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Electrospun gelatin scaffolds incorporating rat decellularized brain extracellular matrix for neural tissue engineering

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ABSTRACT

The fabrication of an instructive bioabsorbable scaffold is one of the main goals for tissue engineering applications. In this regard, genipin cross-linked gelatin scaffolds, produced by electrospinning, were tested as a platform to include decellularized rat brain extracellular matrix as an active agent to provide fundamental biochemical cues to the seeded cells. This approach is expected to furnish a suitable natural-based polymeric scaffold with sufficient temporal stability to support cell attachment and spreading, also providing tissue-specific signals that can contribute to the expression of the requested cellular phenotype. We first demonstrated the effectiveness of the proposed decellularization protocol and the cytocompatibility of the resulting brain matrix. Then, the *in vitro* biological assays of the conditioned electrospun scaffolds, using rat allogeneic mesenchymal stromal cells, confirmed their biocompatibility and showed a differentiative potential in presence of just 1% w/w decellularized rat brain extracellular matrix.

1. Introduction

The natural extracellular matrix (ECM), being a complex mixture of structural and functional proteins, can be considered as an inductive means for the development of active tissue engineered scaffolds [1]. This approach might lead to a potential alternative for the treatment of critical affections like those related to the central nervous system (CNS), that, due to the failure of neural regeneration, can result in permanent disability [2]. The development of an effective neural therapeutic strategy is of fundamental relevance since neurological, neurosurgical and psychiatric diseases account for one-third of the burden of all diseases in the developed world [3]. Loss of cells and tissue disrupt the normal brain architecture, inhibiting tissue regeneration, mainly due to a lack of matrix and structural support [4,5]. Indeed, it has been demonstrated that the interaction of cells, either transplanted or migrating endogenous stem cells, with the ECM plays a key role in brain healing and regeneration [6–10]. There is, therefore, a great need to develop new tools to reconstitute the native ECM and the tissue architecture of the damaged CNS. For this aim, it is necessary to bridge the tissue gap and provide supporting substrates. It should be considered that the cerebral ECM has a unique composition as it contains relatively small amounts of fibrous proteins, such as collagen, laminin and fibronectin, and high amounts of linear polysaccharides, such as glycosaminoglycans [11,12]. Appropriate biomaterials, to be processed for mimicking a three-dimensional instructive microenvironment with specific biochemical cues, and promoting cell migration, adhesion and survival, could therefore enhance the success of neural implants [13].

In this regard, the present study proposes a genipin cross-linked gelatin electrospun scaffold incorporating rat decellularized brain extracellular matrix (dBECM) as a potential improvement for cell adhesion, proliferation and differentiation and, as a consequence, for nervous tissue regeneration. In order to provide a morphological contribution to the seeded cells, electrospinning was selected as a cost-effective technique to produce fibrous dBECM-gelatin mats. Based on the same technique, previous studies showed the ability to produce (*i*) electrospun adipose tissue-derived ECM for

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adipose stem cell culture [14], (*ii*) hybrid mats composed of porcine urinary bladder matrix and poly(ester-urethane)urea [15], and (*iii*) hybrid mats by combining electrospun poly(ester-urethane)urea and electrosprayed dermal ECM hydrogel extracted from decellularized adult porcine dermis [16].

A complete evaluation of the effective decellularization of the rat brain was firstly provided. Then, the collected electrospun mats were characterized by means of scanning electron microscopy (SEM) and differential scanning calorimetry (DSC) before and after the cross-linking procedure; cytocompatibility was assessed by *in vitro* testing rat mesenchymal stromal cells.

2. Materials and methods

2.1. Materials

Gelatin powder (type A, from porcine skin, M_w ranging from 50,000 to 100,000), phosphate buffered saline (PBS), Triton X-100, deoxycholate, DNAsi, antibiotic and antimycotic solution, papain, sodium acetate, N-acetyl cysteine and EDTA were supplied by Sigma–Aldrich (Milan, Italy), while paraffin, glutaraldehyde, hamatoxylin and eosin (H&E) by Merck (Darmstadt, Germany). Movat pentachromic stain kit was supplied by Diapath (Bergamo, Italy), 4′-6-diamidino-2-phenylindole (DAPI) by Vector Laboratories (CA, USA) and sodium cacodylate buffer (pH 7.2) by Prolabo (Paris, France). Dulbecco's modified Eagle's medium with low glucose (DMEM-LG) was supplied by Gibco-Invitrogen (Milan, Italy), while fetal bovine serum (FBS) by Hyclone (SouthLogan, Utah, USA). Cell WASH (0.1% sodium azide in PBS), CD54-FITC, CD11b-FITC, CD90-PE, GFAP-Alexa Fluor 647 and isotype-matched mouse MAb were supplied by BD Pharmingen (San Jose, CA, USA), CD44-Alexa Fluor 647 by Biolegend (San Diego, CA, USA), while MHCII-FITC by eBioscience (San Diego, CA, USA). Acetic acid was supplied by Carlo Erba Reagenti (Arese (MI), Italy), Genipin by Wako Chemicals GmbH (Neuss, Germany) and Lympholyte-H by Cedarlane (Burlington, Ontario, Canada). Nucleic Acid Purification Lysis Solution was supplied by Applied Biosystems (Foster City, CA), PCR Tissue Homogenizing kit from PBI International (Milan, Italy) and Master Pure™ DNA Purification kit from Epicentre Biotechnologies (Madison, WI). Fastin™ elastin assay kit and Blyscan Glycosaminoglycan Assay kit were provided from Biocolor (Carrickfergus, UK).

All materials and reagents were used as received.

2.2. Study design

Male Brown Norway rats ($n = 25$) (Charles River Laboratories Italia S.r.l., Calco, Italy), weighing 230–320 g, were used as donors of brain tissues and bone marrow. All animals received care in compliance with the "Principles of laboratory animal care" formulated by the National Society for Medical Research and the "Guide for the care and use of laboratory animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised in 1996. The study was approved by the Animal Care and Use Committee and the Bioethics Committee of the University of Florence (Italy). Rats were individually housed and maintained at an environmental temperature of 25 ± 2 °C and on a 12/12 h light/dark cycle. Animals were acclimated for 7 days before experiments.

Whole brains were harvested from donor rats. Brains ($n = 15$) were used for structural (H&E and Movat staining), morphologic (scanning electron microscopy, SEM), matrix content (elastin and glycosaminoglycan quantification) and effectiveness of decellularization (DAPI staining, nuclear counting, DNA quantification) evaluation. Decellularized brains ($n = 3$) were used for *in vitro* cytocompatibility studies. Afterwards, brains ($n = 7$) were decellularized, completely dehydrated and used to condition the gelatin scaffolds. Femurs and tibiae were harvested from donor rats ($n = 10$) for bone marrow harvest and used for MSC ($n = 5$) and MNC ($n = 5$) isolation.

2.3. Decellularization process

After harvesting, brain tissues were stored in cold PBS, containing 1% antibiotic and antimycotic solution, and subsequently processed using a modification of a previously described detergent-enzymatic method (DEM) [17], to obtain a complete tissue decellularization without losing most of the brain matrix. Briefly, samples were frozen (at −80 °C) and thawed completely four times, incubated with Milli-Q water (72 h at room temperature), and then processed twice as follows: 1.0% Triton X-100 (60 min), water (30 min), 4.0% deoxycholate (60 min), water (30 min), 2000 KU DNAsi in 1 M NaCl (60 min) and water (30 min). All the decellularization steps were performed using agitated baths at 60 rpm. After the last washing step, brain samples were stored in PBS containing 1% antibiotic and antimycotic solution at 4 °C.

2.4. dBECM characterization

2.4.1. Histological analysis

Parts of brain samples (native and decellularized) were fixed for 24 h in 10% neutral buffered formalin solution in PBS (pH 7.4) at room temperature. They were washed in distilled water, dehydrated in graded alcohol, embedded in paraffin, and sectioned at 5 μm thickness. Adjacent sections were deparaffinized, rehydrated and stained with H&E to evaluate tissue decellularization and morphology. To evaluate the tissue morphology, each sample was also stained with the Movat pentachromic stain kit, according to the manufacturer's protocols.

2.4.2. Assessment of cellular content

To evaluate the remaining cells after DEM, adjacent sections (5 μm thickness) were deparaffinized, rehydrated and stained with DAPI, a fluorescent nucleic acid stain (VECTASHIELD Mounting Medium with DAPI; excitation wavelength 350 nm, emission wavelength 460 nm) for 30 min at room temperature in darkness, and analyzed by fluorescence microscopy.

2.4.3. DNA quantification

To assess DNA quantification within native and decellularized brain matrices, samples ($n = 3$ for each condition) were resuspended in 200 μl Nucleic Acid Purification Lysis Solution and homogenized using PCR Tissue Homogenizing kit in microcentrifuge tubes. DNA was isolated using Master Pure™ DNA Purification kit, which is based on a gentle salt-precipitation protocol to allow a rapid purification of nucleic acids, and successively stored at −80 °C. DNA (diluted 1:50) was quantified by measuring the absorbance in a BIORAD spectrophotometer (*SmartSpec™ Plus spectrophotometer, BIORAD, Milan, Italy*).

2.4.4. Scanning electron microscopy

To qualitatively evaluate decellularized matrix structure, brain (native and decellularized) matrices were fixed with 3% (v/v) glutaraldehyde in a buffered solution of 0.1 M sodium cacodylate buffer (pH 7.2). After rinsing in cacodylate buffer, specimens were dehydrated through an ethanol gradient, critical point dried, sputter coated with gold and observed by means of scanning electron microscopy (SEM; JCM-5000 NeoScope, Nikon).

2.4.5. Elastin content measurement

Insoluble elastin was extracted from native and decellularized samples ($n = 4$ for each condition) as soluble cross-linked polypeptide elastin fragments, using the hot oxalic acid extraction technique. Wet samples (mean weight 187 ± 65 mg and 145 ± 30 mg for native and decellularized samples, respectively) were mixed with oxalic acid (0.25 M) and boiled in a water bath for 1 h. The supernatant was collected by centrifugation, and the sediment was submitted to a second and third extraction under the same conditions. Soluble elastin content in the oxalic extracts was determined using the colorimetric Fastin™ elastin assay kit, based on a fastin dye reagent (5,10,15,20-tetraphenyl-21,23-pophrine tetrasulfonate), following the manufacturer's instructions. Briefly, samples were added with elastin precipitating reagent, incubated for 15 min and centrifuge. The Dye Reagent was then added to allow the formation of elastin-dye complex. After incubation (90 min) and centrifugation, the elastin-dye complex was dissolved by incubation with the dye dissociation reagent for 10 min. Absorbance was measured at 513 nm on a Epoch Microplate Spectrophotometer (*BioTek, VT, USA*). Replicate samples were averaged and corrected by subtracting the blank average, and elastin content was determined from a standard curve constructed using five concentrations (5–25 mg) of α-elastin. Final values were expressed as mg of elastin per wet weight.

2.4.6. Sulfated glycosaminoglycan content measurement

Native and decellularized samples ($n = 4$ for each condition) were digested with 0.2 mg/mL papain in 0.2 M phosphate buffer (pH 6.4) containing 0.1 M sodium acetate, 5 mM N-acetyl cysteine and 10 mM EDTA at 65 °C overnight. Total sulfated glycosaminoglycans (GAG) was quantified using the Blyscan Glycosaminoglycan Assay kit, based on 1,9-dimethyl-methylene blue binding, following the manufacturer's instructions. Briefly, samples were added with Blyscan dye reagent, incubated for 30 min and centrifuge. The insoluble GAG-dye complex was then dissolved by adding the dissociation reagent and incubated for 10 min. Absorbance was measured at 656 nm on a Epoch Microplate Spectrophotometer (*BioTek, VT, USA*). Replicate samples were averaged and corrected by subtracting the blank average, and GAG content was determined from a standard curve constructed using five concentrations (1–5 μg) of GAG. Final values were expressed as μg of GAG per dry weight.

2.5. Scaffold fabrication

dBECM was lyophilized and stored dry until use. dBECM powder (1% w/w with respect to gelatin) was firstly ultrasonicated for 10 min in a mixture of acetic acid/deionized water (9:1), then gelatin powder was added (14% w/v). Neat scaffold was prepared by dissolving gelatin at the same concentration in the same solvent mixture. The resulting solution was then poured into a glass syringe to be electrospun through a blunt tip metallic needle (18G) onto a metallic target in the following conditions: 12 kV applied voltage (Spellman, USA), 0.4 ml/h feed rate (KD Scientific, USA), and 10 cm needle-to-target distance.

All samples were vacuum dried for 48 h and stored in a desiccator.

2.6. Cross-linking procedure

Genipin was dissolved in ethanol at 0.5% w/v and cross-linking was carried out by soaking the electrospun gelatin mats, neat and dBECM loaded, into the alcoholic