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Transparent, resilient human amniotic membrane laminates for corneal transplantation

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ABSTRACT

This study evaluated a new technique to toughen and optically clarify human amniotic membrane (AM) tissue, which is naturally thin and clouded, and determined the suitability of the altered tissue for corneal transplantation. The technique created a tissue laminate by repeatedly depositing wet layers of AM and dehydrating them, followed by chemical cross-linking to tighten integration at the layer interfaces and within the layers, thereby improving the physical properties of the laminates by increasing light transmittance and mechanical strength. Interestingly, this improvement only occurred in laminates with at least 4 layers. Cross-linking also improved the resistance of the laminates to collagenase degradation, such as occurs in corneal melting. This study also confirmed that the AM tissue was biocompatible by inserting AM monolayers into the corneal stroma of rabbits, and by performing lamellar keratoplasty in rabbits with cross-linked AM laminates. The laminates were sufficiently thick and resilient to need only one set of sutures, whereas in previously described multi-layer AM transplantation technique, each layer required separate sutures. The current findings are a promising advance in the engineering of novel biomaterials and the alteration of existing tissues for medical use.

1. Introduction

For many years, new techniques to patch or graft human amniotic membrane (AM) onto the cornea have promised to improve the treatment of severe ocular surface disorders [1–3]. Recently, the medical uses of AM tissue have multiplied rapidly, and research into new potential uses for this tissue has accelerated. AM tissue now has clinical relevance in engineered biomaterials, in which it is used as a substrate in the construction of oral or corneal epithelia from stem or transient amplifying cells [4,5]. The anti-inflammatory, anti-angiogenic, and anti-bacterial nature of AM tissue has aided its successful clinical use [3,6]. However, other intrinsic properties have limited its applications. Most importantly, its low mechanical strength makes it difficult to handle, and its thin structure and low optical clarity make it difficult to use as a substitute tissue in corneal stromal transplantation, a procedure for which substitutes are urgently necessary due to severe shortages of donor corneas.

Collagen is the most abundant protein in the AM and is the primary structural component of AM tissue [7,8]. The hierarchical molecular, fibrillar, and supra-fibrillar architecture of collagen is reflected in various tissue-specific functions [9,10]. The cornea is an illustrative example, as collagen molecules in corneal tissue self-assemble into uniquely thin, uniformly spaced fibrils (32 nm in diameter), larger fibers (1–2 μm in thickness and 5–100 μm in width) and stacked lamellar arrays [11–14]. These nano- to micro-size structures are thought to permit both high levels of light transmission [13,15,16] and create mechanical strength [17,18]. This lamellar architecture in the cornea is similar to other mechanically resilient fibrocartilages, such as the meniscus [19] and intervertebral discs [20], but it is absent in the AM. Recent studies have demonstrated that resilient artificial collagen laminates, which resemble the corneal stroma, can be created from purified collagen

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[10,21]. Techniques from such structural engineering, especially lamination, may also be applicable in the construction of biomaterials from fibrous tissues such as the AM. In particular, they promise to enhance the mechanical properties of the AM, and extend the potential range of its applications.

In addition to lamination, previous studies have shown that slow dehydration can increase light transmittance in opaque collagen-rich tissues [21–25], including both the AM [26] and other proteins, such as boiled egg white [27]. These studies speculated that dehydration narrowed the spaces between collagen fibers, thereby reducing irregular light scattering. Additionally, cross-linking through photo irradiation [28] or chemical reagents has been widely studied as a way of improving the mechanical properties of collagen tissues [29–31]. Several recent reports have shown that cross-linking treatments can increase light transmittance in fibrous tissues such as the AM [32–34].

The present study explored a new tissue engineering method to create transparent, resilient AM laminates from human AM samples (Fig. 1). Our method relied on a newly developed multistep process that included dehydration, which was necessary to overcome the mechanical difficulty of handling and processing the soft, thin AM samples. The method also included chemical cross-linking of the dehydrated AM laminates to improve their optical and mechanical properties. Evaluation of the finished AM laminates used a variety of methods. *In vitro* examinations were used to evaluate the optical and mechanical properties of the laminates and to reveal their interior microstructure, as well as to test the resilience of the cross-linked laminates to the enzymatic degradation of interlayer adhesion. Experimental transplantation of the laminates into the corneas of rabbits was used to test their biocompatibility and to evaluate the feasibility of their use in surgical procedures requiring suturing.

2. Materials and methods

2.1. Materials

Wako Pure Chemicals (Osaka, Japan) provided Dulbecco's phosphate buffered saline (PBS), N-hydroxy succimide (NHS), 4% paraformaldehyde, 99% ethanol, glycine and collagenase type I. Thermo Scientific (Rockford, IL, USA) provided 1-Ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC). Dako Japan (Kyoto, Japan) provided a hydrophobic felt-tip pen (Dako Pen). Santen (Tokyo, Japan) provided levofloxacine (CRAVEIT). Otsuka Pharmaceutical (Tokyo, Japan) provided 0.9% NaCl solution. AS ONE (Osaka, Japan) provided trepans (3.0 and 5.0 mm in diameter). Matsunami Glass Ind., Ltd. (Osaka, Japan) provided slide glass and cover glass. Alcon Japan (Tokyo, Japan) provided balanced salt solution (BSS) and 10-0 nylon sutures. Natsume (Tokyo, Japan) provided 4-0 nylon sutures.

2.2. Preparation of wet human AM samples

The AM samples used in this study were collected from patients at the Department of Obstetrics and Gynecology, Tohoku University Hospital, Sendai, Japan, after full local ethics committee approval and in compliance with the Declaration of Helsinki. The dissected AM specimens were washed with PBS and stored at −80 °C with 1.5 mol/L dimethylsulfoxide for 1–6 months. Frozen specimens were thawed at 4–8 °C before further examination and washed by immersion in normal saline on a laboratory shaker for at least 2 h, in order to remove any remaining blood. They were then washed by immersion in distilled water in a beaker on a laboratory shaker for 1 h, in order to remove residual salt from the saline immersion. After thawing, the wet samples were cut into rectangular pieces approximately 15 × 15 mm in size.

2.3. Lamination of the AM samples

The AM laminates were constructed by laying individual AM samples on top of one another while an optical clarifying process (specifically, dehydration) was used after each layer was applied, as shown in Fig. 1 [26]. Briefly, the first layer comprised a wet AM sample that was dried with an electric fan at room temperature for 15–30 min after it had been laid on a glass slide. Another wet AM sample was then placed over the dried first layer, and the drying procedure was repeated. This lamination process was continued until the desired number of layers was reached, ranging from 2 to 8.

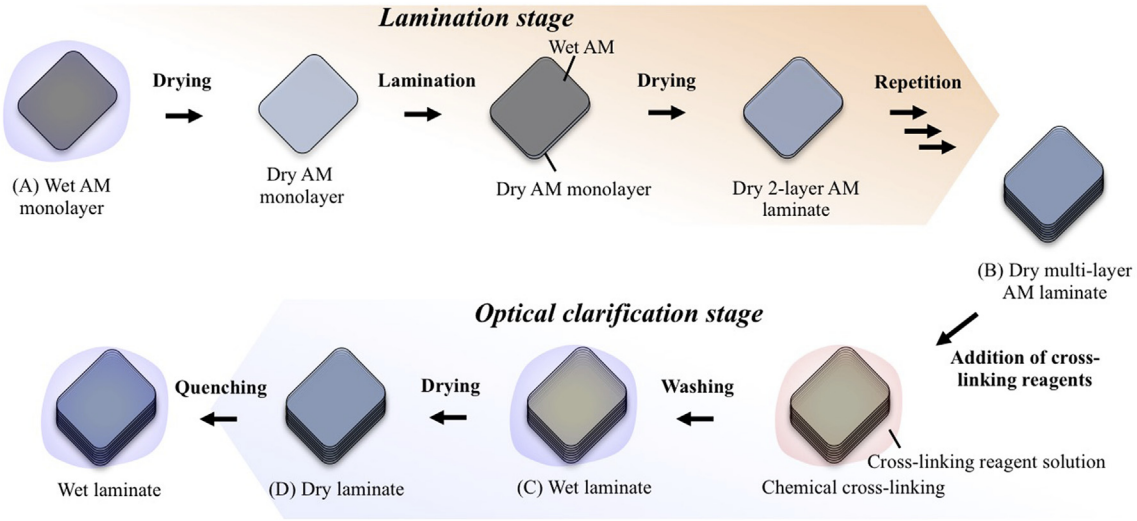

Fig. 1. Schematic illustration of the proposed method for producing transparent, resilient human amniotic membrane (AM) laminates. The method includes two main steps: lamination of the AM samples and optical clarification with cross-linking reagents. In the first step, a single wet AM sample is placed on a glass surface and allowed to dry (A). After drying, another wet AM sample is placed on the initial sample and allowed to dry. This process is repeated until the desired number of layers is reached. In the second step, the layers are chemically cross-linked (B), washed of excess reagent (C), and allowed to dry again (D). Finally, any remaining active reagent in the tissue is quenched with a glycine solution.