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Characterization of a new decellularized bovine pericardial biological mesh: Structural and mechanical properties

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ABSTRACT

Implants made from naturally-derived biomaterials, also called biological meshes or biomeshes, typically derive from decellularized extracellular matrix of either animal or human tissue. Biomeshes have many biomedical applications such as ligament repair, bone and cartilage regeneration and soft tissue replacement. Bovine collagen is one of the most widely used and abundantly available xenogenic materials. In particular, bovine pericardium is widely used as extracellular matrix bioprosthetic tissue. The efficiency of a pericardial mesh to function as scaffold depends on the quality of the decellularization protocol used. Moreover, the biomesh mechanical features are critical for a successful surgical repair process, as they must reproduce the biological properties of the autologous tissue. Different methods of physical, chemical, or enzymatic decellularization exist, but no one has proved to be ideal. Therefore, in the present study, we developed a novel decellularization protocol for a bovine pericardium-derived biomesh. We characterized the biomesh obtained by comparing some ultrastructural, physical and mechanical features to a reference commercial biomesh. Quantification revealed that our novel decellularization process removed about 90% of the native pericardial DNA. Microscopic and ultrastructural analysis documented the maintenance of the physiological structure of the pericardial collagen. Moreover, mechanical tests showed that both the extension and resilience of the new biomesh were statistically higher than the commercial control ones. The results presented in this study demonstrate that our protocol is promising in preparing high quality bovine pericardial biomeshes, encouraging further studies to validate its use in tissue engineering and regenerative medicine protocols.

1. Introduction

Tissue engineering is a multidisciplinary field that includes the production of scaffolds used as supporting materials to restore, maintain or even improve tissue anatomy and function (Zonari et al., 2012). Membranes typically derive from either animal tissue or synthetic polymers. Biological meshes, also called "biomeshes", are constituted of pure collagen matrix derived from human, porcine, or bovine tissue through a decellularization process. Grafted biomeshes act as a regenerative framework that supports remodeling and new collagen deposition. Once implanted, the ideal biomesh is gradually and fully integrated into the host tissue, promoting cellular and vascular regeneration and *de novo* formation of tissues similar to the normal ones (Pascual et al., 2012).

Biomedical research and tissue engineering scientists have identified the essential prerequisites of an ideal biomesh (Wang et al., 2006): a biodegradable material with satisfactory biomechanical behavior, efficient host tissue incorporation, high cell compatibility, and a low inflammatory response (Scheidbach et al., 2004). The characteristics of each graft are unique and depend on the tissue source and the specific protocol used for decellularization (Keane et al., 2012). Usually tissues are processed to remove all components, cells and debris, that can cause an inflammatory immunoreaction following implantation, while retaining as much as possible the three dimensional ultrastructure and

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composition of the native extracellular matrix (ECM) (Schmidt and Baier, 2000). In addition, biomaterials used in reconstructive surgery should be highly compatible with the function of the native tissue, providing mechanical properties matching the tissue to be replaced (Rohrnbauer and Mazza, 2013, 2014).

At present, biomeshes are mainly obtained from five different types of tissue: bovine pericardium, human cadaveric dermis, porcine small intestine submucosa, porcine dermal collagen, and bovine dermal collagen (Bellows et al., 2013). Over the last decade, bovine pericardium-derived biomeshes have been evaluated in numerous studies. In particular, bovine pericardium was used for repairing abdominal defects in a rat model (Zuki et al., 2007), in replacement of aortic valve in the ovary model (Amaral et al., 2010), in the reconstruction of the tendons in the chicken (Sungur et al., 2006), and abdominal surgery for the reinforcement/anastomosis healing (Testini et al., 2014). Several methods of decellularization have been introduced to obtain an appropriate biomesh (Booth et al., 2002; Courtman et al., 1994; Kasimir et al., 2003). Decellularization methods of bovine pericardium include mechanical, chemical, detergent, and enzymatic techniques, or a combination thereof. Each of them has different effects upon both the resulting biologic scaffold and the associated host remodeling response and outcome. However, the combination of the different methods can minimize adverse effects on the remaining matrix constituents of the decellularized tissue (Crapo et al., 2011; Keane et al., 2015).

The aim of the present study was to develop a novel protocol of decellularization to obtain a bovine pericardium biomesh with superior characteristics compared to commercially available ones. Therefore, Assut Europe S.p.A. modified a well-established protocol, developed, on a new substrate (bovine pericardium) produced for human application. To characterize our non-cross-linked biomesh derived from bovine pericardial tissue, called Bioripar®, we analyzed its structural and mechanical properties and compared them to a commercial biomesh, named Tutomesh®, RTI Surgical Inc, USA.

2. Materials and methods

2.1. Tissue preparation

For preparation of Bioripar® mesh (Bioripar®, ASSUT Europe, Rome, Italy), bovine pericardial membranes were obtained from a local slaughterhouse. We selected cattle of the same age from the slaughterhouse to get pericardia with a thickness comprised between 0.4 and 0.7 mm. Immediately after slaughter, the tissue was rinsed with distilled water to remove blood and body fluids and dissected to remove the external fat, then transported in a saline solution (0,9% w/v NaCl). Warm ischemic time was less than 2 h.

2.2. Decellularization protocol

The effective removal of antigenic epitopes associated with cell membranes and intracellular components of tissues and organs is necessary to minimize or avoid an adverse immunologic response by allogeneic and xenogeneic recipients of the scaffold material (Badylak and Gilbert, 2008). To remove all cellular material without adversely affecting the composition, mechanical integrity, and biological activity of the scaffold ECM, four stages of processing of the bovine pericardium were performed:

1. Hypertonic solution treatment: tissues were immersed into hypertonic saline solution 7,5% (w/v) for 20 min to destroy cells, and alternately into purified water to wash residues away; this step was repeated three times at room temperature (RT = 25 °C).
2. Treatments with alkaline and neutralizing solutions: tissues were soaked into phosphate-borate buffer (0.2 M, pH 9.0) for 10 min at RT, to remove most of the antigenicity due to the presence of proteins and proteoglycans of cell membranes. Subsequently, tissues were immersed in a 1 M sodium hydroxide solution (pH > 13.0) for one hour and half, then soaked in a 5% boric acid solution (pH 9) for one hour at RT. Since sodium hydroxide and boric acid solutions digest unnecessary protein and proteoglycan residues, they help with antigen elimination. In addition, this stage completely inactivates most of pathogens.
3. Stabilization: after a thorough washing in purified water, tissues were soaked into a bacteriostatic solution containing propylene glycol 30% in ethanol (w/v) for 15 min at RT.
4. Sterilization: after being cut to size, membranes were packaged in double aluminum pouches and sterilized by γ-irradiation with a 21 kGy (Singh et al., 2016). Membranes were then kept at room temperature until use.

The here presented decellularization protocol differs from the one for the Tutomesh® for the following major points: a) it does not require oxidation with H2O2; b) NaOH is used in alkaline treatment and c) acetone is used in the dehydration stage. This last stage is not present in our protocol.

2.3. DNA isolation and quantification

Residual cell material from the decellularized tissue was evaluated using a low-density microarray (GeneTop Meat V kit, LifeLineLab, Italy) for species identification, able to detect DNA traces ($\geq$ 0.001%). To perform a low-density microarray, genomic DNA was purified from 200 mg of the decellularized tissue (three different pieces for each brand) using the QIAamp® DNA Mini kit (Qiagen, Italy), according to the manufacturer's protocol. DNA concentration was calculated by spectrometric measurements and adjusted to a concentration of 20 ng/μL. Samples of 2.5 μL of the purified DNA were analyzed according to manufacturer's instructions and the presence of a specific spot for the bovine zoological species evaluated. All measurements were performed in triplicate.

2.4. Mechanical characterization

The mechanical properties were characterized by two mechanical tests, namely uniaxial tensile test and burst test. These two tests assess the mesh stiffness by using a load applied in-plane (tensile stiffness) and a load applied perpendicular to the mesh (distension) (Deeken et al., 2011).

To compare mechanical properties of Bioripar® to those of a reference commercial biomesh (Tutomesh®), three pieces of each were used. Membranes were extracted from the sterile package and re-hydrated with saline for at least ten minutes, then they were cut into four scaffolds for the tensile test and one for the bursting strength test. Mechanical tests were carried out in an ISO 17025 Accredited Laboratory (Brachi Testing Services Srl, Prato Italy).

Four specimens of each biomesh were prepared as a dogbone shape of 1 cm wide and 6 cm long, with a narrowed central region approximately 0.4 cm wide and 1.5 cm long (Fig. 1A) using a template sharp. The main thickness of each sample was measured in six different points by using a micrometer. To carry the experiments out under conditions as similar as possible to the *in vivo* ones, all samples were drawn from the central part of the biomesh. Specimens were orientated in different directions to simulate *in vivo* applications. This test allows to determine the ultimate tensile stress and strength and to evaluate the anisotropic characteristics of the biomeshes. Each specimen had one extremity clamped onto a grip and tension was tested by means of a constant strain rate until mesh failure. Specimens were subjected to uniaxial tension at a rate of 300 mm/min crosshead spread by dynamometer (Constant-Rate-of-Extending machine, Hounsfield HS10) following the Grab Method (EN ISO 13934-1 EN ISO 13934-2 with different settings). Stress-strain curves were obtained by dividing the recorded load by the specimen cross-sectional area against nominal strain. Tensile stress was