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Establishment of 3-dimensional scaffolds from hemochorial placentas

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ABSTRACT

Introduction: The extracellular matrix (ECM) is a complex, tissue-specific 3-dimensional network that controls cell processes. ECMs derived from various organs are used to produce biological scaffolds comparable to the native microenvironment. Although placentas are often overlooked, they offer a rich ECM for tissue engineering, especially the hemochorial placentas from rodents and lagomorphs that resemble the ones from humans.
Methods: Here we established a protocol for decellularization and investigated the ECM in native and decellularized placentas of guinea pigs, rats and rabbits by means of histology, immunohistochemistry, immunofluorescence and scanning electron microscopy.
Results: Effective decellularization were achieved by immersion in 0.25% Sodium Dodecyl Sulfate for 3 days, resulting in an intact ECM, while cells or nuclei were absent. All species had a high diversity of ECM components that varied between areas.
Discussion: Dense fibrous networks in the junctional zone were strongly positive to collagen I, III and IV, fibronectin, and laminin ECM markers. Noticeable response were also found for the decidua, especially along the maternal vessels. The labyrinth had thin fibers strongly positive for fibronectin and laminin, but not much for collagens. In conclusion, we established an effective protocol to obtain biological scaffolds from animal models with hemochorial placentas that possessed promising values for future purposes in Regenerative Medicine.

1. Introduction

Worldwide more than one million transplantations per year are currently performed. Chronic conditions like chronic renal diseases and cardiac insufficiencies, contribute largely to this high demand for transplantation. Brazil places second with the highest number of transplants performed, losing the first place only to the United States. Despite these remarkable ranking, the Brazilian Association of Organ Transplantation has registered a reduction in the number of potential and effective donors, as well as, a reduction in the transplantation rate [1]. Regenerative medicine provides promising alternatives to overcome these challenges. Thus, strategies to improve the use of tissue bioengineering tools, such as decellularized biological scaffolds that could be repopulated with stem cells into a 3-dimensional tissue could be used, in order to replace and/or regenerate injured organs and tissues [2–4].

A variety of such scaffolds, produced from natural or synthetic biomaterials have been tested [5]. However, only biological scaffolds derived from decellularized tissues/organs preserve the extracellular matrix (ECM). Only they are adequate to meet the complex tissue-intrinsic molecular, structural and mechanical properties that regulate tissue-specific cell behavior inside a native ECM microenvironment [6]. The ECM represents a heterogeneous connective network that supports cell migration, proliferation and differentiation for tissue morphogenesis and homeostasis *in vivo* [7,8]. It is formed by 4 types of macromolecules: (1) the collagen system, (2) elastic fibers, (3) proteoglycans, and (4) multifunctional glycoproteins [9] which all are intracellularly produced by tissue-resident cells, secreted into the ECM via exocytosis and then integrated with the existing matrix [10]. Both, the anatomical origin of the tissue and the applied decellularization method influence the ultrastructural and compositional characteristic of the ECM producing a biological scaffold [10]. Thus, studying the plasticity of different decellularized tissues/organs is important for the success in tissue engineering, since the molecules of ECM should be preserved in order to use for regenerative medicine [11].

Extensive literature is found on organs like heart, kidney, cornea, lung, blood vessels, skin and tendons, ranging from established decellularization protocols up to preclinical studies in animals and clinical

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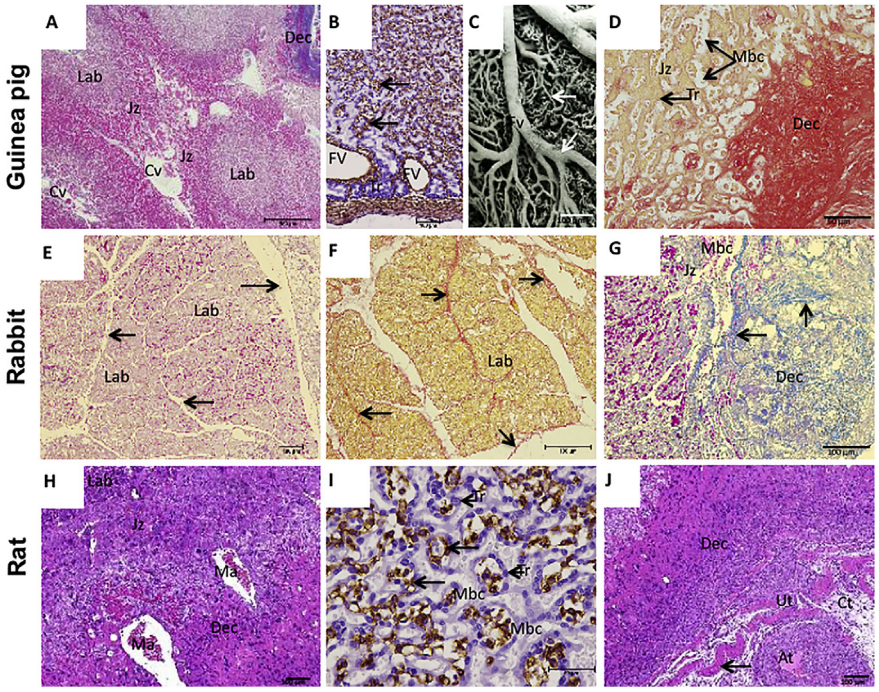

Fig. 1. Native placentas and their ECM in guinea pig [A-D], rabbit [E-G] and rat [H-J]. [A] Masson's Trichrome staining. Lobules containing of outer junctional zone (Jz) and central labyrinth (Lab) with central large vessels (Cv) in the lobules and decidua (Dec). [B] Immunohistochemistry for vimentin. Fetal vessels (arrows, FV) were positive, associated with vimentin-negative trophoblast (Tr). [C] Fetal vascular casts of the labyrinth with intense ramification (arrows) of fetal vessels (Fv). [D] Picrosirius red staining. Collagen bundles are mainly located in the decidua (Dec) and near to junctional zone (Jz), which are formed by trophoblast cells (Tr) and maternal blood channels (Mbc). [E] Masson's Trichrome staining. Deep subdivision of the rabbit labyrinth (Lab) in small lobules by septae (arrows). [F] Picrosirius red staining. A central collagen septae (arrows) branched inside the labyrinth (Lab). [G] Masson's Trichrome staining. Collagen fibers (arrows) with different thickness in the decidua (Dec). Jz = junctional zone. Mbc = maternal blood channel. [H] Hematoxilin-eosin staining. Rat placenta organized in labyrinth (Lab), junctional zone (Jz) and decidua (Dec) compartments. Ma = Maternal arteries. [I] Immunohistochemistry for vimentin. Fetal vessels (arrows) in the labyrinth were positive, lined by trophoblast cells (Tr) that faced towards the maternal blood channels (Mbc). [J] Picrosirius red staining. ECM of the decidua (Dec). The different spongiotrophoblasts (SZ) in the junctional zone and the decidua (Dec) showed intense staining. Lab = labyrinth and arrows = maternal blood spaces in the junctional zone. Scale bars: In A and D: 50μm/In B, C, E, F, G, H and I: 100 μm.

Table 1
Expression of extracellular matrix proteins in the different compartments (labyrinth – Lab, junctional zone – Jz and decidua – dec) of hemochorial placental types.

Marker	Guinea			Rabbit			Rat		
	Lab	Jz	Dec	Lab	Jz	Dec	Lab	Jz	Dec
Collagen I	+	+ +	+ + +	+	+ +	+ + +	+	+ +	+ + +
Collagen III	+	+	+ + +	+	+ +	+ + +	+	+ +	+ + +
Collagen IV	+	+ +	+ + +	+	+ +	+ + +	+	+ +	+ + +
Fibronectin	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Laminin	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

applications in the human [12–23]. However, among various tissues that can be used for tissue engineering, placentas demonstrate a possible tool to be used due to its rich extracellular matrix composition [8,24–27]. Recently, decellularized fetal tissues was shown to possess more coiled fibers and fibronectin than adult bioscaffolds [6]. Fibronectins are multi domain glycoproteins responsible for the recruitment and binding of globular molecules (collagens, fibrin and heparin sulfate) from the extracellular space and for cell surface receptors. In vertebrates they are playing a key role for cell adhesion, migration, growth and differentiation [28–30]. Thus, fetal tissues such as placentas demonstrate be a possible promising tool for tissue engineering purposes in animals [6,8,24,31–33]. According demonstrated by Nagomi et al. (2016) the use of human amnion decellularized in interaction with mesenchymal stem cells were favorable for cartilage repair [7].

In particular, the placenta is a good study model for being an important embryological organ responsible for feto-maternal exchange during pregnancy, easily accessible and ethically acceptable tissue source. Moreover, placentas have abundant ECM including well-preserved endogenous growth factors, such as insulin-like growth factor-1, fibroblast growth factor-2, vascular endothelial growth factor or transforming growth factor-b [34–37]. Furthermore, placentas offer the possibility to perform decellularization of a whole organ with intact 3-dimensional ECM architecture and vascular spaces. The discoidal, villous human placenta is hemochorial [38] and has extensive vascular spaces and branches derived from umbilical cord vessels that are maintained after decellularization. This natural layout facilitates the anastomose to the host [39]. Consequently, placenta-derived scaffolds may provide adequate access to the host vasculature, allowing for *in vivo* survival of other transplanted tissues. Fully decellularized vessel grafts isolated from the chorionic plate of human placenta have been recellularized by endothelial cells to build functional vessel-like structures [40]. Recently, studies have demonstrated the use of decellularized canine placenta scaffolds for recellularization using yolk sac cell with vascular endothelial growth factor (VEGF) for potential use in regenerative medicine [26,33].

Although animal models are essential to further understand and test these biological scaffolds, no model fully resembles the human condition [41,42]. Nevertheless, alike humans, discoidal hemochorial placentas are also found in rodents and lagomorphs [43]. These placentas are labyrinthine in nature and have structurally defined regions, including the labyrinth, junctional zone and decidua. The number of trophoblastic layers in the placental barrier as the border between fetal and maternal blood systems differs in having either three layers (hemotrichorial, e.g. in mice and rats), two layers (hemodichorial, e.g. in rabbits) or just one (hemomonochorial, e.g. in guinea pigs) [38,44–47].

To understand differences and usability of such animal models for producing biological scaffolds, we studied three placental decellularization processes testing protocols from other organs [48,49] in three

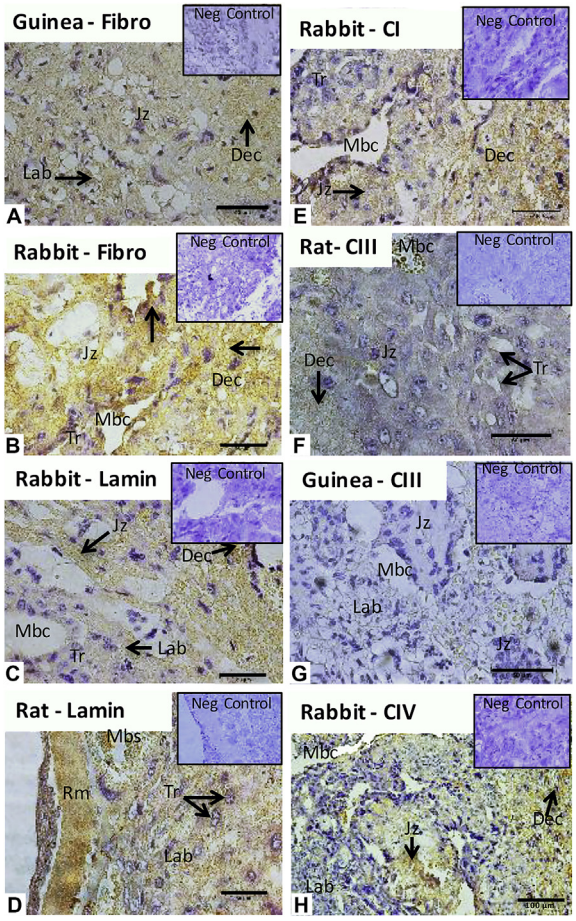

Fig. 2. Immunohistochemistry of ECM components in placental areas. [A] Guinea pig. Abundant expression of fibronectin in labyrinth (Lab), junctional zone (Jz) and decidua (dec). [B] Rabbit. Thicker fibronectin-positive bundles were mainly located in the junctional zone (Jz) and decidua (Dec), surrounding large maternal vessels (arrows). Tr = trophoblast cells. Mbc = maternal blood channel. [C] Rabbit. Laminin expression in decidua (Dec), junctional zone (Jz) and labyrinth (Lab). Ma = maternal artery, Tr = trophoblast cells and Mbc = maternal blood channel. [D] Rat. Expression of laminin in the Reichert's membrane (Rm) and labyrinth (Lab). Tr = trophoblast cells and Mbc = maternal blood channel. [E] Rabbit. Collagen I positive fibers in the junctional zone (Jz) and decidua (Dec). Tr = trophoblast cells. Mbc = maternal blood channel. [F] Rat. Expression of collagen III near to trophoblast cells (Tr) in the junctional zone (Jz) and in decidua (Dec). Mbc = maternal blood channel. [G] Rabbit. Collagen IV expressed in junctional zone (Jz) and decidua (Dec). Discreet expression in the labyrinth (Lab). Mbc = maternal blood channel. [H] Guinea pig. Discreet expression of collagen III in the labyrinth (Lab) and junctional zone (Jz). Mbc = maternal blood channel. Scale bars: In E and F: 50μm/In A, B, C, D, G and H: 100 μm.

species respecting the main placental types: guinea pig (*Cavia porcellus*), rat (*Rattus norvegicus*) and rabbit (*Oryctolagus cuniculus*). Special attention is drawn to the composition and arrangement of the ECM in the placental regions before and after decellularization, studied by means of scanning electron microscopy (SEM), histology, immunohistochemistry and immunofluorescence. To control the decellularization protocols, corrosion casts were used to preserve and study the placental ECM architecture. Finally, we discussed characteristics to validate our bioscaffolds from placentas as potential microenvironments for purposes in the future using stem cells to recellularization in regenerative medicine applications.

2. Materials and methods

2.1. Samples

We studied the ECM before and after decellularization in mature placentas that had all main regions. In addition, two stages of the guinea pig were used. Placentas of rats (n = 8, 18–20 days), guinea pigs (n = 8, 30–40 days and near term) and rabbits (n = 6, 28 days of gestation) were obtained from the Animal Facilities at the School of Veterinary Medicine and Animal Science. Estimations of the gestational age were done on the basis of crown-rump-lengths [50]. Experiments were approved by the Committee of Bioethics (Protocol 13.045).

2.2. Decellularization protocols

Decellularization was performed in whole placentas of guinea pigs and rabbits with agitation at 165 rpm (Biomixer, TS-200A VDRL Shaker). Due to the small size and sensibility of rat placenta, uterine wall, fetal membranes and the placenta were processed in total by the same procedure. First, both umbilical arteries and veins were cannulated and washed with PBS solution to remove blood cell components. Samples were immersed in detergent Sodium Dodecyl Sulfate (SDS)/ 10 mM Tris HCl at pH 7.8, supplemented with 1% antibiotic solution (Penicillin G 10.00 U mL, 25 mg mL, Streptomycin 10.000 mg mL, Invitrogen, Carlsbad, CA, USA) for 3 days.

In order to identify the best protocol, we tested different SDS concentrations (1.0, 0.5, 0.25, and 0.1%), combined with Triton X-100, EDTA and trypsin. SDS solution was replaced once a day. Then, 3 washes in PBS with 1% antibiotic solution were done (20 min each), followed by immersion in 5 mM EDTA (Ethylenediamine Tetraacetic Acid) with 0.05% trypsin (Invitrogen, Carlsbad, CA, USA) for 1 day and immersion in 1% Triton (Sigma, St. Louis, Mo. USA) for 1 additional day. Three final PBS washes were performed to remove residual detergents, followed by 3 washes in 70% alcohol and subsequently processed for further analysis.

2.3. Histology

Native and decellularized placentas, fixed in 4% paraformaldehyde, were transversally divided; then dehydrated in increasing concentrations of ethanol solutions (70–100%), diaphanized in xylene (2 times) and embedded in paraffin. The paraffin blocks were cut into 5 μm sections by an automatic microtome (Leica, RM2165, Nussloch, Germany), stained with Hematoxylin-Eosin (HE), Masson's Trichrome or Picrosirius red, and analyzed by an Olympus BX40, Zeiss KS400 microscope (Carl-Zeiss, Oberkochen, Germany).

2.4. Immunohistochemistry

Native placentas were subjected to immunohistochemistry using primary antibodies for: vimentin (dilution 1:400, mouse, sc-73259, Santa Cruz Biotechnology, Santa Cruz, California, USA), collagen I (dilution 1:100, rabbit, Rockland 600-401-103S, Limerick, Pennsylvania, USA), collagen III (dilution 1:100, mouse, Quartett 1-CO078-05, Berlin, Germany), collagen IV (dilution 1:100, rabbit, Quartett 1-CO083-01, Berlin, Germany), laminin (dilution 1:100, rabbit, Bioss bs-8561R, Woburn, Massachusetts, USA), and fibronectin (dilution 1:100, rabbit, Novus Biologicals NBP1-91258F, Littleton, USA), following a protocol formerly used by our group [51]. Incubations with the secondary antibody were performed using the Dako AdvanceTM HRP kit (Dako, K4065, Carpinteria, California, USA) for 30 min at room temperature. Negative controls were performed using IgG (Goat anti-Mouse IgG – AP 308F, Chemical International, Temecula, California, USA) to substitute the primary antibody.

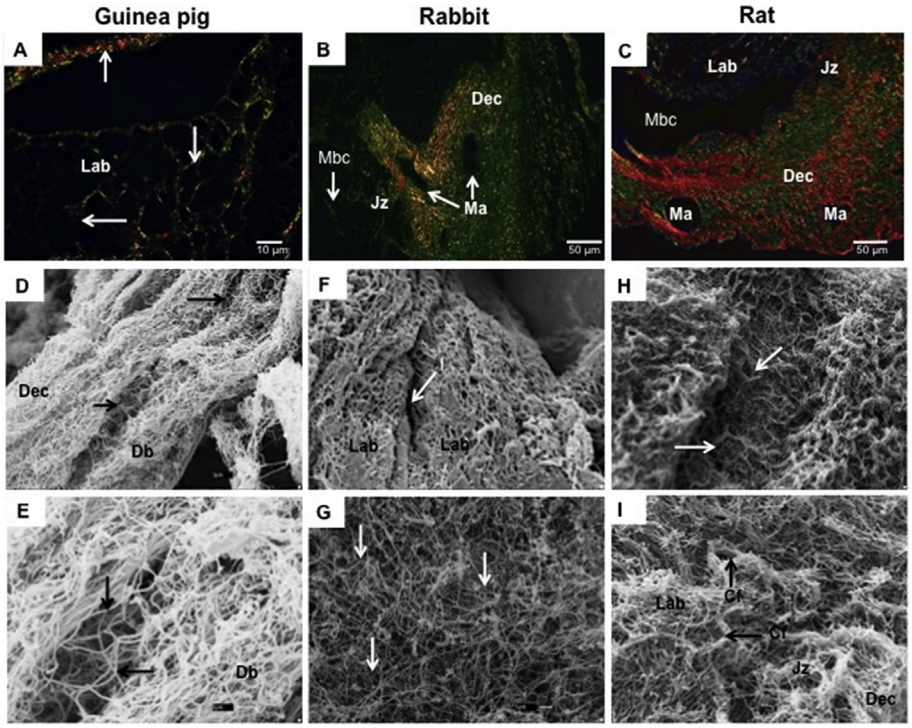

Fig. 3. Distribution and arrangement of ECM fibers in native placentas [A-C] Picrosirius red staining under polarized light microscopy. [D-I] NaOH corrosion in scanning electron microscopy (SEM). [A] Guinea pig. Birefringence of collagen fibers in the labyrinth (Lab) along spaces previously occupied by vessels (arrows). [B] Rabbit. Different intensities of birefringence fibers in the junctional zone (Jz) near to maternal blood channels (Mbc) and decidua (Dec) around maternal arteries (Ma). [C] Rat. Labyrinth (Lab) showed thinner fibers with birefringence. Thicker fibers were distributed mainly in decidua (Dec) and junctional zone (Jz), surrounding maternal arteries (Ma) and maternal blood channels (Mbc). [D,E]. Guinea pig. Thin, dispersed (arrows) collagen fibers in the middle of the lobules. Dense bundles (Db) in the outer areas of lobules and decidua (Dec). [F,G]. Rabbit. Deep invaginations (I) formed the subdivision of the labyrinth (Lab) that were composed by very thin collagen fibers (arrows) with multiple connections. [H,I] Rat. A "honeycomb-shape" arrangement (arrows) in the labyrinth with circular and thin collagen fibers (Cf). Dense bundles of collagen occurred in the junctional zone (Jz) and decidua (Dec). Scale bars: In A: 10μm/In B and C: 50μm/In D, E, F, G, H and I: 1 μm.

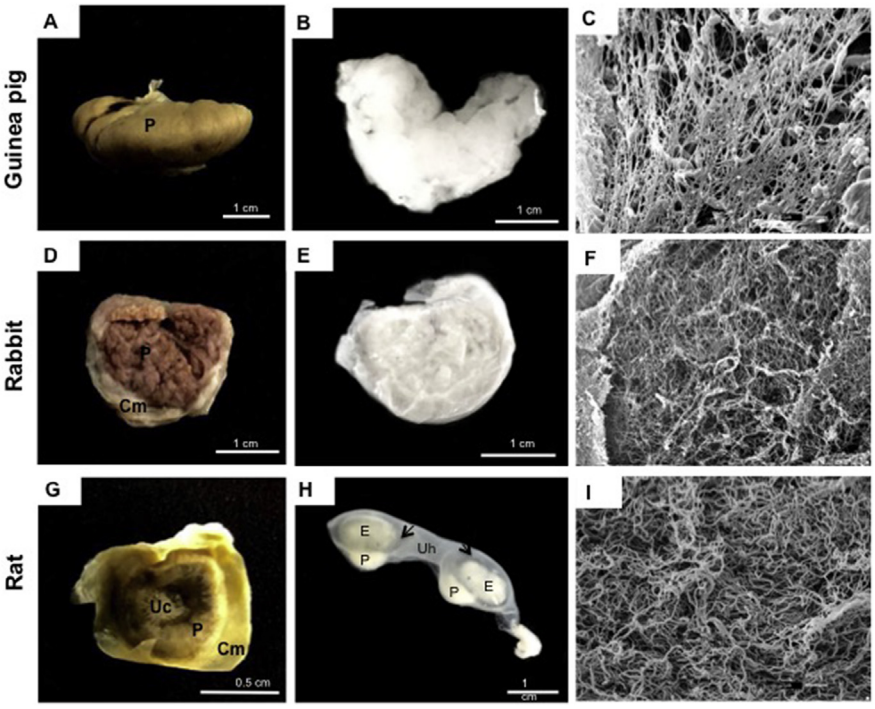

Fig. 4. Macroscopy of native and decellularized placentas of guinea pig and SEM respectively [A, B, C], rabbit [D, E, F] and rat [G, H, I]. Appearance and color of chanced after decellularization (0.25% SDS) into a homogeneous, white-translucent aspect. P = placenta, V = blood vessels, Am = amniotic membrane, Uh = uterine horn, and E = embryo. By SEM **[C, F, I]** the preservation of the collagen fibers in the placenta after the process of decellularization is observed. Scale bars: In A, B, C, D and H: 1cm/In G: 0,5cm/In C, F and I: 1 μm.

2.5. Immunofluorescence

Decellularized and native placentas using the same antibodies for collagen I, III and IV, laminin and fibronectin that are specific for ECM. Sections were rehydrated in ethanol and submitted to peroxidase blockage in 3% hydrogen peroxide (v/v) in ethanol for 20 min. They were placed in 0.1 M citrate buffer at pH 6.0 and submitted to microwave irradiation at 700 MHz for 15 min (3 times of 5 min each). Sections were equilibrated with in PBS, and non-specific binding was than blocked using 10% normal horse serum solution (NHS) and 1.5% Triton (Sigma, St. Louis, Mo. USA). Then, the samples were incubated with primary antibodies for 48 h at 4 °C in a humid chamber. After this, the samples were washed with PBS (3 times) and were incubated with the secondary antibody (dilution 1:500, donkey anti-rabbit IgG Alexa 488, Molecular probes, Oregon, USA) for 1 h at room temperature. Then, tissues were washed in PBS and immersed in 2.6-diamino-2-phenylindole dihydrochloride (DAPI) for 5 min to identify nuclei. Analyses were performed with a Nikon 80i fluorescence microscope (Olympus FluoView™ FV10SW Laser).

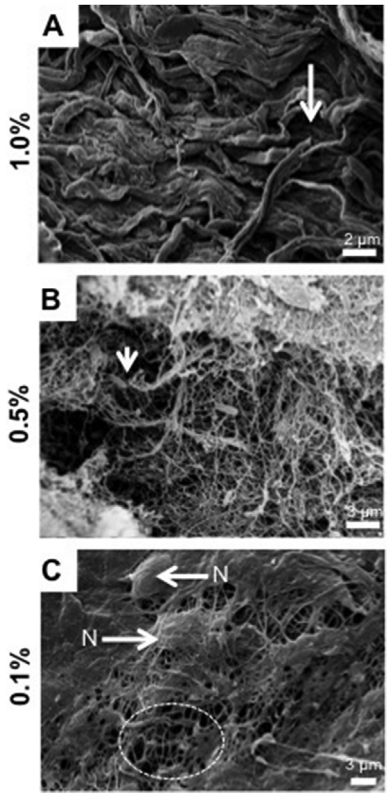

Fig. 5. Scanning electron microscopy analysis of extracellular matrix using different concentrations of SDS [A] 1.0%, [B] 0.5% and [C] 0.1% for decellularization of guinea pig, rat and rabbit placentas, respectively. **[A,B]** Although the higher concentrations of SDS remove the cells, it also resulted in disrupted, disorganized and retracted ECM components (arrows). [C] In contrast, the removal of cells was not completely using low concentration of SDS (0.1%) and cell nuclei were present (N) between preserved ECM fibers (circle). Scale bars: In A 2µm/In B and C: 3 µm.

2.6. Vascular casts

To show the abundance of fetal vessels in the labyrinth, 2 guinea pig near term placentas were injected with Mercox CL-2R (Okenshoji Co., Ltd, Tokyo, Japan) as described by Ref. [52]. After cannulation of a fetal vessel from the umbilical cord, Mercox resin was injected under manual control. Parenchymal tissues were than digested by immersion in 20% NaOH solution at 50–60 °C, rinsed in distilled water and dried in an oven at 37 °C. The casts were refrigerated in 20% gelatin and processed and analyzed by SEM.

2.7. Corrosion casts

To directly control the results of the decellularization protocols, corrosion casts of the tissues that preserved the collagen architecture [53] were done for all 3 species. Samples were immersed in 10% aqueous solution of NaOH for 8–10 days at room temperature, followed by immersion in distilled water for 5–10 days (until the samples were transparent). Then, samples were washed 3 times (10 min each) in 0.1M PBS buffer, pH 7.4, immersed in 1% aqueous solution of tannic acid for 2 h, and post fixed in 2.5% osmium tetroxide (EMS®, Hatfield, Pennsylvania, PA) for 3 h.

Samples then were freeze-fractured after freezing in liquid nitrogen and processed for SEM analysis.

2.8. Scanning electron microscopy (SEM)

The vascular casts and the freeze-fractured placental tissues after corrosion as well as the decellularized placentas were fixed in 2.5% glutaraldehyde in 0.1M PBS buffer, pH 7.4, and post fixed in 2% osmium tetroxide (EMS®, Hatfield, Pennsylvania, USA) for 10 min at 4 °C.

The tissues were dehydrated in crescent series of ethanol (70–100%). After drying by critical point (Balzers Union® Critical Point CPD 020, Liechtenstein, Germany) with liquid CO_2, the material was sputtered with gold in a sputter coater (Emitech®, K500, Ashford, Kent, Great Britain) and analyzed using a SEM microscope (LEO VP 435; Carl-Zeiss).

3. Results

3.1. Native placentas and their ECM components

The guinea pig placentas (3.76 ± 0.17 cm in diameter) were highly lobulated with a central labyrinth (Fig. 1A), build by the endothelium of fetal vessels (vimentin+) and the trophoblast lining of the maternal blood spaces (Fig. 1B, C). The junctional zone surrounded the lobes (Fig. 1A, B), containing trophoblast components and maternal blood spaces (Fig. 1D) as well as a few, dispersed regions of ECM (Fig. 1D). Only in the decidua a rich ECM including collagen fibers was present (Fig. 1A, D).

The bi-lobulated rabbit placentas (2.58 ± 0.13 cm in diameter) were subdivided into smaller lobules of the labyrinth (Fig. 1E). A central, collagen-rich septum ran inside each lobe (Fig. 1F). It was continuous to thick areas of collagen fibers belonging to the decidual ECM (Fig. 1G).

The rat placentas (0.65 ± 0.08 cm in diameter) were not lobulated (Fig. 1H). The labyrinth was the most prominent placental area and contained fetal vessels and trophoblast lining of the maternal blood spaces (Fig. 1I). Only the junctional zone and the decidua contained a dense ECM in the rat, including the walls of maternal blood spaces (Fig. 1J).

Immunohistochemistry revealed a largely similar pattern of expression of markers for ECM in the investigated species (Table 1). Inside the labyrinth, thin fibers, positive for fibronectin (Fig. 2A and B) and laminin (Fig. 2C) occurred between the fetal endothelium and the trophoblast. In contrast, collagen I, III and IV had a low expression in the labyrinth (Table 1). A very thick fibronectin and laminin positive basement membrane, i.e. Reichert's membrane was found at the fetal side of the labyrinth in the rat (Fig. 2D). The dense ECM in the junctional zone that was located between the trophoblast along the maternal blood spaces expressed fibronectin and laminin (Fig. 2A-C), but also collagen I (Fig. 2E), collagen III (Fig. 2 F, G) and collagen IV (Fig. 2H) with different intensities (Table 1). In the decidua, all applied ECM markers were strongly positive, especially around the maternal vessels (Fig. 2 A-C, E-G, Table 1).

Picrosirius red staining confirmed a presence of thick collagen bundles in the decidua, surrounding the maternal vessels as well as collagen bundles arranged in multiple orientations in the junctional zone (Fig. 3A–C). These areas had intensities of birefringence varying from greenish yellow to yellowish and reddish orange (Fig. 3B, C). Inside the labyrinth, only thin collagen fibers surrounded the spaces occupied by trophoblast, fetal vessels and maternal blood spaces (Fig. 3A, C). Also, the Reichert's membrane and the basal membrane of the chorioallantoic placenta showed an intense staining (Fig. 3A).

In the corrosion casts of guinea pig placentas, the well-preserved collagen fibers of the ECM were arranged in dense bundles in the outer areas of the lobes (Fig. 3D). Central areas had large spaces between the thin, circular-arranged fibers that followed the course of the vasculature (Fig. 3E). In the rabbit, the small lobes of the labyrinth contained very thin collagen fibers, organized in all directions with multiple connections (Fig. 3F, G). In the rat labyrinth, a circular arrangement of thin collagen fibers was observed (Fig. 3H), situated in areas where formerly fetal endothelial and trophoblast cells occurred. Dense bundles of collagen fibers were found only in the junctional zone and decidua

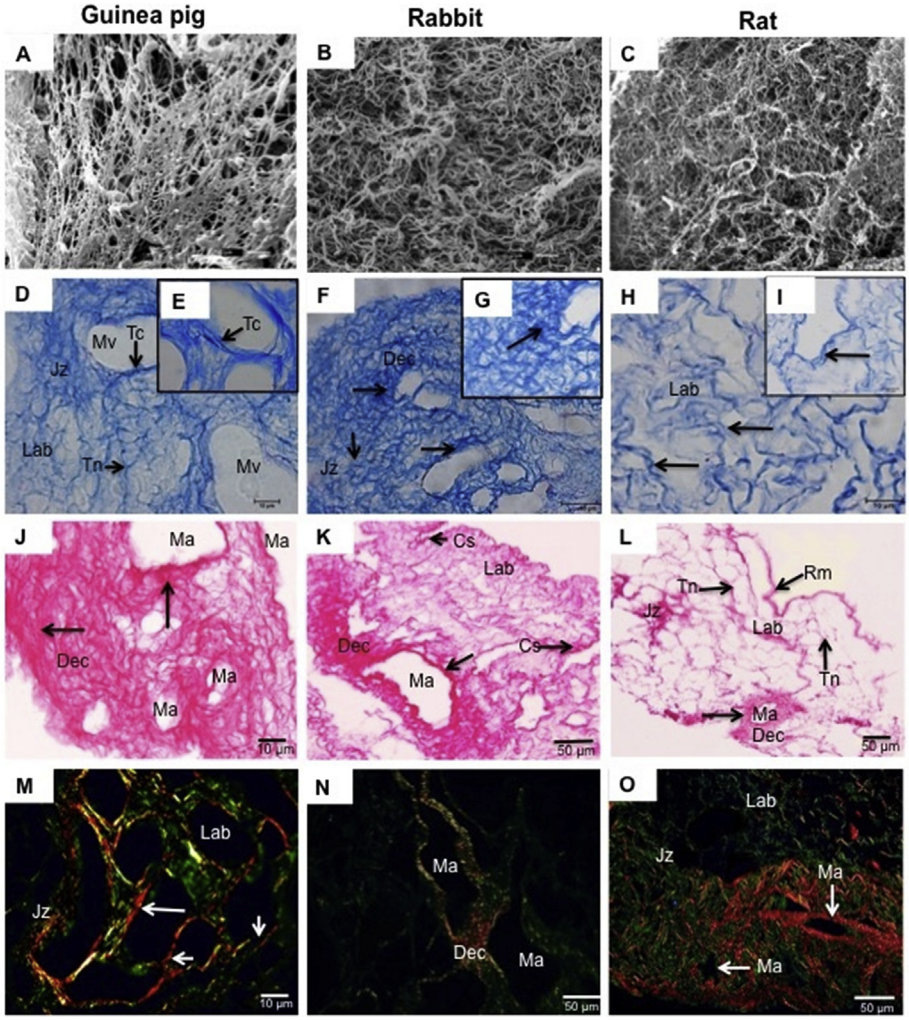

Fig. 6. Ultrastructure and histology of ECM after decellularization. [A-C] SEM of guinea pig, rabbit and rat ECM, respectively, showed the absence of cells and maintenance of the structure and arrangement of collagen fibers, evidencing vasculature preserved after the process of decellularization (arrow). [D, E] Guinea pig. Masson's Trichrome staining. Thick collagen fibers (Tc) near to spaces previously occupied by maternal vessels (Mv) in the junctional zone (Jz). Thin collagen fibers (Tn) in the labyrinth (Lab). [F,G] Rabbit. Masson's Trichrome staining. Thick bundles of collagen fibers (arrows) in the junctional zone (Jz) and decidua (Dec). [H, I] Rat. Masson's Trichrome staining. Labyrinth (lab) with very thin collagen fibers (arrows). [J-L] Picrosirius red staining of guinea pig, rabbit and rat, respectively. Thick bundles of collagen fibers (arrows) in the decidua (dec), near to original spaces of maternal arteries (Ma). In the rabbit, central septae (Cs) of lobes and junctional zone (Jz). Thinner collagen fibers (Tn) occurred in the labyrinth (Lab). Collagen fibers of the Reichert's membrane (Rm) were strongly positive in the rat. [M-O] Picrosirius red staining samples under polarized light microscopy of guinea pig, rabbit and rat matrices, respectively. In the labyrinth (Lab) birefringent collagen fibers with different colors (arrows) along spaces formerly occupied by vessels and trophoblast cells (arrows). The junctional zone (Jz) and decidua (Dec) had dense bundles of birefringent collagen fibers along large maternal vessels (Ma). Scale bars: In A, B, C: 1µm/D, J and M: 10 µm/In F, H, K, L, N and O: 50 µm/In E, G and I: 20 µm.

(Fig. 3I).

3.2. Decellularized placentas and their ECM

Among pilot protocols, decellularization was best achieved by 3 days in 0.25% SDS, 1 day in 5 mM EDTA/0.05% trypsin and 1 day in 1% Triton. It worked for guinea pig and rabbit placentas and intact uteri of the rat (Fig. 4A–F). Tissues were white-translucent with gelatinous consistence after decellularization, but maintained the anatomical arrangement and size (Fig. 4B,D,F).

Severe problems occurred when using other concentrations. In higher concentration of SDS (1.0%), the placental disks almost dissolved after 1 or 2 days of immersion. We found that cells were removed, but that the ECM was disrupted, disorganized and contained signals of retraction (Fig. 5A). In such material, it was not possible to identify placental regions. Medium concentration of SDS (0.5%) preserved the ECM only slightly better. In particular, thin collagen fibers were absent and the thicker ones were disconnected (Fig. 5B). In contrast, by low concentration of SDS (0.1%) a removal of cells was incomplete (Fig. 5A).

Only using 0.25% SDS resulted in the integrity of the ECM, i.e. the presence of thin and thick collagen fibers in their natural arrangement, while cell nuclei, cell remnants or debris were absent (Fig. 6A-C). Masson's Trichrome staining showed a blue network of collagen fibers in guinea pig (Fig. 6D and E), rabbit (Fig. 6F and G) and rat (Fig. 6H, I) placenta, likewise varying in intensity in the placental areas. Picrosirius red staining resulted in thick collagen bundles maintained in the matrices of junctional zone and decidua, whereas thin fibers were found in the labyrinth (Fig. 6J, L). In the decidua, dense collagen fibers originally belonging to the tunica layers of the maternal arteries maintained a circular arrangement (Fig. 6K, L). In the labyrinth, thin collagen fibers remained intact with reddish orange fibers in the border, completed by green fibers in the middle in polarized light microscopy (Fig. 6M). The different intensities of birefringence in the decidua and junctional zone were also preserved, especially the intensities varying from yellowish to reddish orange on collagen fibers surrounding the spaces occupied by the maternal arteries (Fig. 6N, O).

Complete removal of cellular components in our successful protocol was confirmed by confocal analysis after DAPI staining (Fig. 7). Also, immunofluorescence showed a structural integrity of the ECM in decellularized placentas and was observed a similar expression pattern of ECM markers for all analyzed species, according to the placental compartment. Fibronectin was present in all placental compartments for the three analyzed species, as demonstrated for guinea pig (Fig. 7A) and rat (Fig. 7B), similar to the laminin that had a lower intensity and was found along the basement membranes and in the borders of large placental vessels (Fig. 7C). Collagen I occurred surrounding the wall of vessels and near to gaps formerly occupied by trophoblast (giant) cells in the junctional zone (Fig. 7D and E), similar to collagen III that also was found along former decidual vessels (Fig. 7F) and in the trabecular septa of rabbit labyrinth (Fig. 7G). Collagen IV occurred in the Reichert's membrane and along large maternal blood spaces (Fig. 7H).

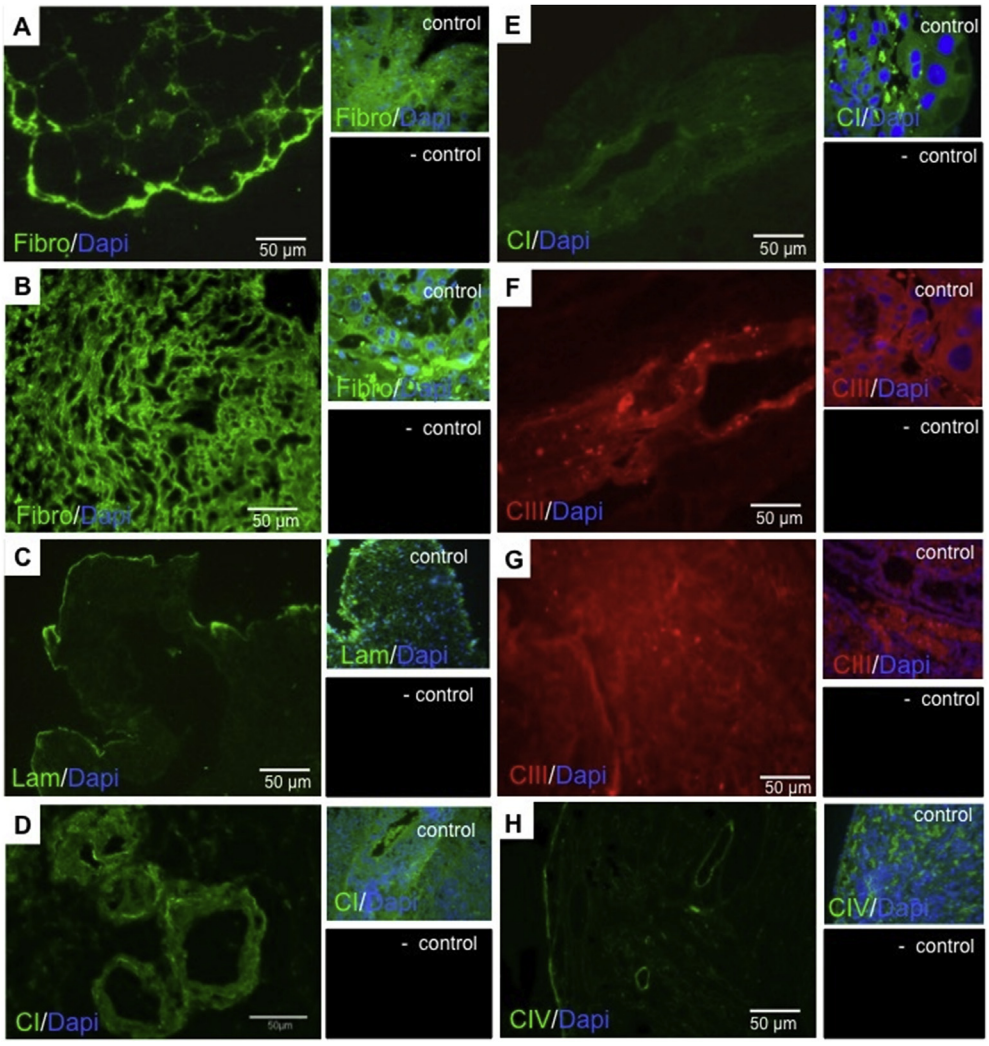

Fig. 7. Expression of extracellular matrix markers in decellularized rodent and lagomorph placentas matrices using confocal microscopy. [A-B] Fibronectin, guinea pig and rat, respectively. Expression of fibronectin occurred in all placental compartments. [C] Laminin, rabbit. Laminin is present especially in basal membranes. [D-E] Collagen I, guinea pig and rat, respectively. CI was observed surrounding large vessels and in the junctional zone. [F-G] Collagen III, rat and rabbit, respectively. Collagen III mainly expressed in junctional zone and decidua near to trophoblastic giant cells, including in the trabecular septae of labyrinth. [H] Collagen IV, guinea pig. Collagen IV occurred in basal membranes and surrounding large blood vessels. Scale bars: In A–H: 50 µm.

4. Discussion

Rich collagen scaffolds were obtained from hemochorial placentas of guinea pig, rat and rabbit. Our most efficient protocol with 0.25% SDS provided a complete cell and DNA removal with intact arrangement and integrity of the ECM after 5 days of immersion, following the standards for decellularization [48,49,54] and confirming that protocols containing SDS lead to bioscaffolds [6,49,55]. Decellularization indeed allowed a preservation of internal vascular arrangement in our scaffolds, which was formerly identified as an important factor for successful transplantations [39,56]. Also, our data showed variations in structure, arrangement, type and abundance of ECM molecules.

Similar to other fetal tissues [6], our placental matrices are rich in fibronectin. The presence of this multidomain glycoprotein directly influence a variety of fundamental processes such as adhesion, migration, proliferation and differentiation of cells [28–30,57]. Fibronectin was frequent in the junctional zone and decidua, where trophoblast is in contact to endometrial ECM. Thus, it may lead to the adhesion of trophoblast cells to maternal components inside hemochorial placentas [9,58]. In addition, our decellularized placental matrices preserved laminin as another glycoprotein with abundant distribution, confirming results on other species [59–61]. Laminin is produced by cytotrophoblast of the villi in human placentas [62] and by giant cells of the junctional zone in rodents [63]. It is important for implantation and placental differentiation [9], likely performing cell adhesion [64]. It is common also in other fetal membranes, i.e. amnion and chorion [65,66].

The collagen system is mainly involved with tissue flexibility and specificity. It is found near implantation sites and in intercellular spaces in early placentas of rats and mice [67,68], and is later related to stromal architecture remodeling [69–71]. As in our results, the distribution and abundance of collagen types vary in the placenta of human and macaque monkey [9,60,61]. We also found collagen IV in the basal membranes, where it contributes to the development and maintenance of the ECM and may fix other basal membrane components [72]. Expression of collagen III is associated with trophoblast along the large maternal vessels and blood spaces in junctional zone and decidua, confirming a similar pattern of type III pro-collagen in rats of days 9.5 and 13 of gestation [73]. Collagen III is present in the mouse decidua and endometrium [74], related to decidualization processes and forming thick, irregular collagen fibrils [75,76] as found in our analysis. Likely it forms tissues that need greater elasticity instead of resistance [9].

We observe the similar expression and distribution the ECM components in native and decellularized tissues, confirming that the proteins of rodents and lagomorph placentas was preserved after the decellularization process, aiming use the scaffolds for cell growth in culture and tissue remodeling of damage areas in tissue engineering, as observed by Ref. [8]. Likewise to human placentas [40], vascular decellularized animal placenta may be used for 3-dimensional stem cell cultivation and small diameter vessel replacement in engineering approaches that will be available for experimental tests. The human

placenta has been used clinically for tissue regeneration, however using their extracellular matrix content to produce gel, extracts or dry sheet [8,77–81] and not their original 3D structure as a graft or a cell seed vehicle. More recently, pieces of human placenta were produced aiming liver regeneration [82]. Also, fetal attachments (membranes and umbilical cord) is more intensely used in clinical approach than the chorioallantoic placenta [83,84].

For that, the following aspects should be considered: (1) As described for other organs [10,49] the composition and abundance of ECM molecules vary according to the applied decellularization protocols [49,54,85] and even subtle changes in the protocols may affect the cell removal efficiency [49]. Our results showed that placentas are very sensitive in that regard. (2) Since growth and development of placental areas are dynamic in rodents [45], the gestational age of the material may have an effect to the volume of fetal and maternal tissues, as well as the distribution of ECM molecules. (3) Scaffolds derived from decellularized rodent and lagomorph placentas are medium/small-sized and may be especially useful as grafts for surgical use to replace significant tissue loss. (4) Decellularized bioscaffolds have more antigen expression and are less immunogenic that synthetic scaffolds [86–88]. (5) Our data showed that scaffolds could be derived from all types of placental barrier that are varying in the number of trophoblast layers between the maternal and fetal circulations. Since the cell layers are removed by decellularization, a similar structure resulted with relatively thin ECM fibers for the labyrinth and more prominent ones in the other areas. (6) Differences between species in the overall structure of the placenta may be relevant for applications. The lobulated structure of placental stroma in the guinea pig resulted in extended areas of thin ECM fibers with wide spaces in between, resembling a lung-like tissue. The decidua was the most relevant area in the small-sized decellularized placenta of the rat that maintained a dense bundle of ECM fibers. Consequently, scaffolds derived from rodents placentas should be used in damaged tissues that need resistance, such as cartilages. (7) The anatomy and natural diversity of ECM components in the placental scaffolds of these animal models may contribute to applications, e.g. tests in regard to growth and proliferation of different cell types and their potential for migration, the establishment of anastomosis with various host tissues and the usability for *in vivo* studies and transplants. (8) The potential applications of placental matrices are not restricted to Regenerative Medicine [24,89–91]. In addition, a decellularized ECM may also contribute to cell therapy studies, as they may increase as endogenous stem cells and can be used as a vehicle for repair in damaged tissues and organs [92].

5. Conclusion

In conclusion, we successfully established a decellularization protocol to obtain biological scaffolds from the hemochorial placentas of three animal model species that preserved their ECM components not only in the stroma, but also by maintaining the vascular spaces. Consequently, such placental scaffolds could be a promising tool to develop microenvironments for subsequently introduced (stem) cells to produce vital grafts with easy access to the vascular system of a host's inner organs. Thus, once we validate our decellularized placenta scaffold as biological biomaterial, future studies are needed to evaluate the biocompatibility and future use in cell therapy.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.04.002.

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