Development of a Cost-Effective and Simple Protocol for Decellularization and Preservation of Human Amniotic Membrane as a Soft Tissue Replacement and Delivery System for Bone Marrow Stromal Cells

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The aim of this study is to develop a simple andcost-effective method for decellularization and preservation of human amniotic membrane (HAM) as a soft tissue replacement and a delivery system for stem cells. The HAM is decellularized (D) using new chemical and mechanical techniques. The decellularization scaffold is evaluated histologically and fully characterized. The cell adhesion and proliferation on the scaffold are also investigated and the biocompatibility of D tissues is evaluated in vivo. The histological studies reveal that the cells are successfully removed from the D tissue. The DNA extraction shows more than 95% cell removal ($p = 0.001$). The in vitro results indicate that the decellularisation process does not deteriorate the mechanical properties of the tissue, whereas it increases the in vitro biodegradation value ($p < 0.05$). In the D samples, there is no significant cytotoxicity, and no changes are found in the rate of cell proliferation ($p > 0.05$). Immunohistochemistry staining indicates that all the tested components remain unchanged within the D tissues. The count of inflammatory cells show that the decellularization process slightly increases the biocompatibility of the tissue after 7 days post-surgery. The results indicate that scaffold proves to be reproducible, rapid, and cost-effective, with a potential role for clinical application.

1. Introduction

Human amniotic membrane (HAM) is a tough and thin range from 0.2 to 0.5 mm in thickness tissue. This consists of three main layers: 1) An epithelium layer that is composed of a single layer of epithelial cells derived from ectoderm. The apical surface of this layer is bathed in amniotic fluid. These cells are adhered to a basement membrane. 2) A basement membrane layer that endows tensile strength and mechanical property to the HAM. This layer derives from the basement proteins such as collagen types I, III, and IV, laminin, fibronectin, etc. and 3) A connective tissue layer as the thickest layer of the HAM. This layer is composed of two sub layers; the fibroblast layer in which sporadic fibroblasts are settled and surrounded with a loose reticulum network, and a spongy layer that is connected to the underlying chorion membrane.[1–3]

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Possessing many favorable properties, such as inexpensiveness and easy availability makes the HAM a potential biomaterial scaffold for tissue engineering applications, especially for soft tissue engineering. In addition, this material consists of many extracellular matrix (ECM) proteins, cytokines, and growth factors that enhance cell proliferation and function as well as antibacterial property.[2,4] The HAM has been used widely in surgical interventions such as wound dressing,[5,6] neurosurgery,[7] ophthalmic surgery,[8] and vagina surgery.[9] However, using the HAM as an allograph has some limitations associated to this group of grafts such as graft rejection. To overcome this, the HAM has been decellularized, this makes it a better cell proliferation supporter with less immunogenicity.[2,10] On the other hand, success in any transplantation depends on the collection and preservation of tissue engineered constructs before surgery.[2,11,12] Many researches have been conducted for the preservation and decellularization of the HAM with various degrees of success, in which almost all of them are detergent- or enzyme-based techniques.[13–16] Despite relatively favorable reports, the previous decellularization techniques are time-consuming and very expensive. Moreover, using enzymes in these procedures might negatively affect the ECM microstructure. For example, Wilshaw et al.[14] have developed a novel detergent-based protocol for the decellularization of the HAM using protease inhibitors, sodium dodecyl sulphate (SDS), tris-buffered saline (TBS), aprotinin, DNase, and RNase. They were then determined of its effect on biomechanical and cytotoxicity properties of the tissue. Although they successfully decellularized the tissue without significant effect on biomechanical behavior and cytotoxicity, their protocol was costly and time-consuming.

In this study, we aimed to develop a new simple, reproducible, and cost-effective method for the removal of cells from HAM without using any enzymatic agent, and then its preservation at room temperature. The effects of the treatment on biomechanical behavior, biodegradation, rat bone marrow stromal cells (BMSCs) viability, cytotoxicity, adhesion, and biocompatibility of the tissue were determined in vitro and in vivo in comparison with those of fresh HAM. Furthermore, the presences of the most important proteins in the ECM (collagen types I, III, and IV) were studied. In this paper, the natural fresh and decellularized HAMs were coded as N and D, respectively.

2. Results and Discussion

2.1. Biomechanical Evaluation of the Samples

To ensure that the processing of HAM is based on clinical standards, biomechanical tests were conducted. The results of the biomechanical measurements are shown in **Table 1**. This amended protocol demonstrated that the tissue could be decontaminated and decellularized without deteriorating its biomechanical properties. Although the decellularized samples appeared to be thinner and weaker than natural fresh samples, there were no statistically significant differences between both samples ($p > 0.05$). These results were also confirmed by the data obtained from suture retention strength tests that showed there was no meaningful difference between both samples. However, the decellularization process slightly decreased suture retention strength. Low mechanical property of HAM has been reported before. Numerous attempts have been made to develop strategies for improvement of mechanical property of HAM. For example, chemical or physical cross-linking of HAM increased its mechanical property.[17]

2.2. In Vitro Biodegradation Tests

The in vitro biodegradation test showed that approximately 35% of the sample mass was degraded during the first week of incubation in PBS solution. However, there was no significant difference between the weight loss values of both N and D samples (independent sample *t*-test, $p > 0.05$), as shown in **Figure 1**a. However, the biodegradation rate of T tissue showed a significant decrease after 4 d incubation in enzymatic solution (independent sample *t*-test, $p < 0.05$) compared with the fresh HAM sample. According to the results, around 71% and 96% of the N and D tissue masses, respectively, were degraded by the enzymatic solution after up to 7 d (Figure 1b). Ma et al.[18] showed that more than 90% of HAM mass was degraded by collagenase during the second week of incubation. In addition, some clinical observations indicated that the time for the biodegradation of HAM varied, ranging from a few days to a few weeks, depending on the severity of the local inflammation and some diseases with an accelerated collagenolytic activity.[19,20] Some methods have been developed to increase mechanical property of HAM.[17,21] For instance, Fujisato et al.[17] cross-linked HAM with radiation and glutaraldehyde (GA). In general, the results indicated that the decellularization process slightly affected the biodegradation property of the decellularized sample in vitro. Then, they investigated the effects of cross-linking on physicochemical and biodegradation properties of HAM. They found that cross-linking with GA affected the mechanical biodegradation properties of HAM more than radiation. Attempts for development of a strategy for enhancement of mechanical and biodegradation properties of HAM are undertaking.

2.3. Microscopic Analysis

Hoechst dye is used to stain DNA through its binding to the minor grooves of the double-stranded DNA. This dye is excited by UV, resulted in the emission of blue light.[22] A certain area

Table 1. Comparison of biomechanical properties of freeze-dried human amniotic membrane (HAM) samples, natural versus decellularized HAM.

Samples	Freeze-dried HAM natural	Freeze-dried HAM decellularized	Significance
Thickness [μm]	97 ± 8.2	74.0 ± 3.9	$P > 0.05$
Maximum load value (N)	1.30 ± 0.17	1.10 ± 0.24	$P > 0.05$
Suture retention strength [mN]	512 ± 63	481 ± 49	$P > 0.05$
Strain deflection at break [mm]	7.30 ± 0.49	6.90 ± 0.61	$P > 0.05$

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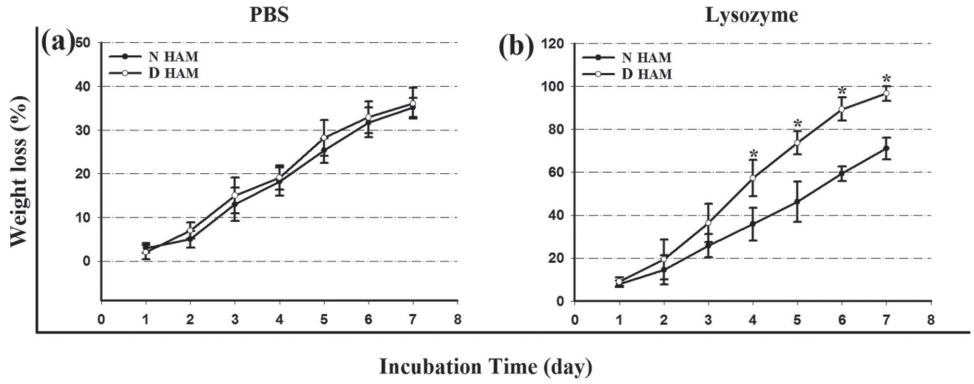

Figure 1. Time course of weight loss of N and D tissues in a) PBS and b) enzymatic (lysozyme) biodegradation solutions. An asterisk (*) indicates significant difference ($p < 0.05$) in biodegradation value compared with N sample.

of the fresh HAM was decellularized and placed on a glass slide so that the border of the fresh and decellularized interface was observable by a microscope. **Figure 2** shows the sample stained with Hoechst 33258 dye. As can be seen, the line between the fresh and decellularized areas is clearly discriminated under both light and ultraviolet light. The results clearly indicated that the cells were successfully removed from the tissue during the decellularization process.

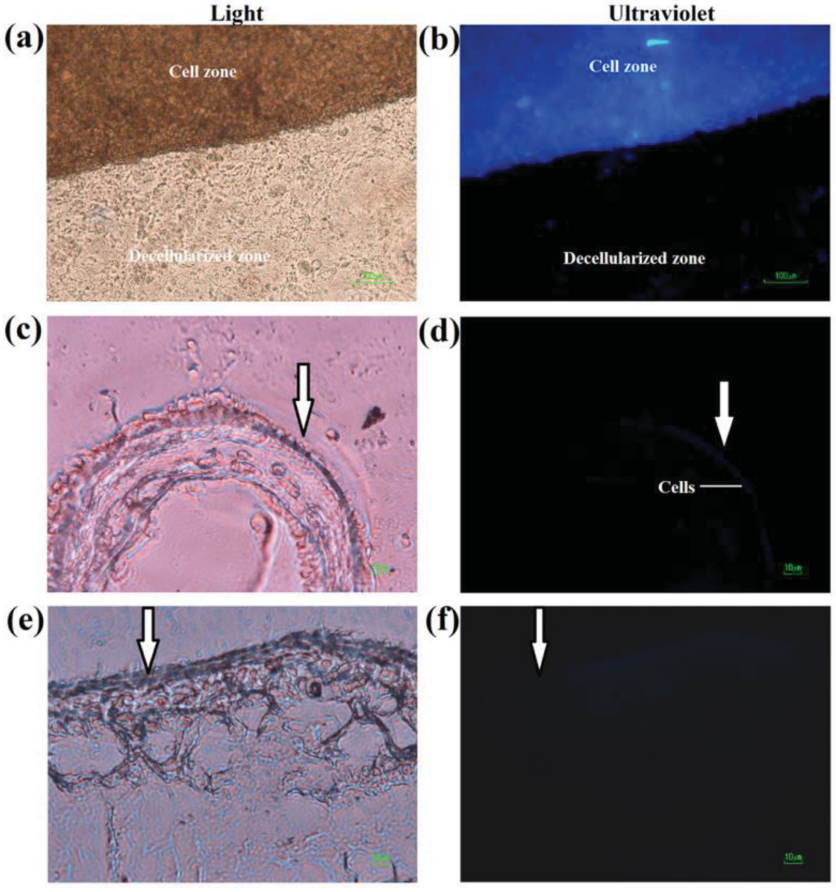

Figure 2. Both fresh and decellularized areas of the tissues were placed in glass slide and then stained with Hoechst (light blue). The samples were then viewed under a) light microscope and b) ultraviolet (UV). c–f) Paraffin-embedded tissues were sectioned on a microtome and then stained with Hoechst 33258 dye to detect cells within the tissue. c,d) and e,f) show the tissues before and after decellularization process, respectively. The epithelial cells were clearly visible within the fresh tissue in d) Hoechst 33258 dye-stained section. The white arrows indicating the apical surface of tissues.

2.4. DNA Extraction

The DNA content present in the tissue was isolated, quantified, and compared with those of the DNA isolated from the fresh tissue, as shown in **Figure 3**. According to the DNA quantification assay, a meaningful decrease in the DNA content of the D tissue was found in comparison with the N tissue (independent sample *t*-test, $p = 0.001$). The DNA content was found to be 159.87 ± 3.20 and 5.8 ± 2.2 μg mL^{-1} for HAM before and after decellularization, respectively, showing more than 95% DNA removal from the matrix (Figure 3a). The data were also confirmed with visualizing the DNA in 0.8% agarose gel (Figure 3b). As can be seen, no residual DNA was visible in the decellularized tissue gel electrophoresis (lane D). Similar results[14] have recently reported the development of an acellular HAM tissue by a detergent-based protocol. They found that the DNA content of HAM before and after decellularization was 3.980 ± 0.2 μg mg^{-1} and 0.2 ± 0.02 μg mg^{-1}, respectively (around 95% DNA removal), which is consistent with our report.

2.5. Histological Investigation

Both N and D HAM samples were investigated histologically to establish whether the cellular components were removed successfully from the tissues. For this purpose, the tissues were embedded in paraffin and serially sectioned (1–10 sections) in slides of 4 μm thickness. The sections were stained

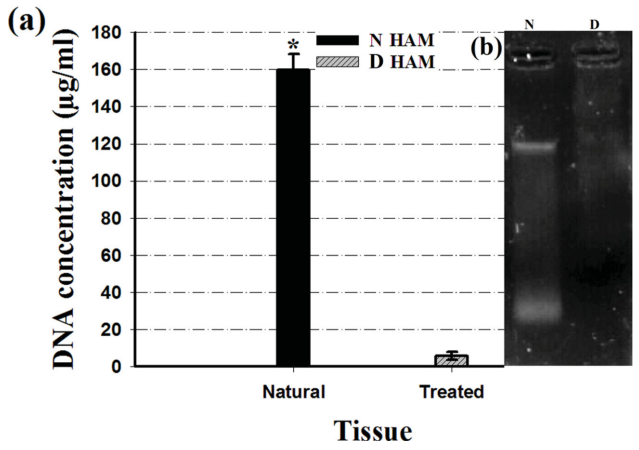

Figure 3. The content of DNA in N and D samples. a) There was a significant difference between the amount of DNA content for different samples (independent sample *t*-test, $p = 0.001$). It was found that more than 95% of the DNA content was removed during the decellularization process. b) The DNA was visualized with 0.8% agarose gel. A p value of <0.05 was defined as the level of significance. Each sample was repeated six times. An asterisk (*) indicates statistically significant difference with treated tissue.

with H&E and Hoechst 33258 dye and then viewed under the light and a fluorescent microscope, respectively. As can be seen in **Figure 4**, the H&E-stained sections showed cell removal from the matrix. In addition, Hoechst 33258 staining showed no residual DNA within the tissue after the decellularization, as shown in Figure 2c-f.

The IHC analysis was also performed to investigate whether the basement membrane proteins remained intact after decellularization. For this purpose, the presence of human collagens types I, III, and IV, as the most important basement membrane proteins, was investigated before and after decellularization. The proteins were detected using rabbit monoclonal primary antibodies and visualized by secondary rabbit antibodies conjugated with FITC. The nuclei were stained with DAPI. The results obtained from the IHC are shown in **Figure 5**. It was found that the quantity and distribution of all the tested components remained unchanged within the decellularized tissues.

2.6. In Vitro Cellular Response

2.6.1. Cell Viability and Cytotoxicity

The BMSCs were cultured on both the N and D tissues and their cell viability was evaluated by the MTT test after 24, 48, and 72 h culture periods. The data were normalized to positive control that represented 100% cell viability. The results obtained from the MTT test revealed that the tissues (both N and D samples) did not change the rate of cell proliferation for up to 72 h (independent sample *t*-test, $p > 0.05$) (**Figure 6**c). In addition, the cytotoxicity assay showed no significant difference between the LDH-specific activity in both N and D tissues in comparison to positive control (independent sample *t*-test, $p > 0.05$) in various culture periods (Figure 6d). The results indicated that the decellularization process did not confer any cytotoxicity to the tissue. These observations conform to previous studies, which reported the noncytotoxicity of both natural and decellularized HAMs.[14]

2.6.2. Cell–Tissue Interaction

The morphology of the BMSCs on both the N and D tissues was observed by the SEM. For this purpose, the cells were cultured on the tissues and maintained for 48 h for the study of cell adhesion. The SEM images are shown in Figure 6a,b. As can be seen, the cells were attached actively on both N and D tissues. The growth and expansion of the cells are clearly seen in both tissues, indicating no detectable effect of the decellularization process on the cell adhesion. These data also confirm the MTT and LDH results that show favorable biocompatibility for both N and D tissues in vitro.

2.7. In Vivo Biocompatibility

Upon implantation, the vascularized connective tissue in the site is injured and leads to the stimulation of inflammatory responses. It is important to keep in mind that these responses occur following tissue injuries. Indeed, the quality and quantity of the body immune response against an implanted biomaterial represent its biocompatibility property. It has been reported

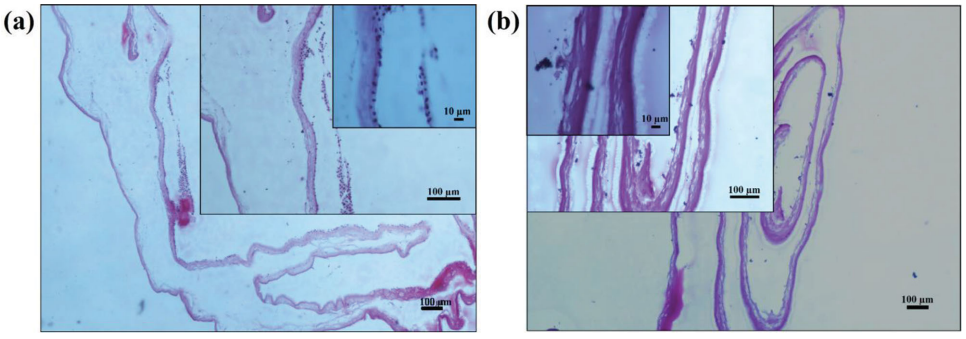

Figure 4. Hematoxylin and eosin (H&E) staining. a) N and b) D HAM samples stained with H&E and photographed under light microscope. The cells or cell fragments were successfully removed from the matrix after decellularization.

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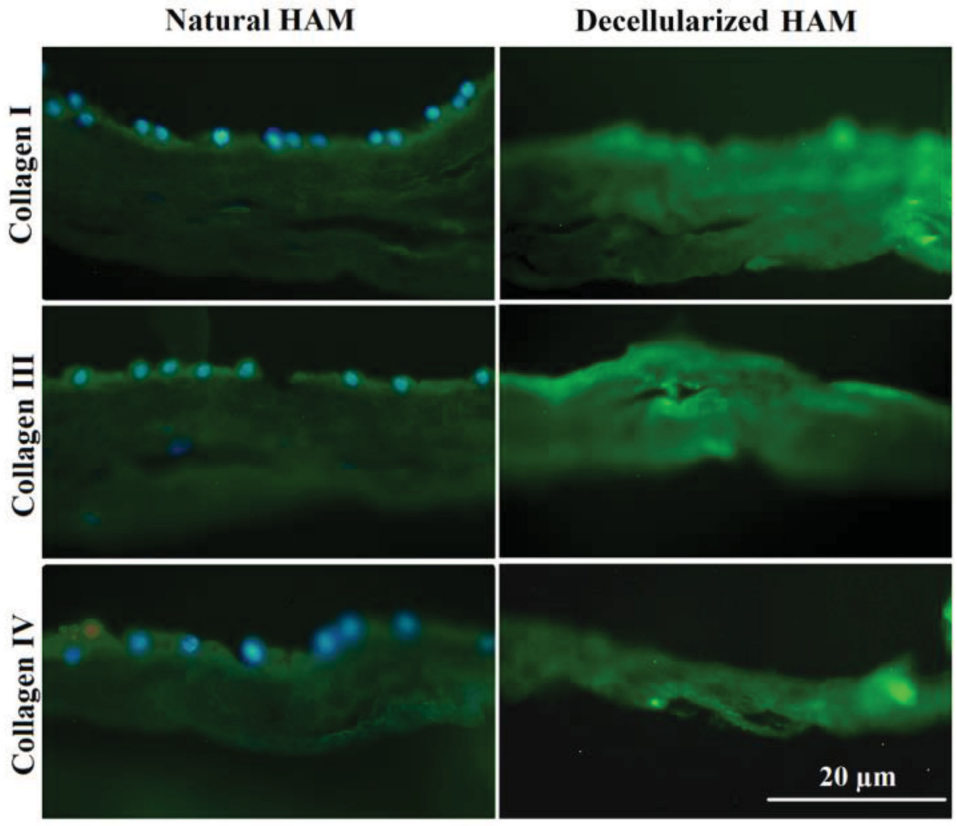

Figure 5. IHC staining for human collagen types I, III, and IV (green) located in the basement membrane of N and D HAM samples. The cells were counterstained with DAPI (blue). The DAPI-stained epithelial cells are apparent along the apical surface of the tissues.

that MQ correspond to the wound healing and biodegradation of the implanted biomaterials.[23] In addition, the LC could be attached to the biomaterial in vivo and increases the risk of rejection.[24] PC are also the cells involved in the production of antibodies against foreign materials. Therefore, the number of the inflammatory cells such as LC, MQ, and PC is an accurate indicator in the investigation of local tissue response against biomaterials.[24–26] For this purpose, the freeze-dried samples were subcutaneously implanted in rats and removed for H&E staining (**Figure 7**a) and the cells were counted after 1 and 4 weeks post-surgery (Figure 7b). The number of the inflammatory cells including LC, MQ, and PC was counted and compared at the site of implantation (Figure 7b).

The severity of the inflammatory response against any biocompatible material is usually resolved after 2 to 4 weeks, depending on the extent of the injury and the physicochemical properties of the materials. It has been found that HAM does not express HLA-A, B, and C.[27] This membrane has an immunomodulatory activity by which protect the baby from mother's immune system. Akle et al.[28] transplanted amniotic epithelial cells to seven volunteers and then investigated the survival of the cells after 7 d. According to their report, acute inflammatory rejection did not occur in the transplanted samples. Possessing some promising features has made HAM a suitable dressing scaffold for tissue engineering, especially soft tissue engineering. For example, HAM contains some immunoregulatory factors as well as natural collagen and growth factors. Many efforts showed the potential application of such membrane in wound dressing,[5] ocular,[8] vagina,[9] etc.

As mentioned earlier in this article, HAM contains two types of cells that limit its applications. Many strategies have been made to remove all cellular components from HAM not only to increase its biocompatibility but also provide a universal biological substrate on which to seed various cell types.[13,29] Almost all of these strategies have used enzymatic or chemical agents that make them costly and time-consuming. On the other hand, these agents are immunogen and required to be fully removed after processing. With proposed protocols, we expected that the decellularized HAM shows a high biocompatibility.

The results obtained from cell count showed a significant difference (one-way ANOVA, $p < 0.05$) in the average number of LC in the implanted area of the N tissue sample in comparison with the control and the D tissue, whereas no significant difference in the count of MQ and PC was observed between all three groups for up to 7 d post-surgery. However, after 28 d, no significant difference was seen (one-way ANOVA, $p > 0.05$) in the cell count of the LC, MQ, and PC between the experimental groups. In addition, the total cell number in the implanted area (cellularity) did not significantly change between the groups for up to 28 d.

According to the results, although the number of inflammatory cells in the site implanted with the natural HAM was a few more than those of in the decellularized HAM, after 1 week post-surgery, however the difference was not significant in

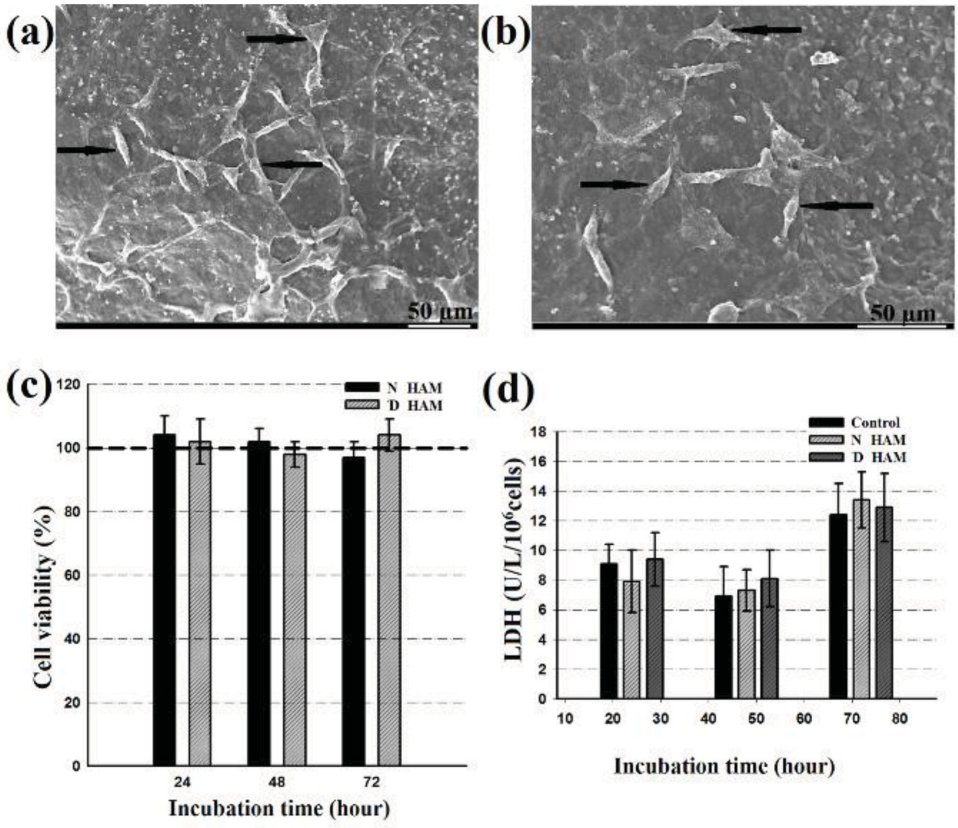

Figure 6. SEM images of the morphology of the BMSCs grown on a) N and b) D HAM samples. The cell viability and cytotoxicity of both tissues were assayed by c) MTT and d) LDH-specific activity tests, respectively. There was no significant difference between MTT (independent sample *t*-test, $p < 0.05$) and LDH (one-way ANOVA, $p < 0.05$) results for both samples. Black arrows indicated the cultured cell on the samples.

comparison to the control sample up to 4 weeks. In confirmation with other studies,[2,14,28] our results confirm that both N and D HAM samples had high biocompatibility property.

3. Conclusion

HAM as a biological substance, stand alone or in combination of other materials has widespread applications in tissue engineering, especially soft tissues. This membrane has an immunomodulatory property that makes it a promising substrate for tissue engineering. There are two types of cells, epithelial, and fibroblast, within the HAM that have made its applications problematic. Therefore, development of a strategy for fully removal of these cells from HAM is worthy. Decellularized HAM could mimic ECM and act as a biological substrate on which the various cell types be seeded. In the present study, a cost effective and simple procedure for the decellularization and preservation of HAM has been developed for tissue engineering applications. The removal of cells or cellular components (H&E, Hoechst, and DAPI staining), and the presence of intact collagen types I, III, and IV indicated that the decellularization process slightly increased the biocompatibility of the tissue. All of the results obtained from this study suggested that the presented method could be considered as a viable strategy for the development of decellularized HAM for preservation at room temperature and would have huge application for tissue engineering organs as biological scaffold. This consortium is working on number of application using this scaffold, including skin[30] and small intestine.[31]

4. Experimental Section

Tissue Collection and Decellularization: All of the human placentas were obtained from consenting mothers ($n = 6$) upon their cesarean-section deliveries. All donors were screened serologically for the possibility of infectious diseases such as human immunodeficiency virus types II, human hepatitis virus types B and C, syphilis, gonorrhea, toxoplasmosis, and cytomegalovirus and I. The placentas were placed in a container containing sterile phosphate buffer saline (PBS) supplemented with antibiotics, including 50 μg ML^{-1} penicillin, 50 μg mL^{-1} streptomycin, 100 μg mL^{-1} neomycin, and 2.5 μg mL^{-1} amphotericin B, and antimycotic (fongison) (all from, Gibco, Carlsbad, CA, USA), then transferred to the laboratory for the decellularization process. The human placenta collection was handled in accordance with the Declaration of Helsinki.[32] All of the procedures were performed under sterile conditions. Fresh placenta was washed with sterile distilled water three times. Blood residual was discarded from the placenta and washed several times with sterile distilled water. The HAM was separated from the chorion layer and transferred to a sterile PBS (PH 7.4) supplemented with antibiotic and antifungal agents. The HAM was then treated with 0.2% EDTA for 30 min at 37 °C. Thereafter, the tissue was treated with 0.5 M NaOH for 30 s. The tissue was then transferred to a 5% ammonium chloride and shook vigorously. The cells were discarded from the HAM with vigorous

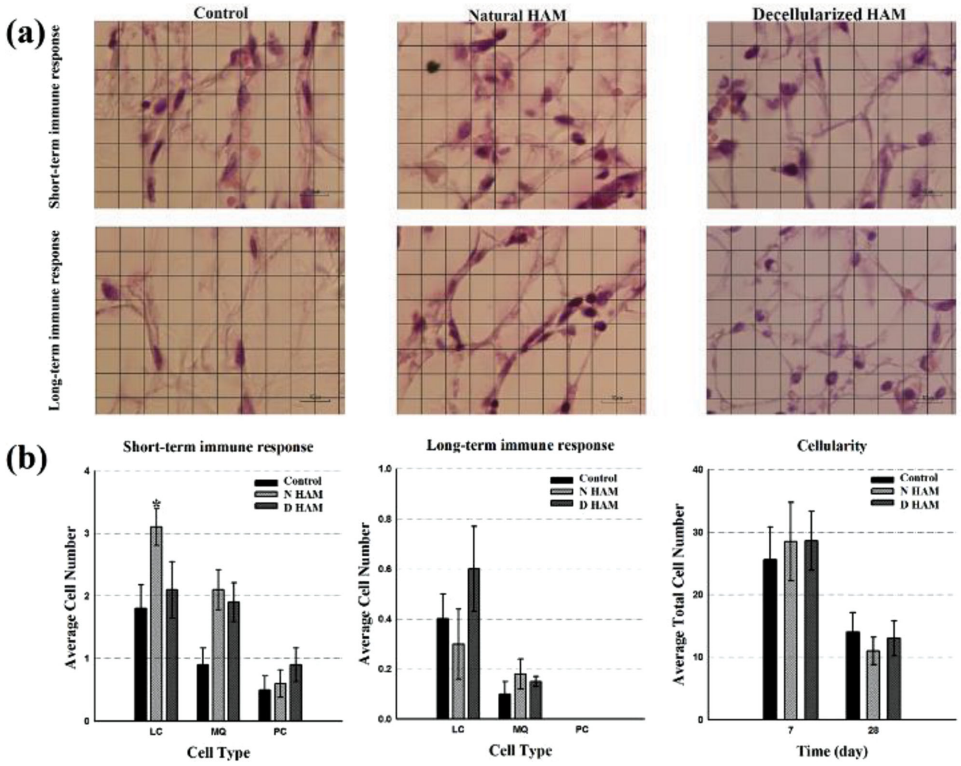

Figure 7. a) H&E staining of three experimental groups; Control, N and D HAM samples after short-term (1 week) and long-term (4 weeks) post-surgery. b) Average cell number of lymphocytes (LC), macrophages (MQ), and plasma cells (PC) after 1 (left) and 4 (middle) weeks post-implantation as well as cellularity in the implanted site. An asterisk (*) indicates significant differences (one-way ANOVA, $p < 0.05$)

shaking and scraping (placed on a lam and scraped), followed by a final wash with sterile PBS three times. The D tissue was transferred to an 80° C environment and then dried through sublimation. The freeze-dried membrane was sealed with nylon and preserved at room temperature until further analysis. All steps were done under aseptic conditions.

Biomechanical Behavior: The biomechanical behavior of the freeze-dried N and D HAMs ($n = 5$) was investigated on a universal tensile testing machine at a crosshead speed of 10 mm min^{-1} with a specified sample size (length = 20 mm and width = 10 mm). During the whole duration of the biomechanical testing, the samples stayed wet with PBS. For conducting the suture retention strength testing, 2 mm from the one end of each sample was sutured by a 5–0 nylon suture and the other end was clamped onto the holder. The samples were then loaded to failure, and the test runs were discarded if the failure did not occur near the center of the samples. The average thicknesses of the samples were measured with a Mitutoyo 547–400S Digimatic IDC thickness gage.

In Vitro Biodegradation: The biodegradation properties of the N and D HAMs ($n = 5$) were evaluated by enzymatic (0.1 wt% solution of lysozyme in Dulbecco's modified Eagle's medium (DMEM) culture medium) and PBS biodegradation solutions, in vitro. For this purpose, the samples were placed into a 10 mL of biodegradation solution after weighing with the accuracy of 0.1 mg (Mo). All the samples were then incubated at 37 °C for 7 d. For each chemical series, three samples were removed after 1 d, washed with distilled water, and dried at room temperature for 72 h. Then, the samples were weighed (M_d), and percentage of weight loss was determined on the basis of the following equation:

$$\text{Weight Loss}(\%) = 100 \times (M_o - M_d) / M_o$$

Microscopy Analysis: A certain zone of HAM was decellularized and then washed with sterile PBS. The tissue was placed on a glass slide and stained with Hoechst 33258 dye (1 mg mL^{-1}, Calbiochem, Chandlers Ford, UK) to detect the presence of residual DNA within the tissue. The samples were viewed under light and ultraviolet (UV) light.

DNA Extraction and Agar Gel Electrophoresis: DNA was extracted from six samples of N and D HAMs (50–60 mg) and quantified. Briefly, DNA was extracted from HAM according to a standardized method provided by the DNA extraction kit (Qiagen, USA) following the manufacture's instructions. The sample was purified and dissolved in 50 µL of distilled water. DNA was then quantified by determining its absorbance at 280 nm wavelength. In addition, the samples were electrophoresed in 0.8% agarose gel to confirm the absence of DNA in the treated tissue.

Histological Study: For histological analysis, the samples ($n = 3$) were fixed with 10% natural-buffered formalin (Sigma), dehydrated through a graduated series of increasing ethanol up to 100% and embedded in paraffin wax. The paraffin-embedded tissues were sectioned at 4 µm and then stained with hematoxylin and eosin (H&E), and Hoechst 33258 dye (1 mg mL^{-1}, Calbiochem, Chandlers Ford, UK). Existence of human collagen type I, III, and IV in both N and D tissues was assayed by immunohistochemistry (IHC). For this purpose, the sections were deparaffinized and rehydrated through descending strengths of alcohols. After trypsin/EDTA treatment for an antigen retrieval step, the samples were blocked in a blocking solution containing 1% bovine serum albumin (BSA), 0.2% Triton X-100, and 0.05% Tween-20. Subsequently, the sections were incubated with primary antibodies specific for human collagen I (Rabbit polyclonal to Collagen I, abcam, ab34710), human collagen III (Rabbit polyclonal to Collagen I, abcam, ab83829) and human collagen IV (Rabbit polyclonal to Collagen I, abcam, ab6586) for 1.5 h at room temperature. The sections incubated with the appropriate nonspecific normal rabbit polyclonal IgG isotypes served as a negative control. Thereupon washing with PBS, the sections were treated with anti-rabbit secondary antibodies conjugated with fluorescein isothiocyanate (FITC) for 1 h at room temperature. The cell nuclei were then counterstained with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich).

BMSCs Isolation and Culture: The BMSCs were isolated from rats and expanded following well-established protocols.[33] Briefly, three- to four-month-old Sprague-Dawely rats weighing 200–300 g were sacrificed by CO_2 asphyxiation. The tibia and femurs of the rats were dissected. Bone-marrow contents were then flushed into a 15-mL tube by a 25-gauge needle loaded with 5 mL low-glucose DMEM supplemented with 10% fetal bovine serum, 1% pen/sterp, nystatin, and amphotericin B, 2×10^{-3} M glutamax, 1×10^{-3} M L-glutamine, and 1% nonessential amino acids (all from Gibco, Carlsbad, CA). Thereafter, the bone marrow plugs were transferred into a cell-culture flask and maintained as described above. The culture medium was changed every 3–4 d. The cells were subcultured at 80%–90% confluence and used at passage three for the following in vitro and in vivo examinations.

Cell Viability and Cytotoxicity: The cell viability assessment was evaluated on the basis of the mitochondrial function of living cells by the reduction of tetrazolium salt (MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazolium bromide). The MTT assay was carried out by a procedure described in our previously published work.[34] To perform the MTT test, the BMSCs were cultured on the N and D HAMs for 24, 48, and 72 h and their proliferation rates were compared with those of the cells cultured on standard culture plates (as the positive control). The negative control was prepared with supplemented DMEM without tissues and cells in each well (OD*nc*). A blank optical density (OD) value was derived from each sample reading. The OD was measured using an ELISA (enzyme-linked immunosorbent assay) reader at a wavelength of 590 nm with a reference filter of 620 nm (OD*s*). The absorbance value was defined by the following formula:

$$\text{Absorbance value} = \text{OD}s - \text{OD}nc$$

The cytotoxicity was also evaluated by measuring the LDH-specific activity in the medium in which the cells were cultured on the samples for 24, 48, and 72 h. For this purpose, the medium was collected and the LDH activity in medium was measured with an LDH kit (Zist Shimi kits, Co No: 10–503 and 10–533–1), based on the P-nitro phenyl phosphate conversion to P-nitro phenol. The UV absorbance of NADH, as an index of NADH concentration, was quantitated on a Biotek EL800 absorbance plate reader at 490 nm. At the same time, the cells were ruptured via freeze thawing (three times) and the total LDH activity in the medium was measured. LDH data were normalized for 10^6 cells.[35]

Cell–Tissue Interaction Study: The BMSCs were cultured on both N and D samples and maintained for 2 d in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For investigating the cell tissue interaction, the morphology of the cells on both N and D samples was viewed by the scanning electron microscope (SEM, Philips XL30, the Netherlands). After the incubation, the samples were prepared for the SEM analysis, by a previously published protocol.[36] Briefly, the cell–tissue complexes were fixed with gutaraldehyde 2.5% solution and dehydrated in a graded concentration (30%, 50%, 70%, and 100%) of acetone (Merck). In the next step, the samples were treated with osmium tetroxide (OsO_4) 0.1% (Sigma, USA) and then freeze-dried. For taking the SEM images, the samples were coated with gold by sputtering, observed, and analyzed by the SEM at an acceleration voltage of 15 kV.

In Vivo Biocompatibility: For the in vivo biocompatibility study, the tissues were subcutaneously implanted in rat models by a procedure described before.[37] Briefly, an animal (5–6-week-old Sprague-Dawley adult male rats) was anesthetized with an IP injection of ketamine hydrochloride (75 mg kg^{-1}) and xylazine (10 mg kg^{-1}) (both from Sigma, USA). Hair was removed from the back of the rat and the site was wiped with ethanol 70%. The tissue (N or D, around 10×10 mm) was placed in the left hemiback of the rat and then sutured with a gut suture. A negative control was prepared from the right hemiback incision that was sutured without placing any tissue. The implanted area was marked. After short-term (1 week) and long-term (4 weeks) post-surgeries, the animal was sacrificed by CO_2 asphyxiation and the tissue was removed and processed for H&E staining, as described above. The number of lymphocytes (LC), macrophages (MQ), plasma cells (PC) as well as cellularity in the implanted area was counted. The surgery was performed according to The Guiding Principles in the Care and Use of Animals.[38]

Statistical Analysis: Distribution of the data was assessed by the Kolmogorov–Smirnov test. The data were expressed as a means of ±SD and analyzed statistically by independent sample *t*-tests, one-way ANOVA and Tukey's test where appropriate. All of the data obtained from the MTT assay were normalized to a positive control (as 100% cell viability). A p value of <0.05 was defined as the level of significance. The SigmaPlot 11.0 θ software was used for plotting graphs.

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