatocytes on Poly-L-lactic acid (PLLA) matrices in liver-injured patients confirming the expected functional result of hepatic cell matrix implantation at the small bowel mesentery.[19] Numerous reports of animal investigations have proven the good tolerance of such polymers implanted between the mesenteries of the small bowel.[20] Histological analyses showed the survival of hepatocytes and co-seeded islet cells for up to 1 year and the ingrowth of capillaries into the implants after 3 weeks. In our phase I clinical trial with such a PLLA implants (called type I) in liver-injured patients, we have proven the clinical feasibility and safety of the scaffold. A phase I clinical study is inherently a feasibility study that cannot prove the efficacy of the method. Schwarz and colleagues published a similar study with over 50 patients using the same type I of PLLA scaffolds.[21] We observed clinically relevant improvements in a number of clinical and laboratory indicators of liver function such as albumin and cholinesterase for some patients.[19] The results were encouraging but showed the many limitations of our type I scaffolds. One of the characteristics of the PLLA base material is its hydrophobicity. To achieve cell adhesion, it is mandatory to coat the scaffold structures with collagen. Collagen as one component of the ECM, facilitates the adhesion, signaling, and growth of epithelial and mesenchymal cells. Collagen has been successfully used before for surface modification of PLLA scaffold to improve wettability and cell adhesion.[22] Collagen as a fundamental component of ECM improves cell adhesion and interaction as well as the 3D architectural scaffold structure for optimal cells functionality. However, when the scaffolds were coated with collagen, low cell adhesion and viability were observed. It was also noticed that in some matrices, the watery cell solutions showed some bubbles on the surface of the scaffold instead of coating the entire matrix. We could only implant 30 million of hepatocytes. Therefore, before initiating the necessary phase II clinical trial, we focused our attention on the improvement of poly (lactic acid) (PLA) and poly (glycolic acid) (PGA) scaffolds to create a more functional hepatocyte matrix implant type II (HMI). Fortunately, we found a PLLA/collagen scaffold published by Chen.[22,23] He had developed a method to cover all walls of the scaffold structures with a nano-thick collagen layer. The area covered by collagen with this method is superior to the coating method of the type I scaffold. In fact, only the basic structure of the scaffold of PLLA is guaranteeing stability. All surfaces are covered by collagen that serves as a natural surface for the hepatocytes and other seeded cells to adhere. The cells are not in direct contact with the PLLA. We planned to use this type II scaffold, with some minor modifications in our further clinical phase II trial. For safety reasons, we had to test the new type II scaffold in a preclinical study, to show improvements of attachment rates and growth of the implanted cells.

In this study, we evaluated the performance of 3D PLLA scaffolds coated with nano-thick collagen in-vitro as HepaRG cell carrier and later, in-vivo as HMI in our phase II trial.

2 | MATERIALS AND METHODS

2.1 | Scaffold fabrication of wall coated matrices (0.5% and 1% collagen)

The PLLA scaffolds were prepared by the salt-leaching method using sieved sodium chloride (NaCl) particles as porogen.[23,24] PLLA was purchased from Sigma (St. Louis, MO). The PLLA was dissolved in chloroform to prepare 20% (wt/vol) solution. NaCl particles (9 g) of diameter ranging from 355 to 425 μm, were sieved and mixed with the PLLA solution in chloroform (5 ml). The NaCl/PLLA mixture was put into an aluminum pan (40 mm diameter) and air-dried in a fume hood at room temperature for more than 24 hours (to evaporate the chloroform), and placed in a vacuum drying service (Yamato, Tokyo, Japan) at –0.1 mPa for another 3 days. The dried NaCl/PLLA block was soaked in 200 ml deionized water to leach out the NaCl particles and the water was changed every hour for 20 times with a total soaking time of 48 hours. After air-drying of the washed scaffolds, PLLA scaffolds with porosity around 90% were obtained as per method of Sugiyama et al[22] The scaffolds were cut into disks of 1 cm diameter and 2 mm thickness for further experiments.

2.2 | Surface coating with collagen

The PLLA scaffolds were immersed in the 0.5% (wt/vol) and 1% (wt/vol) type I porcine collagen solution (Wako, Osaka, Japan) in the ratio of 2% (wt/vol).[22,23] Matrices were directly de-aerated under a reduced pressure (20 Pa) with a vacuum freeze dryer FDU-2200 (EYELA, Tokyo, Japan), in order to fill the scaffold pores with collagen solution throughout the whole scaffold. The collagen/PLLA constructs were placed onto 100 μm cell-strainer membranes (Falcon, Durham) and centrifuged at 2000g for 10 minutes at 4°C to remove excess collagen solution from the pores. After centrifugation, very thin layers of collagen solution were left on the pore surfaces. To determine the amount of collagen solution that remained in the PLLA scaffolds, the

dried uncoated scaffolds were weighed before immersion in the collagen solution and the wet collagen-coated scaffolds were weighed after centrifugation. The differences between the two weights indicated the amount of collagen solution that was left in the PLLA scaffolds. The collagen-coated scaffold disks were frozen at −80°C for a minimum of 4 hours and later freeze-dried under vacuum (<5 Pa for more than 24 hours) to freeze-dry the thin coated collagen layers on the scaffold pore surfaces.

2.3 | Plasma surface treatment and sterilization

PLLA collagen-coated disks were subsequently treated with plasma for surface cleaning and activation with O_2 for 10 minutes (custom-made plasma treatment system, Diener electronic GmbH, Ebhausen, Germany). The matrices were then sealed in Tyvek bag, and sterilized in the Diener machine with 30% H_2O_2 solution at 30°C for 12 hours. Samples were then transferred into 12-well plates and stored under vacuum at 4°C until use.

2.4 | Scanning electron microscopy

To analyze disks morphology, collagen distribution into the pores as well as the interaction between the scaffold and the cells, the matrices were processed for scanning electron microscopy analysis. Three samples per scaffold type were washed in phosphate-buffered saline (PBS) (Sigma), and fixed in 3% glutaraldehyde (Sigma) in PBS for 2 hours at 4°C. Thereafter, they were washed in PBS and subsequently in water, and dehydrated at room temperature in increasing concentrations of ethanol (Thommen Furler, Bueren, Switzerland) up to 100%. Samples were chemically dried with hexamethyldisilazane (HMDS) (Sigma), for 24 hours at room temperature in a fume hood and coated with gold particles using the Quorum Q150R S sputter device (Gala Instrument GmbH, Bad Schwalbach, Switzerland). Each sample was then observed under a Phenom scanning electron microscope (Schaefer-Tec AG, Langen, Germany) and the images were obtained at 5 kV accelerating voltage.

2.5 | Cytocompatibility tests

In order to test the scaffolds cytocompatibility, the PLLA collagen-coated matrices were seeded with primary dermal fibroblasts from a rat and cells viability was analyzed. One male Sprague Dawley Rat (National Agency of Drug and Food Control, Jakarta), 12 weeks old, was used as the donor for fibroblasts. The fibroblasts were isolated from the rat's skin. All surgical procedures were conducted according to the protocols approved by the Tarumanagara University Institutional Animal Care and Use Committee (IACUC). IACUC approval number 003.KEPH/UPPM/FK/VI/2019. Surgical procedures were performed after intraperitoneal anesthesia (ketamine 10%, 40–80 mg/kg Body Weight [BW], and xylazil 2%, 5–10 mg/kg BW). Under aseptic conditions, the abdominal skin was dissected and removed, washed five times with PBS (Gibco, Grand Island, NY) containing 2% antibiotic-antimycotic (Sigma) and five times with Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) solution (Sigma) containing 2% antibiotic-antimycotic. The biopsy was then digested for 5 hours with 30 ml of 200 U/ml collagenase type I (Gibco) solution in a ratio of 5% (wt/vol) at 37°C, 5% CO_2 and 95% relative humidity. The dermis side of the skin was gently scrapped with cell scraper. The cells were sieved (1 mm and 100 μm mesh size), washed twice, and suspended in Dulbecco's Modified Eagle's medium (DMEM) medium (Gibco) completed with 20% fetal bovine serum (FBS) (Gibco), 2 mM L-Glutamine (Sigma), 1 mM Sodium pyruvate (Sigma), 10 mM (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) (HEPES) (Sigma), and 2% antibiotic-antimycotic solution (Sigma) (fibroblasts proliferation medium, FPM). The fibroblasts were cultured and sub-cultured in complete FPM (doubling time of 16 hours) and finally seeded onto PLLA collagen-coated matrices (three matrices/type) at the density of 500,000 cells/sample. As an internal control, the same number of cells (500,000 fibroblasts) was seeded directly into wells pre-coated with 0.5% (wt/vol) type I porcine collagen solution (Wako, Osaka, Japan). Cell-seeded scaffolds and blanks (matrices without cells) were cultivated in FPM at 37°C, 5% CO_2 and 95% relative humidity, 24 and 48 hours after the seeding. Fibroblasts not attached to the matrices were collected from the media and counted. The experimental internal control, cells seeded directly in the wells (2D culture samples). The disks and the experimental internal control on the wells were analyzed for cell viability/adhesion rate with Cell Counting Kit-8 viability assay(CCK-8) (Sigma), based on the reduction of a tetrazolium salt into soluble formazan exclusively inside viable cells. For analysis, culture medium was replaced with CCK-8 reagent diluted 1:10 in FPM medium and samples were incubated for 4 hours at 37°C and 5% CO_2. Medium absorption was measured in the Multiskan reader at 450 nm wavelength (Multiskan Ex, Thermo Scientific). Results were expressed as fold change relative to control percent adhesion (cells adhered on the wells, 100%).

2.6 | Cell culture and seeding of hepatocytes on the scaffolds

After assuring the cytocompatibility of scaffolds with rat's primary fibroblasts, the next step was to test their suitability for the human hepatocytes. To achieve the goal, the human hepatic progenitor cell line "HepaRG cells" was used (Thermo Fisher Scientific, Life Technologies, Carlsbad, CA). Cells were handled following the manufacturer's guidelines. HepaRG were thawed in Thaw, Plate, & General Purpose Medium (TPG medium, Thermo Fisher Scientific) and counted to determine their viability with the Trypan blue exclusion method. Cell suspensions with viability higher than 90% were used for the following studies. HepaRG were seeded at the density of 500,000 cells per disk (150 μl cell suspension per scaffold, pre-wetted, and not pre-wetted with TPG medium) and samples were incubated for 1 hour at

37°C, with 5% CO_2 and 95% relative humidity before adding 1 ml TPG medium per well. 24 hours after the seeding, the samples were transferred in new wells and cultivated for 24 and 48 hours in Maintenance/Metabolism Working Medium (MMW medium, Thermo Fisher Scientific). Samples were analyzed for cell morphology and distribution (SEM and MTT assay), viability and adhesion rate (MTT and Presto Blue assay), and albumin secretion (ELISA) at the following time points: 48 hours (24 hours in TPG + 24 hours in MMW medium) and 72 hours (24 hours in TPG + 48 hours in MMW medium). As blanks, matrices without cells were cultivated in the same experimental conditions mentioned above. To assure HepaRG quality, cells were seeded directly onto scaffolds coated with collagen type I to analyze their capability to adhere and to observe their morphology (internal control).

2.7 | Viability tests

2.7.1 | MTT assay

Forty eight and 72 hours after seeding of scaffolds, the samples were harvested and the medium was replaced with 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) in PBS. The samples were incubated for 2 hours at 37°C and 5% CO_2. Only viable cells are able to reduce the MTT to insoluble formazan salts that stain the cells blue, revealing their viability and distribution in the collagen-coated PLLA matrices. Samples were transferred in PBS for light microscopy analysis (Olympus 1X81 microscope equipped with the Olympus DP72 camera; Olympus, Tokyo, Japan).

2.7.2 | Presto Blue assay

The HepaRG-seeded samples, as well as blanks, were analyzed for cell viability with the Presto Blue assay (Life Technologies), 48 and 72 hours after the seeding. The Presto Blue assay is based on a resazurin solution containing a non-fluorescent, cell permeable compound that turns fluorescent after being metabolized inside the cell. Only viable cells reduce the compound into the highly fluorescent resorufin. For analysis, the culture medium was replaced with Presto Blue reagent diluted in 1:10 ratio in MMW medium. The samples were incubated for 45 minutes at 37°C and 5% CO_2, and media fluorescence was measured in the FLUO star OPTIMA reader at 550 nm ± 10 nm excitation wavelength and 590 nm ± 10 nm emission wavelength (FLUO star OPTIMA; BMG Labtech, Ortenberg, Germany). The number of cells was extrapolated from standard curves that were set up with HepaRG cells seeded at defined densities and cultivated up to 48 and 72 hours. The adhesion rate was calculated as the percentage of cells adhered versus the initial amount of seeded cells (500,000 cells per disk).

2.8 | Albumin secretion

Albumin production was determined with enzyme-linked immunosorbent assay (ELISA) assay (Abcam, Cambridge, UK) to evaluate the HepaRG functionality. Supernatants were harvested at 48 and 72 hours of culture and centrifuged at room temperature for 10 minutes at 3000*g*, before their analysis, in order to remove probable debris. Albumin amount was normalized with the number of HepaRG cells populating the same matrices.

2.9 | Feasibility of implementation to clinical trial phase II

The aim of this phase II clinical trial was to prove the efficacy of this autologous cell transplantation technique more definitively.

The trial was conducted as previously described.[19] The protocol was approved by the Health Research Ethics Committee, National Institute of Health Research and Development, Republic of Indonesia (No: LB.02.01/2/KE.064/2019). Eligible patients were older than 18 years and had a clinical diagnosis of liver cirrhosis with Child-Turcotte-Pugh (CTP) score of ≤10 and Model of End-stage Liver Disease (MELD) score of ≤15. Patients having active hepatitis, organ failure (other than liver failure) were excluded from the study. The patients underwent the HMI procedure at Gading Pluit Hospital, Jakarta, Indonesia. The patient group comprised of five males, between 36 and 63 years of age, with an average age of 47.8.

In brief, the procedure included harvesting hepatocytes and islets of Langerhans cells from a patient's own tissues in a first operation. The tissues were processed into single cells in the laboratory. The 3D PLLA collagen matrices were seeded with cells and later implanted into the patient's small bowel mesentery.[19]

In phase I clinical trial, cells were seeded onto 20 mm × 4 mm PLA scaffolds (Phrontier SARL, 2 rue Saint Clair, 76,490 Caudebec en Caux, France) pre-coated with 0.1% type I bovine collagen solution (Sigma) by pipetting the collagen solution (2 ml) onto the matrix. To prevent the matrix from floating on the collagen solution, metal ring was placed on the top of each matrix. After 1 hour, the matrix was transferred in a new well-plate and air-dried for more than 5 hours.

In the phase II clinical trial, we applied our new refined scaffolds, fabricated according to the methods mentioned above, with a diameter of 20 mm and a thickness of 2 mm and covered with 1% (wt/vol) bovine atelocollagen (Koken, Yamagata, Japan). The collagen was switched from porcine to bovine ateocollagen in phase II trial as directed by the Health Research Ethics Committee, considering Indonesia as a Muslim majority country. A very thin collagen layer was produced by centrifugation and freeze-drying. Cell suspensions (the mixture of isolated hepatocytes and islet of Langerhans cells) were co-seeded onto the matrices in 600 μl William E medium (Sigma) completed with 10% autologous patient serum. Engineered cell-scaffolds were cultivated in 1 ml medium for around 63 hours, before being transported to the hospital for implantation. The direct calculation of

adhered cells was not possible so it was calculated by the following formula:

Number of placed cells on the matrices – number of cell remaining in the wells = number of adherent cells on the matrix (transplanted cell number).

2.10 | Data analysis and statistical methods

Results are expressed as means ± SD. The significance between two independent groups was determined by independent Student's *t* test. For multiple comparisons, One-Way Analysis of Variance (ANOVA) was used. Values were considered statistically significant at $p < .05$.

3 | RESULTS

3.1 | Scaffold characterization by SEM

The PLLA collagen-coated matrices fabricated for the study had a diameter of 1 cm and a thickness of 2 mm (Figure 1A). All the pores were covered with a thin layer of collagen (Figure 1B). No relevant difference could be detected with SEM between 0.5% and 1% collagen sheets (Figure 1C,D). The sponge with a porosity above 90% showed macro- and micro-pores of 200–400 μm and 20–60 μm, respectively (Figure 1D). The amount of the collagen solution that remained in the PLLA scaffolds after centrifugation at 2000*g* was 0.016 ± 0.002 g when 0.5% collagen solution was used and 0.019 ± 0.002 g when 1% collagen solution was used (Figure 2).

3.2 | Cytocompatibility tests

There are several requirements for implantable biomaterials in human with special considerations of safety and accuracy. Primary rat dermal fibroblasts, widely used for preliminary cellular cytotoxicity studies, were seeded onto PLLA collagen scaffolds to test their potential as future implants. During the seeding process, the cell suspension immediately and easily infiltrated into the collagen-coated PLLA matrices without any leakage. The increase in cell number, based on cell viability measurements was found to be, 76.8% ± 0.4% (24 hours after seeding) and 67.8% ± 5.4% (48 hours after seeding) for 0.5% collagen-coated matrices and 70.7% ± 0.14% (24 hours after seeding) and 71.9% ± 3.8% (48 hours after seeding) for 1% collagen-coated

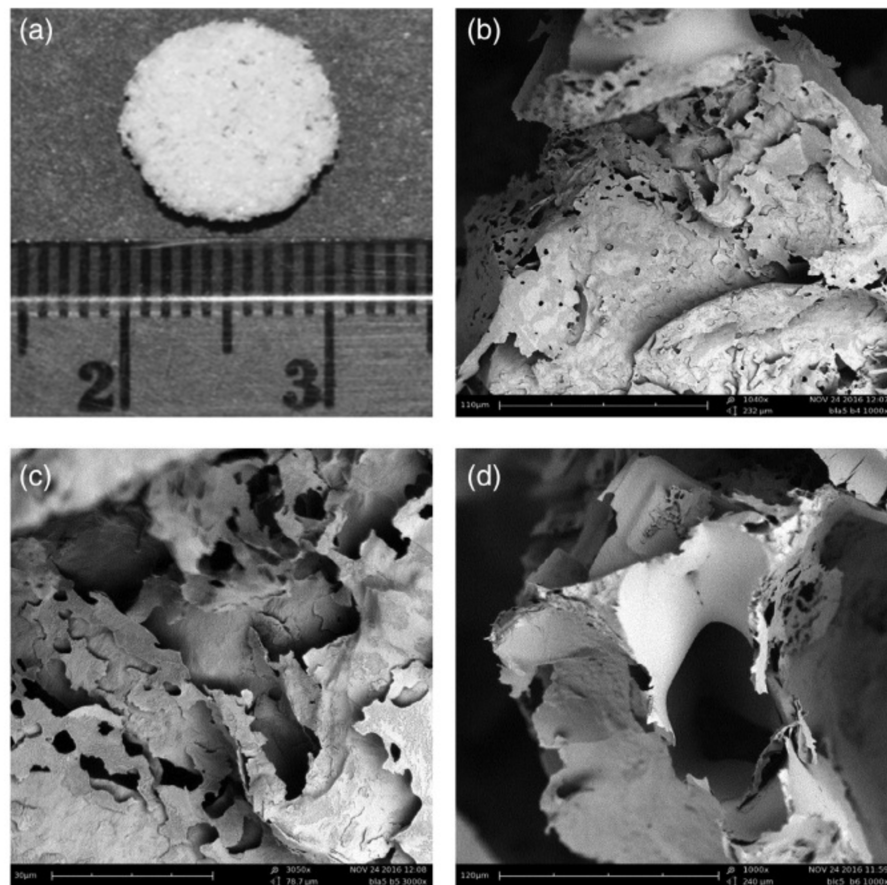

FIGURE 1 (a), Dried PLLA collagen-coated matrix. (b), SEM image of dried PLLA collagen-coated matrix. (c) and (d), SEM images of unseeded scaffolds cultivated in MMW medium up to 72 hours. (c), 0.5% collagen walls coated scaffold; (d), 1% collagen walls coated scaffold

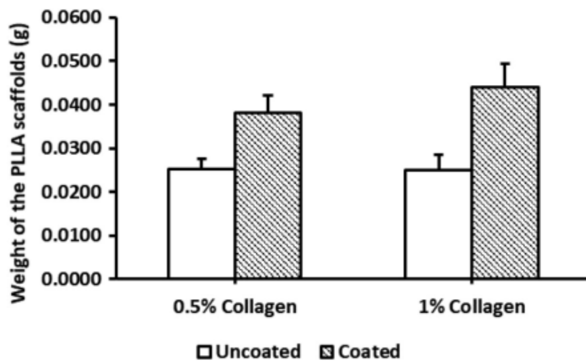

FIGURE 2 Weight comparison of PLLA scaffolds (1 cm diameter, 2 mm thickness) between the uncoated and coated with 0.5% and 1% collagen solution. Data show mean ± standard deviation (SD) (n = 6)

TABLE 1 Percent adhesion folds change in relation to the control (2D cultures)

Samples	Fold change (%) (mean ± SD) 24 h	48 h
0.5% PLLA collagen-coated scaffold	76.8 ± 0.44	67.8 ± 5.4
1% PLLA collagen-coated scaffold	70.7 ± 0.14	71.9 ± 3.8

Note: Samples were analyzed with Cell Counting Kit-8 assay 24 and 48 h after the seeding (n = 3).

matrices, showing no significant influence of collagen concentration on fibroblasts adhesion (Table 1).

3.3 | Cell viability and adhesion rate

The pre-wetting of the matrices with medium, before cell seeding, reduced the scaffolds' suitability for cell attachment. Only 21.02% ± 2.85% and 22.89% ± 3.93% of the initial cell seeded amount (500,000 cells/disk) populated the scaffolds (1% collagen walls coated scaffolds), respectively, 48 and 72 hours after cell seeding. The use of dried matrices improved the adhesion rate up to 80.26% ± 6.89%. The swelling induction of collagen directly with cell suspension increase of four-fold the HepaRG incorporation into the matrices (two independent experiments, n = 3; Figure 3). No significant difference could be detected between the two different collagen densities scaffolds. Moreover, a slight decrease of cells viability characterized the matrices cultivated for 72 vs 48 hours.

3.4 | Cells morphology and distribution

The morphology of HepaRG into the matrices was observed after samples were processed for SEM analysis. Matrices were cultivated for 48 and 72 hours before to be harvested and processed for SEM. In accordance with the seeding method, HepaRG cells were completely incorporated in the collagen sheets and presented as

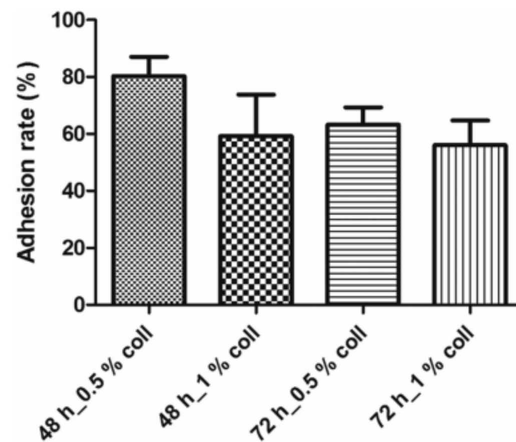

FIGURE 3 Percentage of adhered HepaRG cells in the PLLA matrices enriched with 0.5% or 1% collagen after 48 and 72 hours. Samples were analyzed with Presto Blue assay. Data show mean ± SD for triplicate samples of two independent experiments

round and as spread out morphology (Figure 4). Isolated cells presenting focal adhesion as well as cellular agglomerates populated the entire PLLA collagen coated scaffolds independently from collagen concentration (Figure 4).

In order to observe and analyze cell distribution inside the disks, an MTT assay was performed after 48 and 72 hours of culture to stain the viable cells. As shown in Figure 5, HepaRG remained viable up to 72 hours, in accordance with Presto Blue data (Figure 3), and were homogenously distributed in the entire scaffold (Figure 5).

3.5 | Cells functionality

As an additional assessment of cell viability, cells' functionality was analyzed by measuring the levels of secreted albumin. Albumin, a marker of mature and active hepatocytes, as a general indicator of protein production capacity, was produced from the first 2 days of culture and increased with the time showing higher protein secretion at longer incubation time (72 vs 48 hours). The albumin amount was correlated with the cell number of the same matrices and as shown in Figure 6, HepaRG cultivated in the manufactured scaffolds could reach albumin values up to 1,845.90 ± 157.84 ng/10^6 cells. (1% collagen wall coated matrices, 72 hours).

3.6 | Clinical phase II trial (preliminary data)

We assessed the efficacy of implanted scaffold cultured hepatocytes on liver cirrhosis patients. We implanted a large, viable population of autologous hepatocytes, up to 140 million, almost five times more than in our phase-I trial. In phase II trial, 600 μl cell suspension was seeded per scaffold. The cell suspension immediately and easily infiltrated into the disk without any leakage, while in phase I trial, only

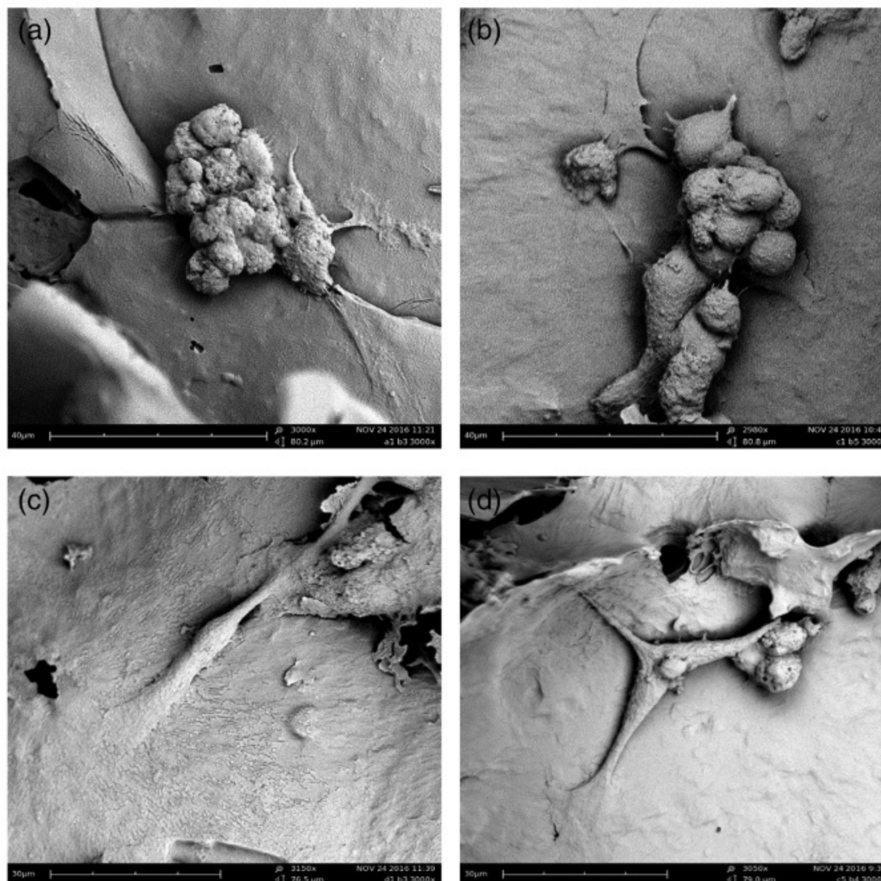

FIGURE 4 SEM images of HepaRG cells 48 hours (a, b) and 72 hours (c,d) post seeding in PLLA collagen coated scaffolds with 0.5% (a, c) and 1% (b, d) collagen coating. Scale bar: 40 μm (a, b), 30 μm (c, d)

250–400 μl cell suspension could be accommodated per disk and about 60% of the scaffolds were hydrophobic, inhibiting the cell suspension infiltration and hindering cell seeding.

The mean adhesion rates of hepatocytes onto the matrices showed a higher trend in phase II trial compared to the phase I trial[19] (phase I: 72.09% ± 10.69% vs phase II: 77.08% ± 10.56%) (Table 2).

4 | DISCUSSION

Basically, there are two methods to implant isolated cells into the body: via infusion into the veins or by seeding on carriers that are placed somewhere in the body. The first method has the disadvantage that isolated cells, such as hepatocytes, do not survive in the bloodstream in sufficient large number and do not attach to the recipient tissue. The second method requires porous 3D scaffolds that are biocompatible. They have shortcomings in the scaffold properties, unless they are templates offering the cells ease of attachment, signaling possibilities, potential growth, and survival.

Hepatocytes are isolated as single cells by mechanical and enzymatic methods, and let adhere and grow on these specific supports-scaffolds, before they are implanted in the liver-injured patients. During isolation, hepatocytes are deprived of their original micro- and macro-environment,[25] therefore, to avoid losing their specific functions they must be anchored and cultured under appropriate conditions[26]; they have to be cultured on an ECM component, such as collagen[26] or commonly used Matri-gel.

However, the use of scaffolds with good mechanical stability is fundamental for an optimal scaffold implantation process, a property that does not characterize such hydrogels. For many years, scaffolds made of degradable synthetic polymers have been widely used because of their biodegradable, biocompatible, and good mechanical strength features. Among them are polyesters, such as PLA and PGA.[17] Török et al could demonstrate that the cultivation of hepatocytes on PLLA matrices, lead to the formation of hepatocyte microtissues, thereby increasing the number of viable cells on the scaffold significantly.[11,22] However, these polymers have disadvantages, the scaffolds surface of these polymers is relatively hydrophobic, which obstructs cell seeding process, therefore, this simple type of scaffolds needs to be functionalized and improved.

The solution of a hybrid scaffold of poly lactide and collagen offered many advantages, combining positive features of both material types, naturally derived polymers (collagen) and synthetic polymers (PLLA). Hybrid scaffolds facilitated cell adhesion and growth.[27]

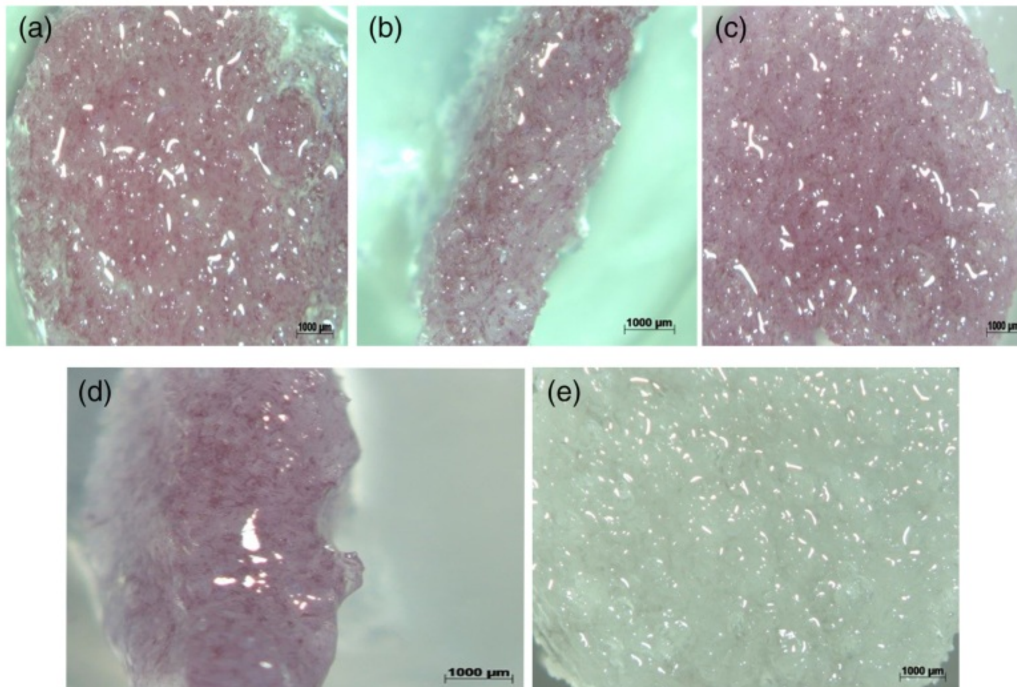

FIGURE 5 HepaRG cells viability and distribution was analyzed with MTT staining 48 hours (a, b) and 72 hours (c, d) after the seeding in PLLA scaffolds enriched with 0.5% (a, b) and 1% (c, d) collagen density. (e) 1% collagen walls coated PLLA matrix cultivated for 3 days. Scale bar: 1000 μm

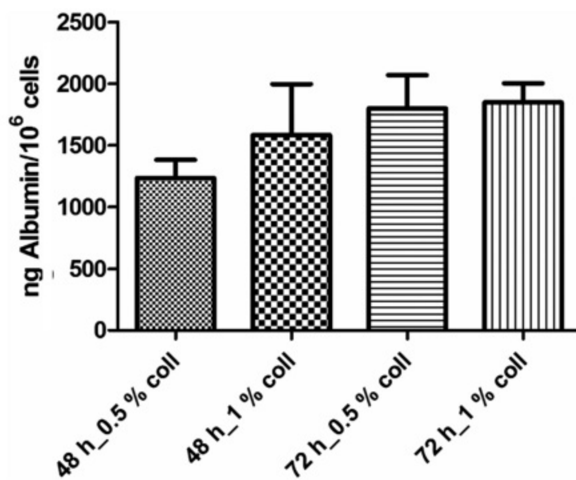

FIGURE 6 Albumin amount produced from HepaRG cells in PLLA collagen coated scaffolds after 48 and 72 hours of culture. The albumin secretion was assessed in the supernatant of the sample with ELISA. The accumulated protein increases with time. Data show mean ± SD for triplicate samples of two independent experiments

One problem of this new hybrid PLLA collagen-coated scaffold was a suitable method for sterilization. Since collagen gets denatured at a temperature above 40°C the sterilization process was difficult to develop as thermal sterilization procedures using high temperatures, would destroy the scaffold surface structure of collagen. Sterilization with gamma rays would also destroy the polymer scaffold and using alcohol is only a decontamination method and not a sterilization method. Hence we developed a low-temperature sterilization process leading to sterile and intact PLLA and collagen scaffolds.

In the present study, we investigated PLLA scaffolds coated in depth with a nano-thick layer of porcine collagen type I. Collagen type I and PLLA are the materials, which are already approved for clinical use. The idea was to use a known skeleton of PLLA for mechanical strength and collagen for ECM environment simulation. The scaffolds' surface was functionalized modifying it with a nano-thick collagen layer according to Chen and colleagues.[23] The overall surface covering collagen manufactures a true template that can facilitate cell seeding and high attachment rates (adhesion rate up to 80%).

Moreover, the scaffolds' surface was further modified with a developed plasma surface activation treatment, which improved scaffolds hydrophilicity by adding O_2 groups to the matrices surfaces.[28] Therefore, the combination of collagen coating and plasma treatment effectively enhanced the performance and surface properties of the matrices. A sterilization technique using a hydrogen peroxide solution at low temperatures was also developed during the present study, to maintain the unaltered collagen structure.

In the present study, 3D PLLA scaffolds were analyzed in a first phase clinical trial with primary rat fibroblasts to assure their cytocompatibility. Not only did the fibroblasts survive, but also could multiply with a fold change of more than 70% for either 0.5% or 1% collagen-coated matrices, showing matrices capability to promote the

TABLE 2 Comparison of cells implanted and adhesion rates between clinical phase-I and phase II trial

	Number of hepatocytes implanted (M ± SD)	Cell density per disk (M ± SD)	Adhesion rates (M ± SD) [%]
Clinical phase-I trial (n = 11)	$26.8 \times 10^6 \pm 15.1 \times 10^6$	$1.5 \times 10^6 \pm 7.6 \times 10^5$	72.09% ± 10.69%
Clinical phase-II trial (n = 5)	$137.8\times 10^6 \pm 102.2 \times 10^6$	$5.2 \times 10^6 \pm 3 \times 10^6$	77.08% ± 10.56%

cell growth. The scaffolds were later analyzed with HepaRG cells, terminally differentiated hepatic cells derived from hepatocellular carcinoma, which represent hepatic progenitor cells in their capacity to differentiate into hepatocytes and cholangiocytes.[29,30]

This cell line combined two important features classifying it as an appropriate tool for scaffold assessment. First, as it is a matter of standardization of test parameters and by using the human cell line the donor to donor variations could be omitted. Second, this cell line was shown to act very similarly with respect to drug metabolism in 3D culture compared to human primary hepatocytes.[31] HepaRG cells have also been used as the biocomponent source for bioartificial livers.[32]

PLLA scaffolds coated with two different collagen concentrations, 0.5% and 1%, did not show any significant difference between both coatings with respect to cell attachment and albumin production, although more cells were detectable after 48 hours on 0.5% collagen -coated matrices. Both types of scaffolds, 0.5% collagen-PLLA matrices and 1% collagen-PLLA matrices, allowed hepatocytes attachment and distribution throughout the 3D structure, with significantly higher adhesion rate values when cells were seeded onto dried scaffolds rather than in pre-wetted ones. Pre-wetted matrices had an adhesion rate of only 21.02% ± 2.85% after 48 hours, while in dried matrices the adhesion rate was up to 80.26% ± 6.89%. After 72 hours of in-vitro culture, the cell number started to decrease. Long-term maintenance of Primary Human Hepatocytes (PHH) in vitro has proven to be challenging. It requires optimum culture condition with special media, supplemented with stimulating factor,[33] certain chemical,[34] or flow culture system.[35] For our clinical application, the cellularized matrices are prepared and transplanted back into the patients no later than 72 hours after the seeding; therefore, we did not alter the conditions of the cell culture. That is why in clinical use the implantation takes place on the third day after harvesting the patients' tissues for cell isolation. On SEM investigation the cubic cells start to migrate on the ECM of collagen showing pedicles. Albumin production increases with time; albumin concentration was higher at longer incubation time (72 vs 48 hours). Therefore, the results indicate that the collagen-coated scaffolds can facilitate the cell adhesion and functionality.

5 | CONCLUSION

The hybrid PLLA collagen-coated scaffolds allowed hepatocytes attachment and distribution throughout the entire 3D structure. Such scaffolds presented high cell adhesion rates and adequate albumin production. This study showed that single-cell hepatocytes, seeded on three-dimensional ECM collagen templates, can be used as HMI acting as artificial mini liver or additional hepatocytes in humans. HMI may serve as a valuable alternative of a hepatocyte transplantation procedure for liver cirrhosis patients. Preliminary results of a phase II clinical trial with the hybrid templates are encouraging with numbers of seeded and attached hepatocytes surpassing a first type of 3D-PLLA scaffold with mean 27 million cells with up to 140 million cells.

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CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In doing so, we confirm that we have followed the regulations of our institutions concerning intellectual property.

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