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Improved tympanic membrane regeneration after myringoplastic surgery using an artificial biograft

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ABSTRACT

Tympanic membrane perforations are due to common otologic problems. The current treatments to heal tympanic membrane perforation, such as myringoplasty, have some disadvantages, including the need for autologous grafting, which is rapidly absorbed by the organism before perforation recovery is complete. To improve the structural and functional tympanic membrane healing after surgery, we propose a new branch of artificial grafts. In this study, we report the development of artificial grafts using electrospun bioabsorbable polymers. Polymers such as poly (L-lactic acid) and poly (lactic-*co*-glycolic acid) acted as the scaffold for cell growth in a co-culture of fibroblasts and keratinocytes. This co-culture promoted the growth of an epithelial-equivalent tissue over the electrospun scaffold, which was used as an alternative graft in myringoplasty. The *in vivo* study was performed in Sprague Dawley rats. Ear endoscopy was performed 30 days after surgery and showed that tympanic membrane perforations treated with artificial grafts healed naturally, completely and with the possibility of maintaining their actual functionality. In conclusion, our study described a new artificial graft created specifically to fulfill the requirements of perforated tympanic membrane healing processes, which are compatibility, proper durability and less intense side effects following myringoplasty.

1. Introduction

Tympanic membrane (TM) perforation is a frequent pathology of varied etiology; perforation can be traumatic, infectious or residual from transtympanic drainage [1,2]. Audiological manifestations of a tympanic perforation may include conductive hearing loss with, tinnitus, ear fullness and otorrhea may also occur [3,4]. The location and size of the perforation and the frequency of hypoacusis (partial hearing loss) generally depend on the infectious agent and the severity of the condition; and hearing loss is a dependent variable of tympanic perforation chronicity [5].

Tympanic membrane treatment aims to heal perforations of the tympanic tissue to eradicate the disease, restore the tympanic aeration, reconstruct the sound-transformer mechanism and create a dry, self-cleaning cavity [6]. Many procedures and materials have been used to attempt closure of tympanic perforations. The literature has focused on two major research themes. The first includes the healing of perforations through new polymer matrices that promote support and serve as a guide for tissue regeneration, using myringoplasty surgery [3,4]. The second aims to induce cell proliferation and migration using mitogen (growth modulating factors) or inducers of blood flow (hemorrheologic agents) [1,7,8]. Currently, myringoplasty or tympanoplasty is the most accepted method. However, relapses are observed in up to 30% of cases [9].

In 1878, Berthold [10] introduced the term myringoplasty to describe the use of a free skin graft to close a perforation. The creators of the current myringoplasty procedure are Zöllner and Wülstein [11], who proposed a classification of surgical procedures based on the location of the graft related to auditory ossicles or the windows of the cochlea. In 1960, Hermann described the use of autologous temporalis fascia grafts to treat tympanic membrane perforation [12,13]. At this time, Goodhill used tragus perichondrium to achieve similar results [14,15]. In 1964, Salen [16] was the first to use nasal septum cartilage as graft material. In 2009, Uçar and Kazkayasi [17] used perichondrium from the nasal septum to repair tympanic membrane perforation. The characteristics and the ease of handling of these grafts meant that the materials are still the most frequently used to repair tympanic defects. However, numerous techniques have been described; the search for the best results continues, from both an anatomical and a functional perspective.

Classical myringoplasty using autologous grafts has certain disadvantages. The most important disadvantages are as follows: the self-implanted tissue has a short lifespan, which may be insufficient to allow complete tympanic regeneration, and serves exclusively as a support to the new cells that may develop over the graft, since the fascia tissue

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does not grow by itself; the need to extract tissues from the patient; and, in general, hearing is not sufficiently recovered in 40% of cases [18]. Insufficient auditory recovery is likely due to the abnormal process of tympanic membrane healing and injury to the ossicles, which are located just underneath where healing occurs.

In recent years, major advances in tissue engineering and regenerative medicine have facilitated the search for new biocompatible and bioabsorbable materials, the so called polymeric scaffolds for cell growth [19–21]. This materials developed in laboratories can be used in the reconstruction of bone defects, in the repair of organs affected by disease, and can replace animal tissues used in *in vivo* testing [22–25]. This new category of materials could find an important application in classic myringoplasty as a better alternative to autologous grafting, once it is possible to recreate all layers that constitute the real tympanic membrane, allowing appropriate functional and anatomical results of the regenerated tympanic membrane.

In tissue engineering, polymeric grafts mimic the extracellular matrix of the tissue on demand, promoting adhesion, cell growth and proliferation. The advantages of these new polymeric grafts are as follows: longevity, which allows for better tympanic regeneration; less inflammatory reactions; elimination of the need to extract patient's tissue to serve as a graft; the facilitation of shorter and simpler interventions that can be performed in a clinic or physician's office; and a structure more similar to natural tympanic membrane, which can improve the recovery of hearing loss.

Teh et al. [26] studied different scaffolds for tympanic membrane regeneration in rats, such as silk fibroin scaffold (SFS) and porcine-derived acellular collagen type I/III scaffold (ACS), compared with two commonly used graft materials, paper patch and Gelfoam. This study showed that both SFS and ACS significantly accelerated acute tympanic membrane wound healing and achieved hearing recovery from an early stage. In contrast, paper patch and Gelfoam lost their scaffold function in the early stages and showed an inflammatory response, which may have contributed to delay in healing. Lee [27] also studied silk fibroin as a biomaterial and the results showed this material displays excellent long-term degradation behavior, which could be an issue when rapid degradation is required, such as for tympanic membrane recovery purposes. Levin et al. [7] also investigated the growth of human tympanic membrane keratinocytes on a scaffold composed of silk fibroin. The results indicated that the biomaterial supported the growth and proliferation of these keratinocytes and silk fibroin scaffolds may enable otological surgeons to successfully close chronic tympanic membrane perforations.

A nonsurgical alternative to treat perforated tympanic membrane was studied by Seonwoo et al. [28]. They used an EGF-releasing chitosan patch for the regeneration of chronic tympanic membrane perforation. Although EGF-CPSs enhanced cell viability and the healing rate of chronic TM perforations compared to spontaneous healing, the healing rate of the EGF-CPS method did not reach those of surgical methods. For this reason, they concluded the treatment could be used for patients suffering less severe chronic tympanic membrane perforations with sizes up to 50% of tympanic membrane. Hence, there is a constant search for better materials to achieve improved healing and hearing.

To fulfill the requirements for tissue regeneration, we developed a tissue-engineered device known as biograft. This biograft was based on electrospun polymeric scaffolds used for cell growth and tissue differentiation. The aim is to repair perforated tympanic membrane through implantation of the biograft after tympanic membrane injury. The healing process with the developed artificial graft is expected to promote improved healing with fewer side effects after myringoplasty.

2. Materials and methods

2.1. Materials and cell sources

Poly (L-lactic acid) (PLLA; IV 3.2 dl/g, M_w 216,000 g/mol) and poly (lactic-*co*-glycolic acid) 50:50 (PLGA; IV 1.0 dl/g, M_w 110,000 g/mol) were supplied by PURAC (Netherlands). Chloroform and dimethylformamide were supplied by Synth (Brazil).

The cell types studied were fibroblasts and keratinocytes. Both cells were supplied by the Clinical Cytopathology Laboratory at the School of Pharmaceutical Sciences at the University of São Paulo, Brazil. Keratinocytes and fibroblasts were isolated from the foreskin of infants and children up to 10 years who underwent circumcision surgery at the University Hospital of the University of São Paulo (Brazil). The rules of the local ethics committee, under registration n° CEP-HU/USP 943/09, SISNEP CAAE 0062.0.198.000–9, were followed.

2.2. Methods

2.2.1. Polymeric scaffold preparation

PLLA, PLGA, and mixtures of PLLA and PLGA (at ratios of 75:25, 50:50, and 25:75) were solubilized in chloroform under constant magnetic agitation for 24 h. After complete solubilization, 10% of dimethylformamide was added to a final concentration of 100 g/l.

The scaffolds were produced from non-woven mats of PLLA/PLGA blends, obtained using electrospinning technique under the following conditions: solution flow rate of 2 ml/min; applied voltage of 25 kV; and capillary/collector distance of 15 cm. After electrospinning, the scaffold was suitably packaged in a vacuum desiccator for complete solvent evaporation. The resulting scaffolds were modeled according to the dimensions of the human tympanic membrane, or 8–10 mm in diameter and 30–90 μm thick [18].

Differential scanning calorimetry (DSC) analyses were performed in a TA Instruments DSC Q-10 calorimeter. DSC curves were typically obtained by heating 5–10 mg of material (10 °C/min) from −20 to 200 °C. This was followed by cooling (20 °C/min) and heating (10 °C/min) in a N_2 atmosphere with a flow rate of 50 ml/min. The second heating curve was used for characterization.

The degradation tests were performed *in vitro* according to the international standard ISO 13781: "Poly(L-lactide) resins and fabricated forms for surgical implants – *In vitro* degradation testing". The membranes were analyzed after incubation periods of 2, 4, 8, 10, and 12 weeks in phosphate buffered saline solution at 37 °C and pH 7.4. Size exclusion chromatography (SEC) and scanning electron microscopy (SEM) were used to analyze the degradation of membranes. Samples of dried membranes were used for the tests. Measurements of $\overline{M}_n$, $\overline{M}_w$, and the polydispersity index (PDI) were performed using a Malvern/Viscotek GPC system composed by GPCmax and TDA-305-040 modules fitted with the three Viscotek T6000 M columns. The mobile phase was chloroform with a flow rate of 0.5 ml/min.

The surface energy of the thin spin coated films was determined by means of the contact angle measurements by applying the sessile drop method. Milli-Q water and diiodomethane (Sigma) were used for the liquid tests (drops of 10 μl). Hysteresis of contact angle ($\Delta\theta$) was determined by measuring the advancing (θ_A) and receding (θ_R) contact angles with water drops of 10 and 5 μl, respectively. Contact angle values were evaluated using the public domain software program ImageJ 1.49 k. Spin-coated films (Headway PWM32-OS-RT90 spin coater) prepared from membranes solutions (10 mg/ml) in chloroform were deposited on Si/SiO2 wafers (1 cm^2) at 3000 rpm. Film thicknesses were monitored by ellipsometry (Ratzeburg DRE-EL02) using a He—Ne laser (632.8 nm) at an incidence angle of 70°. The refractive index was considered to be constant ($\eta = 1.465$), and the deposited film was uniform and isotropic.

For *in vitro* and *in vivo* studies, the membranes were sterilized by immersion in 70% ethanol for 24 h. This was followed by UV-C irradiation for 20 min on each side.

To verify mechanical properties of the polymeric blend, tensile tests were carried out in a Stable Micro systems texture analyzer, Model: TA-HD-Plus, test speed of 1 mm/s, with a cell load of 50 Kg. The specimen dimensions used in the test were 5 cm in length, 3 cm in width and 300 μm in thickness. The test was repeated 10 times.