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Evaluation of decellularization protocols for production of tubular small intestine submucosa scaffolds for use in oesophageal tissue engineering

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ABSTRACT

Small intestine submucosa (SIS) has emerged as one of a number of naturally derived extracellular matrix (ECM) biomaterials currently in clinical use. In addition to clinical applications, ECM materials form the basis for a variety of approaches within tissue engineering research. In our preliminary work it was found that SIS can be consistently and reliably made into tubular scaffolds which confer certain potential advantages. Given that decellularization protocols for SIS are applied to sheet-form SIS, it was hypothesized that a tubular-form SIS would behave differently to pre-existing protocols. In this work, tubular SIS was produced and decellularized by the conventional peracetic acid–agitation method, peracetic acid under perfusion along with two commonly used detergent–perfusion protocols. The aim of this was to produce a tubular SIS that was both adequately decellularized and possessing the mechanical properties which would make it a suitable scaffold for oesophageal tissue engineering, which was one of the goals of this work. Analysis was carried out via mechanical tensile testing, DNA quantification, scanning electron and light microscopy, and a metabolic assay, which was used to give an indication of the biocompatibility of each decellularization method. Both peracetic acid protocols were shown to be unsuitable methods with the agitation-protocol-produced SIS, which was poorly decellularized, and the perfusion protocol resulted in poor mechanical properties. Both detergent-based protocols produced well-decellularized SIS, with no adverse mechanical effects; however, one protocol emerged, SDS/Triton X-100, which proved superior in both respects. However, this SIS showed reduced metabolic activity, and this cytotoxic effect was attributed to residual reagents. Consequently, the use of SIS produced using the detergent SD as the decellularization agent was deemed to be the most suitable, although the elimination of the DNase enzyme would give further improvement.

1. Introduction

The oesophagus as an organ can be affected by a number of medical conditions which may necessitate the need for extensive treatment to correct. These conditions can be congenital, such as oesophageal atresia, which is a paediatric condition where the oesophagus forms incorrectly, or alternatively they can be acquired conditions, such as oesophageal cancer [1–3]. Treatment for these conditions can include complicated surgical procedures, such as gastric transposition or intestinal interposition [4]. These techniques are unfortunately associated with a number of post-operative complications, including stricture formation, anastomotic leakages, dysphagia, dysmotility and gastro-oesophageal reflux, which can itself can lead to abnormal cell growth, potentially leading to neoplasia [5,6]. One approach which may provide an alternative is the use of tissue engineering to creative a viable biomaterial-based replacement for oesophageal tissue.

Clinically successful extracellular matrix (ECM) materials predominantly consist of allogeneic and xenogeneic decellularized tissues. Those tissues commonly in use are urinary bladder matrix, skin, pericardium and small intestine submucosa (SIS), which have all been used for a variety of clinical applications and are derived from human, bovine and porcine sources [7]. The benefits of ECM materials, such as SIS, are related to their effects post-implantation, including the release of growth factors and biologically active cryptic peptides upon degradation, which then influence the important processes of angiogenesis, mononuclear infiltration, cell proliferation, cell migration and cellular differentiation [8,9]. The materials

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are considered to not promote a rejection response but instead elicit a limited immune response restricted to Th2 lymphocytes, in line with graft acceptance, and which differs from the Th1 lymphocyte-mediated response commonly associated with xenogeneic graft rejection [10,11]. There is also an increased presence of alternatively activated M2 macrophages and associated low levels of pro-inflammatory cytokines [12,13]. In summary, the advantages of SIS include relatively rapid degradation by non-immunogenic means and a restricted immune response, in conjunction with the release of growth factors which can contribute towards constructive tissue remodelling and new tissue formation [14].

SIS has successfully been used as an oesophageal patch within *in vivo* models for the repair of the oesophagus [15,16]. However, SIS sutured into a tubular form and used for segmental oesophageal replacement in a canine model did not function well, with stenosis and detachment being the major causes of failure [17]. The mechanical properties, including the stress to failure of the material, were given as one of the principle causes for this failure and it was proposed that, had a biodegradable stent been present during the initial 3 month period, when the majority of failures occurred, it may have been possible to prevent some of the observed failures. This is in accordance with the consensus view that one of the major limitations of ECM materials such as SIS is that they do not possess the mechanical properties for relatively large tissue reconstruction roles or for resisting the stresses present in applications like rotator-cuff injury repair, where clinical trials have been unsuccessful [18,19].

In this work, it was investigated whether SIS could function better for oesophageal tissue engineering if it was produced in an intact tubular form, as opposed to the regular sheet form. It was hypothesized that the lack of a seam would be an advantage not only for the potential for improved performance, but also with a view to allowing further modification of the SIS. The first stage of this was to investigate the creation of a tubular SIS. Peracetic acid under agitation has been the standard decellularization protocol for production of SIS sheets for a number of years [20,21]. However, with the SIS in tubular form, an alternative decellularization protocol was considered necessary to allow for the tissue to be adequately decellularized. Perfusion decellularization protocols have been successfully applied to a variety of tissues, and a large number of protocols and agents exist [22,23]. In this study, the two protocols selected have been used to decellularize a variety of tissues, and these two were compared to peracetic acid under agitation, along with an undecellularized control, to obtain an optimal decellularization protocol for tubular SIS. In addition to this, to draw a fair comparison between the effectiveness of the reagents in the different protocols, peracetic acid was also tested using the perfusion arrangement.

The first method was a perfusion method applied by Ott et al. [24] to decellularize intact rat hearts. This method involved the use of two detergents: sodium dodecyl sulfate (SDS), an anionic detergent, and Triton X-100 (t-octylphenoxypolyethoxyethanol), a non-ionic detergent considered to be non-denaturing [22]. Both detergents are commonly used for cell lysis applications. SDS has been used for a number of years and on a variety of tissue types, including less commonly decellularized tissues, such as the kidney and temporomandibular joint [23]. The use of Triton X-100 has also been quite prominent in the field of tissue engineering and was used for decellularization by Cortijo et al. as early as 1987 [25], when it was applied to a number of different tissues, including the ileum, arteries and muscle tissue. The method used by Ott et al. [26] was replicated by Akhyari et al. and was shown to be one of two better performing methods for whole heart decellularization in a comparison of four different protocols.

The second protocol involved a combination of sodium deoxycholate (SD) and deoxyribonuclease I (DNase). Early work done by Meezan et al. [27] isolated basement membranes from a variety of tissues, including bovine retinal and brain blood vessels, rabbit renal tubules and rat renal glomeruli, all using 4% SD and DNase. A modified version of this method was used in the decellularization of the trachea by Conconi et al. [28]. The method was used in the form of repeated cycles, which had the effect of producing a decellularized donor trachea, which was subsequently implanted into a patient [29]. This method was also then used by Totonelli et al. [30], who applied it to the decellularization of the rat small intestine using a perfusion-based method. In summary, the method described has shown a degree of effectiveness on a number of tissues when used in a perfusion-based protocol. It has been applied to scaffolds which have then gone on to be successfully implanted into a patient, and is therefore highlighted as an effective method for perfusion decellularization.

For any ECM to function *in vivo* it must be adequately decellularized, as the presence of cell membrane epitopes and xenogeneic or allogeneic DNA could result in an adverse immune response upon implantation [31]. In reviewing decellularization, Crapo and Gilbert [23] proposed three criteria for satisfactory decellularization, two of which are suitable for this work, namely: <50 ng of double stranded DNA (dsDNA) per mg of ECM (dry weight) and a lack of visible nuclear material in tissue sections stained with 4′,6-diamidino-2-phenylindole or haematoxylin and eosin (H&E). The third criterion suggested was a < 200 bp DNA fragment length, but this is beyond the scope of this work. It should be stated that these criteria are being used herein as no substantial work has been carried out which establishes the threshold level of cellular remnants in ECM materials that elicits a negative remodelling response. Additionally, should any of the protocols produce a SIS which is mechanically unsound, it would not be considered a suitable protocol.

2. Materials and methods

Frozen samples of porcine small intestine were obtained at a local abattoir from animals that were 6 months in age. The jejunum/ileum samples were cut into lengths of 20 cm and the excess mesentery tissue removed (Fig. 1a). The samples were rinsed thoroughly with deionized water to remove debris.

The SIS was isolated using slight modifications to the well-established method [32,33]. The sections of small intestine were inverted and then gently abraded using moist surgical gauze to remove the inner mucosal layer. The tissue was thoroughly washed with deionized water and then re-inverted, and the serosal layer was then taken off by first creating an incision with a scalpel, then manually removing the layer. Finally, the outer surface was carefully abraded using damp gauze to remove any remnants of the smooth muscle layers. The SIS was then carefully washed twice in deionized water for 5 min, before being stored in phosphate-buffered saline (PBS: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C). Representative tissue samples were collected throughout the process and used as control samples.

2.1. Decellularization

2.1.1. Method 1 (perfusion/immersion)

Prepared SIS samples were placed in individual glass containers, secured to adaptors and connected to a multi-channelled peristaltic pump (Watson-Marlow, Cornwall, UK). The complete arrangement is illustrated in Fig. 2. The input channels were also secured in the same containers. The solutions described below were then placed in the containers to create an isolated chamber and the pump was run to begin perfusion.

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