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Characterization of Decellularized Heart Matrices as Biomaterials for Regular and Whole Organ Tissue Engineering and Initial *In-vitro* Recellularization with Ips Cells

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Abstract

Tissue engineering strategies, based on solid/porous scaffolds, suffer from several limitations, such as ineffective vascularization, poor cell distribution and organization within scaffold, in addition to low final cell density, among others. Therefore, the search for other tissue engineering approaches constitutes an active area of investigation. Decellularized matrices (DM) present major advantages compared to solid scaffolds, such as ideal chemical composition, the preservation of vascularization structure and perfect three-dimensional structure. In the present study, we aimed to characterize and investigate murine heart decellularized matrices as biomaterials for regular and whole organ tissue engineering. Heart decellularized matrices were characterized according to: 1. DNA content, through DNA quantificationo and PCR of isolated genomic DNA; 2. Histological structure, assessed after Hematoxylin and Eosin, as well as Masson`s Trichrome stainings; 3. Surface nanostructure analysis, performed, using SEM. Those essays allowed us to conclude that DM was indeed decellularized, with preserved extracellular matrix structure. Following characterization, decellularized heart slices were seeded with induced Pluripotent Stem cells (iPS). As expected, but – to the best of our knowledge - never shown before, decellularization of murine heart matrices maintained matrix biocompatibility, as iPS cells rapidly attached to the surface of the material and proliferated. Strikingly though, heart DM presented a differentiation induction effect over those cells, which lost their pluripotency markers after 7 days of culture in the DM. Such loss of differentiation markers was observed, even though bFGF containing media mTSR was used during such period. Gene expression of iPS cells cultured on DM will be further analyzed, in order to assess the effects of culturing pluripotent stem cells in decellularized heart matrices.

Keywords: Decellularized matrices; Decellularization; iPS; Tissue engineering

Introduction

Classic tissue engineering strategies, based on solid/porous scaffolds, suffer from several limitations, such as ineffective vascularization, poor cell distribution/organization within scaffold, and low final cell density, among others [1]. Therefore, the search for alternative tissue engineering approaches constitutes an active area of investigation in the field. Decellularized matrices (DMs) have been used for decades [2-5], especially for vascular applications, but recently they have been investigated and considered an interesting biomaterial option for tissue engineering of tracheobronchial airways [5] and heart [6], among others. DMs present major advantages compared to synthetic scaffolds, such as ideal chemical composition, vascularization, and perfect three-dimensional structure [6,7]. Extracellular Matrices have even been used in association to synthetic materials, in order to enhance their performance *in vitro* [8]. Therefore, the use of DMs enables the construction of functional organs *in vitro*, suitable for implantation *in vivo*.

DMs have been extensively tested in humans, as xeno [9], allo or autologous acellular vascular grafts, such as Alloderm (LifeCell) and other acellular products [2,4,10], as well as, more recently, as supporting scaffold for cells [5,11]. Indeed, the most interesting applications of DM may involve both DM production and cell colonization, in order to obtain functional constructs [5,6,11].

The colonization of biomaterials *in vitro* requires a source of cells capable of being grown in large scale, differentiated into various cell types, without generating strong immunological responses [12]. Differentiated cell types have been used as grafts for regenerative medicine, but such strategies are mainly hindered by limitations on cell growth *in vitro*, as well as donor site morbidity [13]. Therefore, stem cells have attracted much attention in the field. Induced Pluripotent Stem (iPS) Cells present all of the aforementioned characteristics, being: i. Pluripotent, ii. Indefinitely expanded *in vitro*, and iii. Potentially compatible with patient [14]. Therefore, iPS cells currently constitute one of the most desirable cell sources for tissue engineering. Studies regarding iPS cell culture in biomaterials and three-dimensional cultures are very preliminary.

Our novel approach was to obtain ECM-preserved decellularized murine heart matrices, seed resultant scaffolds *ex vivo* with human iPS cells, and study their biocompatibility and ability to induce tissue-specific differentiation.

Materials and Methods

Heart decellularization

All rat experiments were performed in accordance with the guidelines for human use of laboratory animals established at our institution (Protocol# 140/11). Rat heart decellularization was

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performed as described by Ott et al. [6], with minor modification. Briefly, Lewis LEW-Tg (EGFP) F455.5/Rrrc rats were systemically heparinized (Billi Farmacêutica Ltda, Brazil) through caudal vein (300 IU/kg) and 10 minutes later, euthanized with 180 mg/kg of ketamine and 45 mg/Kg of xylazine (both from Syntec, Brazil). A medium sternotomy was performed, and pericardium was removed. Aorta was then dissected and had its branches ligated. Caudal and cranial cavas, pulmonary veins, and the pulmonary artery were also ligated. Finally, the heart was removed from chest and aorta was cannulated connected to a perfusion system, consisting of a Pulse-Free Flow Peristaltic Pump (Minipuls 3, Gilson–FRA). Decellularization was achieved though solution perfusion, and was performed with no temperature control, as follows: initially, 50 mL of PBS containing 10 µM Adenosine (Merck SA, USA) was perfused at 10 mL/min rate, in order to prevent undesired cell death and vessel occlusion by cellular debris; then 1.5-2 L of 1% w/v of detergent and cell membrane solubilizer SDS (Sigma, USA) diluted in water, at 5 mL/min rate; next 150 mL of 1% v/v. Triton X-100 (Sigma, USA), which is a gentler detergent compared to SDS, was perfused, and finally, 2 L of Penicilin, Streptomicin and Amphotericin (Sigma, USA) containing PBS was perfused, in order to obtain a DM ready for experimentation and suitable for cell culture applications.

Genomic DNA extraction

DNA content analysis of rat heart decellularized matrices was performed by genomic DNA extraction, using DNAzol reagent (Invitrogen, USA) and following manufacturer's instructions. Briefly, sample was lysed and homogenized by DNAzol reagent addition. DNA was then precipitated and washed using Ethanol in different concentrations, and finally diluted in 8 mM NaOH.

DNA content analysis

In order to assess genomic DNA content of decellularized matrices, the isolated genomic DNA was quantified using Nanodrop Spectrophotometer ND-1000 spectrophotometer (Life Technologies-USA).

PCR

Genomic DNA isolated from rat heart decellularized matrices was used in duplex PCR reactions, in order to assess DNA content, using an amplification method. The triplex reaction consisted of primers that annealed to genomic DNA from both LEW-Tg (EGFP) F455.5/Rrrc rats, as well as their non-GFP counterparts. Primer sequences were: LWS 455 5F: AACCTCCCAGTGCTTTGAACGCTA, LWS 455 5R: GGTGCAAGCCTCAACTTCTTTGT and U3r-4: ATCAGGGAAG-TAGCCTTGTGTGTG. Wild type Lewis rats ($GFP^{-/-}$) presented 438bp amplicons, and $GFP^{+/+}$ Lewis rats presented approximately 129 bp amplicons. Samples of heterozygous cells or containing genetic material from animals of different genotypes presented amplicons of both sizes, even though heterozygous and mixed samples were not used in the present study.

Histological analysis

DMs were fixed using 4% paraformaldehyde (Merck, USA), and embedded in paraffin for histological analysis. 5 µM sections were cut and stained using Hematoxylin and Eosin, as well as Masson`s Trichrome stainings.

Scanning Electron Microscpopy (SEM)

In order to examine the three-dimensional structure of DM, the material was fixed with 2.5% glutaraldehyde (Sigma-Aldrich, USA) in 0.1 M sodium cacodylate (Merck, USA) buffer for 2 h, post-fixed with 1% osmium tetroxide (Merck Millipore, USA) for 2 h, dehydrated in increasing concentrations of ethanol (30-100% ethanol, Merck Millipore), and was critical point-dried. Following, DMs were coated with gold and observed with a scanning electron microscope at 15 kV (JEOL/JSM-6360LV from the Department of Metallurgy-UFMG).

Immunostaining

DM were fixed using 4% paraformaldehyde (Merck, USA) and passed through serial baths of 10, 20 and 30% sacharose (Amresco, USA). Following sacharose baths, DMs were embedded in Tissue-Tek OCT™ Compound (Sakura-NLD) and cut. 5 µM sections were dehydrated and hydrated passing through decreasing and increasing concentrations of ethanol. Following hydration, sections were incubated with primary antibodies, namely: mouse anti-rat collagen I and rabbit anti-rat laminin (both from Abcam, UK) overnight at 4°C. Following incubation, sections were washed with PBS and incubated for 1 h at room temperature with Alexa Fluor (Molecular Probes, USA) secondary antibodies. Sections were then washed and incubated with 2 µg/ml Hoechst 33342 (Sigma-Aldrich, USA) solution for 20 min. Finally, sections were washed in mounted using Hydromount (National Diagnostics, USA), and observed in fluorescence microscope.

iPS cells were also analysed after immunostaining. Briefly, cells were fixed using 4% paraformaldehyde, washed and incubated with mouse anti-human Oct-4 and mouse anti-human SSEA-4 antibodies (from Santa Cruz, USA, and Abcam, UK, respectively). Secondary antibodies and Hoechst were used as already described.

iPS cell culture

FN052 iPS cells, kindly provided by Dr. Stevens K. Rehen, from the National Laboratory of Embryonic Stem Cells (LaNCE-RJ), were cultured in xenofree conditions, using Matrigel® (BD Biosciences, USA) and mTeSR® (Stem Cell Technologies). Once a week, cells were passaged using Dispase (BD Biosciences, USA), followed by mechanical passaging, performed by detachment of needle-cut colonies clumps with a scraper.

Cell seeding and maintenance on DM

iPS Cells were detached from Matrigel, as described for cell passaging, and part of the cell suspension was further treated with trypsin (Invitrogen, USA), to allow cell counting. On average, 5×10^4 cells were plated in 24 wells coated with DM. For 7 days, iPS cells were cultured in mTeSR, and had their medium changed daily.

Cellular viability and proliferation

iPS Cells were seeded on decellularized matrices or on standard culture plate, and their viability and proliferation were determined by the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, which is based on the reduction of tetrazolium salt to formazan crystals, by the dehydrogenase enzymes present in the mitochondria of living cells [15]. After 7 days of cell seeding, the medium was removed and 210 µl of fresh culture media and 170 µl of MTT solution (5 mg/mL in PBS) were added to each well, followed by incubation for 2 h at 37°C in a 5% CO_2 atmosphere. The resulting formazan salts were solubilized with 210 µl of SDS-10% HCl (sodium dodecyl sulfate-hydrochloric acid) for 18 h at 37°C in a 5% CO_2 atmosphere, and the optical density of the solution was evaluated with a microplate spectrophotometer at 595 nm. Cells cultured without the scaffold (on Matrigel) were employed as the control for the proliferation experiment, which evaluated the viability and proliferation of iPS cells cultured with the basal medium and mTeSR, respectively.

Alkaline phosphatase activity

Alkaline Phosphatase is an enzyme associated to pluripotency [16]. The AP activity was evaluated with the BCIP-NBT Kit assay, as described by the manufacturer (Cat. 00-2209, Invitrogen). iPS were seeded on bacterial cellulose and on standard culture plate, and cultured with basal medium at 37°C, 5% CO_2 and humidified atmosphere. After 7 days, the supernatant of each well was removed and washed twice with PBS. Then, 200 µL of BCIP-NBT solution was added to each well and incubated. Two hours later, the insoluble purple precipitants were dissolved with SDS-10% HCl. After 18 hours, 100 µl of solution was transferred to a 96-well plate and the OD was measured at 595 nm. Cells cultured without the scaffold (on Matrigel) were subjected to the same procedure, as a control.

Results

Perfusion-decellularized rat heart matrix characterization

Perfusion-decellularization of rat hearts resulted in virtually completely acellular matrices. Macroscopically, rat hearts which were initially dense and red, gave rise to white-translucid coloured hearts, which maintained their overall three-dimensional structure (Figure 1). Such matrices had virtually no DNA content, as demonstrated by DNA quantification (Figure 2A), as well as PCR amplification experiments (Figure 2B). More specifically, Nanodrop DNA quantification revealed 99% total DNA removal, considering subtraction of blank measurement, which was not zero. PCR amplification was performed, but no DNA amplification occurred.

In contrast to regular hearts, which present cell nuclei and muscle fibers (Figure 3A and 3B), histological analysis of DM revealed an acellular material with preserved vascularization structure (Figure 3C and 3D). Even though cell nuclei were absent in organ stroma and endothelium, observed vessels presented normal structure and diameter, as shown by H&E and Masson`s Trichrome stainings. Masson`s Trichrome Staining also indicated collagen preservation, especially in coronary walls (Figure 3D).

Once again, cellular content was proven absent through SEM.

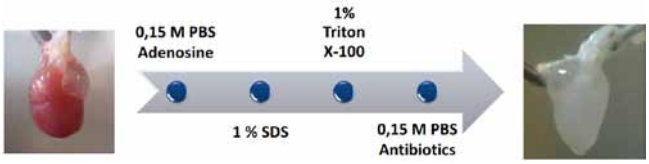

Figure 1: Scheme of decellularization protocol.

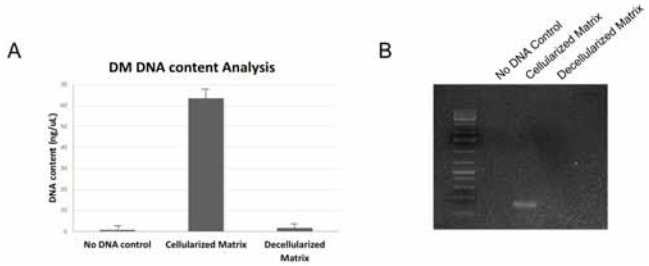

Figure 2: DNA content analysis of DM. In contrast to heart, DM presents virtually no DNA, according to DNA quantification in spectrophotometer (A), and to PCR amplification, using primers for genomic DNA (B).

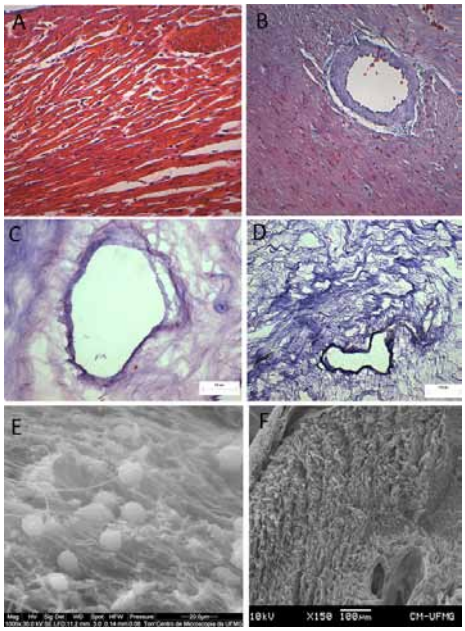

Figure 3: Histological and Scanning Electron Micrographs (SEM) of cellularized and decellularized hearts. Hematoxilin and Eosin (A and C) as well as Masson`s Trichrome Staining (B and D) were performed, in order to show structural and cellular aspects of cellularized (A and B), as well as DM (C and D). SEM (E and F) also demonstrates the contrast between partially decellularized matrix (E) and completely decellularized one (F). SEM also shows structural preservation of DM (F).

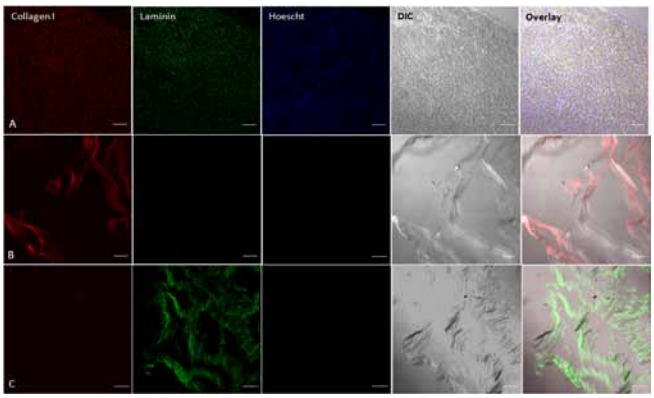

Figure 4: Immunofluorescence assays of cellularized (A) and decellularized (B and C) heart matrices. As depicted, decellularized matrices preserve important ECM components, such as Collagen I (red) and Laminin (green). The pannel also shows the absence of cell nuclei in DM, in contrast to cellularized organs, as shown by Hoechst staining (blue). Scale bars: 100 µm.

Microscpically, a partially decellularized matrix is shown (Figure 3E), as well as the acellular matrix (Figure 3F). SEM analysis allows to observe that collagen fiber orientation is preserved, which indicates the successful production of a decellularized matrix with absent cell content, preserved collagen fibers and three-dimensional structure.

For tissue engineering purposes, though, chemical composition of the scaffold is also important. Therefore, the protein content preservation of two key proteins of extracellular matrix, namely collagen 1 and laminin, was assessed. As observed, similar to control rat hearts, which were not decellularized, DM presented preserved

collagen 1 and laminin (Figure 4). Hoechst staining also confirmed previous results, showing, once again, the lack of cell nuclei in the matrices.

iPS culture on decellularized matrix

Once characterized, DMs were evaluated as scaffolds for tissue engineering purposes. In order to do so, FN052, pluripotent and undifferentiated iPS cells were initially cultured on Matrigel. Their pluripotency was shown by Oct-4 and SSEA-4 expression (Figure 5A), as well as teratoma formation and gene expression (data not shown).

After being seeded on DM, iPS cells readily attached to the DM surface. Indeed, following 7 days from cell seeding, iPS cells were still viable, as indicated by MTT assay (Figure 6). Therefore, decellularization process did not render DM toxic to iPS cells.

iPS cells also expressed Alkaline Phosphatase at 7 days of culture (Figure 6). This is an indicative of the pluripotency state of the cells. However, iPS cells lost Oct-4 and SSEA-4 expression (Figure 5B), indicating that, actually, cells lost pluripotency.. iPS cells were maintained in mTeSR during cell culture on DM. mTeSR contains bFGF, which supports iPS cell pluripotency. The fact that iPS cells cultured on DM for 7 days differentiated in the presence of bFGF, indicates the biocompatibility of DM, but also emphasizes the role of the DM in inducing cell differentiation, as reflected on their decrease on the expression of pluripotency markers.

Discussion

DMs have been used in the field of vascular surgery for decades, as an attempt to prevent calcification of vascular grafts [2]. Since then, DMs have become not only a natural source of acellular biomaterials for vascular and heart valve transplantation, but also a scaffold for whole organ tissue engineering, which requires recellularization [5,6,11].

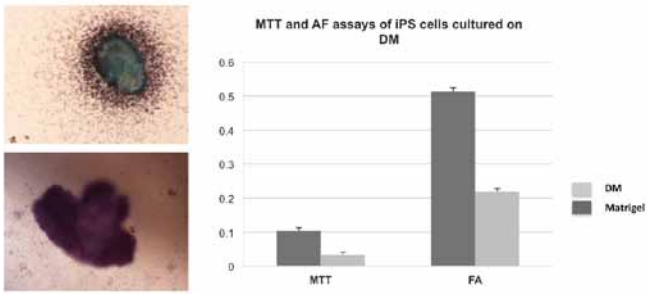

Figure 5: MTT and AF assays of iPS cells cultured on DM. As depicted, iPS cells are able to metabolize MTT and to produce alkaline phosphatase. The former is indicative of cell viability and the latter, pluripotency, when expressed in high levels.

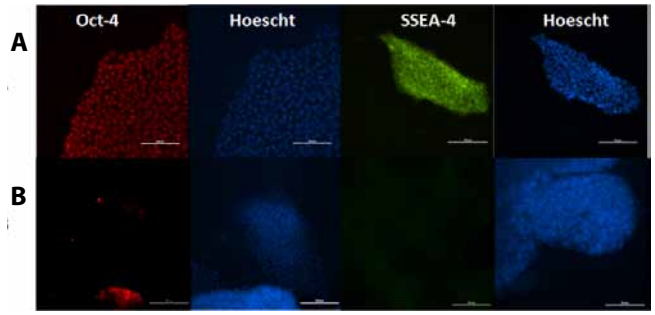

Figure 6: Expression of pluripotency markers by FN052 cultured on Matrigel® (A) and DM (B). As depicted, FN052 decrease expression of pluripotency markers after 7 days of culture on DM. Scale bars: 100 µm.

The paper published by Jungebluth et al. [5] in 2011, describing the recellularization and successful implantation of a decellularized human trachea in a patient, demonstrated the feasibility of such strategy in the clinic. Conversely, the paper published by Ott et al. [6] in 2008 was also considered a milestone in the field, when demonstrated the recellularization of much more complex organs (rat hearts) using neonatal cardiomyocytes and the obtention of a functional organ.

Neonatal cells are not the ideal cell type for DM recellularization, though. In general, neonatal cell sources are limited and hindered by ethical aspects. In addition, those cells are usually allogeneic and suffer senescence *in vitro* [17,18].

In the present study, we characterized rat heart decellularized matrices, in order to assess their suitability as a biomaterial for whole organ tissue engineering purposes. As decellularization methods vary widely in the literature [19], we chose to reproduce with minor modification the protocol established by Ott et al. [6], as they achieved functional organs at the end of recellularization. In contrast to Ott et al. [6] though, we aimed to assess iPS cell behavior in the acellular matrix.

iPS cells constitute pluripotent stem cells produced from adult cells [20]. The production of such cells constitutes an extremely recent technique, whose creator has already been laureate with the Nobel Prize in Medicine or Physiology [21]. Currently, they are considered a desirable tool for large scale cell production for regenerative medicine and tissue engineering purposes, among others.

Unfortunately, even though the pluripotent potential property of iPS and embryonic stem cells is beyond dispute, so far they can`t reconstitute tissue three-dimensional complexity, without a supporting scaffold.

The choice of studying decellularized matrices as scaffolds for iPS cell seeding derives from their role *in vivo*, which includes several effects over cell cycle control, such as proliferation, migration and differentiation [22-24]. Indeed, not only ECM composition contributes to cell events, but also ECM acts as a reservoir of growth factors, which are gradually released during ECM turnover. Specifically, it has been shown that ECM components improve stem cell differentiation toward specific cell types [25]. Furthermore, three-dimensional cell culture also promotes effective differentiation of pluripotent stem cells [26]. Conversely, DM has also been shown to induce iPS cell differentiation in a kidney model [27].

In the present study, we have shown that heart DM constitutes a biocompatible biomaterial for iPS cell culture. During the period tested, iPS cells were still able to metabolize the MTT substrate, indicative of functional mithocondria, and indirectly related to cell proliferation. On the other hand, even though our results indicate that DM allowed iPS cell viability, it also provides strong evidence that DM induced iPS cell differentiation.

Genetic control of pluripotency is strictly regulated by key transcription factors [28], which include Oct-4, Sox-2 and Nanog. Even though further aspects of such event must be further investigated, the loss of Oct-4 is sufficient to indicate the differentiation and loss of pluripotency of cells. For instance, the mechanism of differentiation induced must be clarified, as it may have been induced by ECM chemical composition, or by growth factor release.

The fact that DM induces iPS cell differentiation may be seen as a positive effect of the ECM over those cells, due to their potential to form teratomas *in vivo*. If such event is prevented by the interaction of iPS cells with the DM, it may constitute a suitable biomaterial for tissue engineering based on pluripotent cells.

As already stated, iPS cells are considered an exciting new tool for tissue engineering purposes, along with DMs. Therefore, studies of the interaction of both tools *in vitro* constitute an important step for achieving whole organ formation *in vitro*.

Acknowledgements

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