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인조고막용 키토산 패치 지지체의 생체역학적 특성 및 독성 평가

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Biomechanical Properties and Cytotoxicity of Chitosan Patch Scaffold for Artificial Eardrum

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Abstract

The objectives of this study were to prepare a new artificial eardrum patch using water-insoluble chitosan for healing the tympanic membrane perforations and to investigate biomechanical properties and cyotoxicity of the chitosan patch scaffold (CPS). Tensile strength and elongation at the rupture point of CPSs were 2.49-74.05 MPa and 0.11-107.06%, respectively. As the biomechanical properties of CPSs varied with the concentration of chitosan and glycerol, the proper conditions for the CPS were found out. SEM analysis showed very smooth and uniform surface of CPSs without pores at x1000. The result of MTT test showed that CPSs had no cytotoxicity.

Keywords : Tympanic membrane perforations, Artificial eardrum, Chitosan patch scaffold, Biomechanical property, Cytotoxicity test

1. INTRODUCTION

Tissue engineering is an interdisciplinary field of biomedical engineering that combines the principles of engineering, agriculture science, materials science, biological science, and medicine for the design and production of replacement tissues or organs. Many of the problems inherent in tissue and organ transplantation, surgical reconstruction, and implantation of nonmaterials can be alleviated with tissue engineering.

In any scaffold application for tissue engineering, the selection of materials is great importance. Chitosan (poly-β-(1->4)-D-glucosamine) is an aminopolysaccharide obtained by the alkaline decetylation of chitin. Chitosan is the second most abundant natural polymer, being second only to cellulose, and has characteristics good cell affinity, antifungal function and wound healing. Accordingly, chitosan and its derivatives have been studied for numbers of biomedical applications such as these include wound dressings, drug delivery systems and space filling implants.

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Tympanic membrane (TM) perforation is a common problem in otology. The TM perforation is caused by a physical factor or disgust at mid-ear, usually presenting with conductive hearing loss and chronic infection. Although the TM has a remarkable ability for regeneration and spontaneous healing, perforations of large size, chronic perforation, and otitis media may require suitable treatment.

Heermann (1961) performed successful autografts for closure of TM perforations using temporalis fascias. These days an autologous autograft including muscle fascias or perichondriums are most commonly used for treatment of TM perforations, and the success rate of autografts reaches between 88% and 97% in most studies. Today, autologous treatment's success rate is between 88% and 97% in most studies. However, surgery involves higher costs, the need for general anesthesia in some patients and the microsurgical skills of the surgeon.

Blake (1887) introduced the simple technique of the paper-patch graft that guides the migrating epithelium to a scaffold from the borders of the perforation. This technique is still practiced for acute and traumatic perforations, even though the paper patch has the disadvantages of non-biocompatibility, non-transparence, non-flexibility, easily detachability, and non-resistance to infection. Actually, the closure rate of paper patches was just around 50% in the cases of chronic perforations, which shows the limitation of this technique. And several scaffolds that could stimulate tympanic regeneration, guide eardrum growing, or replace the perforated defects have been introduced until now. The biocompatible scaffolds such as polylactide (PLA), poly (glycolic acid) (PGA), collagen, Seprafilm® (hyaluronic acid and carboxymethylcellulose) or calcium alginate were tried for closure of TM perforations. However, most of these studies were lack of analysis about biomechanical properties of new materials from biomaterials' point of view. Unfortunately, there are no adjustable alternatives to surgical procedures or the paper-patch technique for treatment of TM perforations until now.

Thus, the development of a novel scaffold for TM perforations treatment was required. A study on biomechanical properties and cytotoxicity of the new scaffold was also needed.

The objectives of this study were to prepare new artificial eardrum patch using water-insoluble chitosan for healing the TM perforations and to investigate biomechanical properties and cytotoxicity of the chotosan patch scaffold.

2. MATERIALS AND METHODS

Fig. 1 shows the basic concept for a new eardrum patch. The chitosan patch scaffold (CPS) guides the migrating epithelium as a CPS from the borders of the perforation.

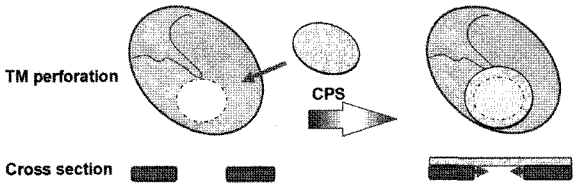

Fig. 1 Basic concept for a new eardrum patch.

A. Materials

Water-insoluble chitosan was purchased from Taehoon Company (Seoul, Korea, Mw; 200,000 DD; 89%). The following materials were used to prepare for the CPS: water-insoluble chitosan, acetic acid and glycerol.

B. Preparation of chitosan patch scaffold

In preliminary experiments a scaffold needed high flexibility for epithelium migrating to the scaffold from the border of the perforation. Thus CPSs were prepared with chitosan and glycerol. Acetic acid of 2% was used to make chitosan solution for dissolving water-insoluble chitosan. The chitosan solution was prepared by adding 3 g or 5 g

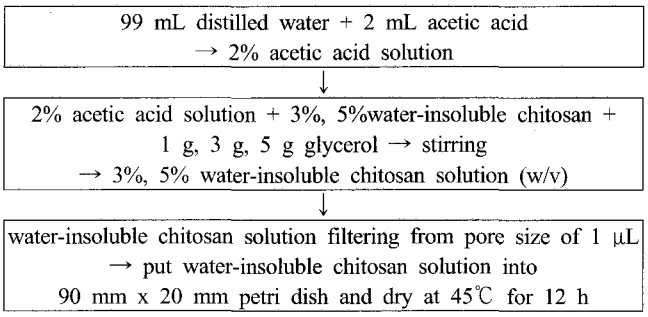

Fig. 2 Preparation process of chitosan patch scaffolds.

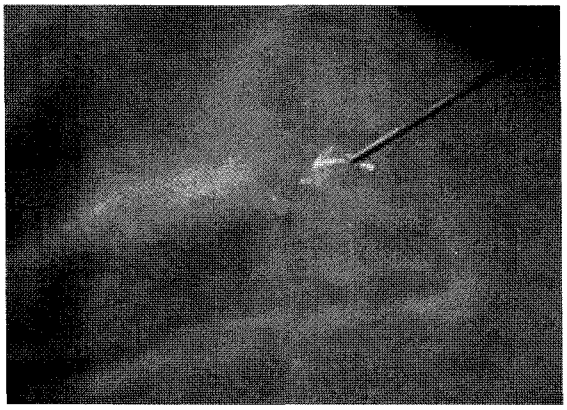

Fig. 3 Chitosan patch scaffold.

of the chitosan to the acetic acid of 100 mL. The chitosan solution was mixed with, respectively, 1 mL, 2 mL, and 3 mL of glycerol as a plasticizer. And chitosan solution was filtered in pore size of 1 μl. Some amount of the prepared chitosan solution was poured into a petri dish to make a CPS with the thickness of 0.03-0.04 mm. The chitosan solution on the petri dish was dried at 45℃ for 12 h.

C. Thickness of chitosan patch scaffold

The thickness of CPSs was measured by a micrometer (Mitutoyo., Japan) with the precision of 0.01 mm and scanning electron microscope (JEOL, JSM-5410LV, Japan). The measurement of the thickness was tried five times at the same place of the CPS. The calculated thicknesses were used for determining the stress values.

D. Mechanical properties

CPSs, 2 mm wide and 70 mm long, were cut from the sample of experiment on petri dish (90 mm x 20 mm), and the mechanical properties were measured by a texture analyzer (Stable Micro Systems Ltd., London, England). The cross-head speed was set at 500 mm/min. The test was repeated 8 times. The average and standard deviation of the properties were calculated. The peak loads and displacement at the breaking point were recorded for the CPS. Tensile strength and elongation of a CPS were calculated using the following formulas:

$$\text{Tensile strength} = F_{max} / A \quad (1)$$

$$\text{Elongation} = (L / L_0) \times 100$$

where:

F_{max}: maximum load of the CPS at the rupture point, N.

A : cross- sectional area of the CPS, m^2.

L_0 : original length of the initial gage length of the CPS, 50 mm.

L : Increase in the length at the rupture point of the CPS, mm.

E. Morphological analysis

The surface property of the CPSs was analyzed by a scanning electron microscope (JEOL, JSM-5410LV, Japan) at an accelerating voltage of 25 kV. The surfaces of the CPSs were coated with gold using a sputter-coater (JEOL, JFC-1100E, Japan).

F. Cytotoxicity assessment of chitosan patch scaffold

We assumed that the amount of toxic matters extracted from the CPS was equal to a toxic quantity of 100%, if the CPS of 1 cm^2 were inserted to a body. A control group was set to be a medium without CPS and an experiment group was set be a medium including CPSs. The extract concentrations in each group were 0, 20, 40, 60, 80, and 100%, and the sizes of CPSs placed in 2 mL culture medium were 0.08, 0.32, 0.732, 1.28, and 2 cm^2. This experiment was repeated 8 times. The extraction was performed in the culture conditions of 37℃, CO_2 concentration of 5%, and relative humidity of 95%. A cytotoxicity test of CPSs was performed by MTT based tests. The optical density (OD) was measured at 540 nm wave length using an ELISA reader (VERSAMAX reader, molecular device, USA). The measured optical density value was used in terms of IC_{50} in this study. IC_{50} was defined as the optical density for 50% concentration inhibition. Therefore, it was considered that IC_{50} value was as same as the optical density when the total cell number was reduced to 50%. This definition is expressed as followings:

$$\text{OD for } IC_{50} = \text{OD for 0\% extract concentration} / 2 \quad (2)$$

where, IC_{50} = optical density (OD) for 50% concentration inhibition

Cytotoxicity tests were conducted with MTT (3-(4, 5-

Dimethyl-2-thiazoly (-2.5-d-iphenyl-2H-terazolium bromide))) (M-5655, SIGMA, USA), dimethyl sulfoxide (DMSO (K31902850, MERCK, Germany)), and HaCaT cells (human spontaneously immortalized non-tumorigenic and highly differentiating keratinocyte cell line).

G. Statistical analysis

The statistical analysis was carried out using the SAS Statistical Analysis System for Windows Ver. 8.2 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was used to compare the means for mechanical properties.

3. RESULTS AND DISCUSSION

A. Mechanical properties

Mechanical properties are of primary important for determining the performance of scaffold for artificial eardrum. The thickness of CPSs was about 0.03-0.04 mm. Fig. 4 shows the thickness of CPS by SEM. The tensile strengths were in the range of 2.49-74.05 MPa. Table 1 shows the mechanical properties of CPSs. The tensile strengths of CPSs varied much depending on the concentration of chitosan and glycerol. The tensile strength of the scaffold with the 5% chitosan and 1% glycerol was the highest compare with that of the other CPSs. The tensile strength of CPSs showed a tendency to increase at the high concentration of chitosan.

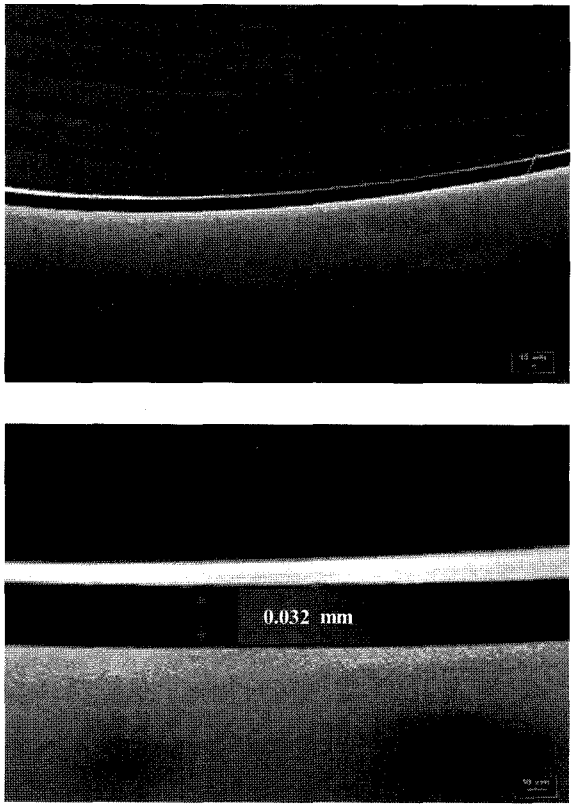

Fig. 4 Result of thickness of a chitosan patch scaffoldby SEM.

Table 1 Mechanical properties of the chitosan patch scaffolds

Samples	Tensile Strength (MPa)	Elongation (%)
3%c + 1%g	36.32 ± 3.42^{b}	42.24 ± 3.25^{d}
3%c + 3%g	6.55 ± 3.52^{c}	107.06 ± 5.82^{a}
3%c + 5%g	2.49 ± 0.13^{c}	0.1067 ± 0.014^{e}
5%c + 1%g	74.05 ± 7.07^{a}	47.36 ± 6.13^{d}
5%c + 3%g	25.57 ± 3.86^{b}	89.22 ± 5.98^{b}
5%c + 5%g	26.11 ± 2.97^{b}	79.651 ± 6.02^{c}

Means in the same column followed by same letter are not significantly different (p=0.05) different by Duncan's multiple range test, c: chitosan, g: glycerol.

The elongations were in the range of 0.11-107.06 %. The elongation of CPSs was also different according to the concentration of chitosan and glycerol. CPSs had the highest elongation when 3% glycerol was added to 3% chitosan, but had the lowest at the 5 % glycerol and 3% chitosan.

The concentrations of chitosan and glycerol had a great effect on the tensile strength and the elongation of CPSs. An ideal patch scaffold for artificial eardrums needs the characteristics of high elasticity with proper strength to let the patch adapted well on the eardrums. We considered that elongation property was more important than the tensile strength in the situation of always movable eardrums due to sound transmission. In other words, the CPSs with high elasticity had better attached to the eardrums than those with high tensile strength. According to these results, the CPSs with 3% chitosan and 3% glycerol showed suitable tensile strength and high elongation. Accordingly, we recommended that the CPSs in this composition seemed to be the most ideal among several candidates for regeneration of TM perforations.

B. Morphological analysis

SEM analysis showed very smooth and uniform surface of CPSs regardless of the concentration of chitosan and glycerol. Fig. 5 shows the surface of CPS without pores by

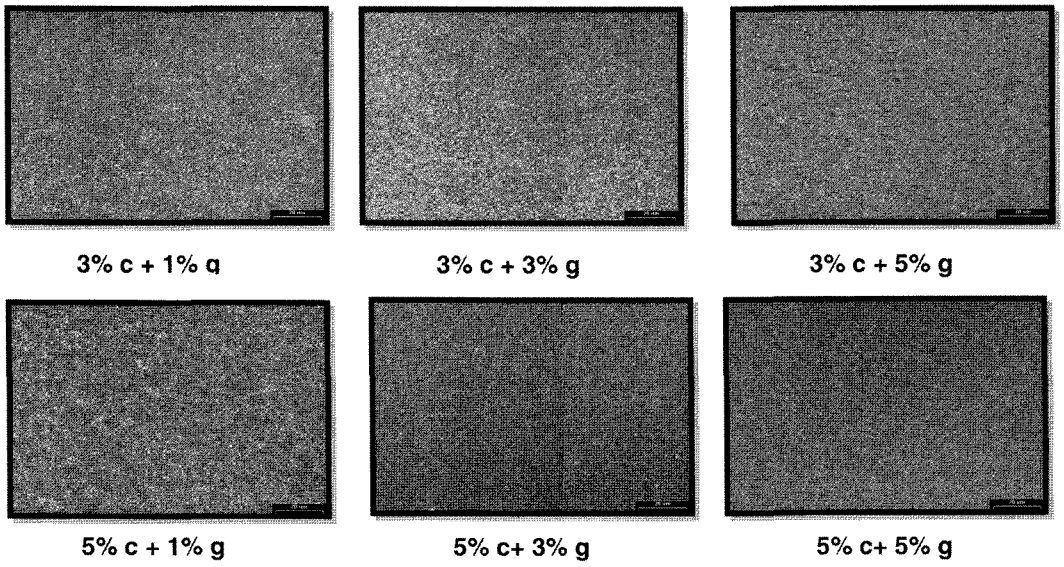

Fig. 5 Uniform and smooth surface structure of chitosan patch scaffolds by SEM, c: chitosan, g: glycerol.

SEM x1000. Bacteria and foreign bodies in the ear canals could be more easily seeded and impacted in the materials with irregular surfaces and coarse structures than the regular and smooth structures. Accordingly, in the morphological and structural points of view, the smooth and uniform surface of CPSs was more resistant to the infection than the irregular surfaces.

C. Cytotoxicity assessment of chitosan patch scaffold

Fig. 6 and Fig. 7 show results of MTT cytotoxicity tests for the extract of CPSs according to the concentration of chitosan and glycerol. The value of average optical densities at the 0% extract on medium was 0.945. Accordingly, the value of IC_{50} could be 0.4748 in this study. Any values in the graph did not reach to this value, 0.4748 as IC_{50}. In addition, the optical densities at the 100% extract concentration were similar to those of at the 0% extract concentration for CPSs. Therefore, results showed that CPSs had no cytotoxicity.

4. CONCLUSIONS

CPSs were prepared using water-insoluble chitosan for a

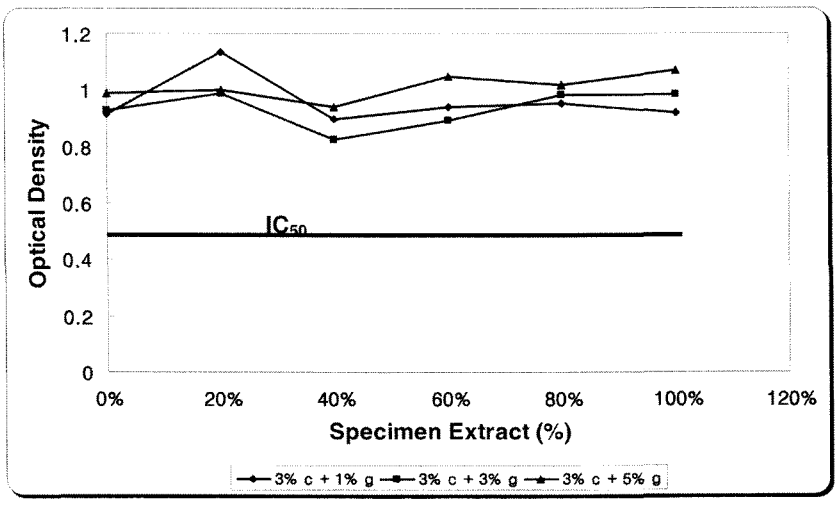

Fig. 6 Results of MTT cytotoxicity test for 3% chitosan patch scaffolds, c: chitosan, g: glycerol.

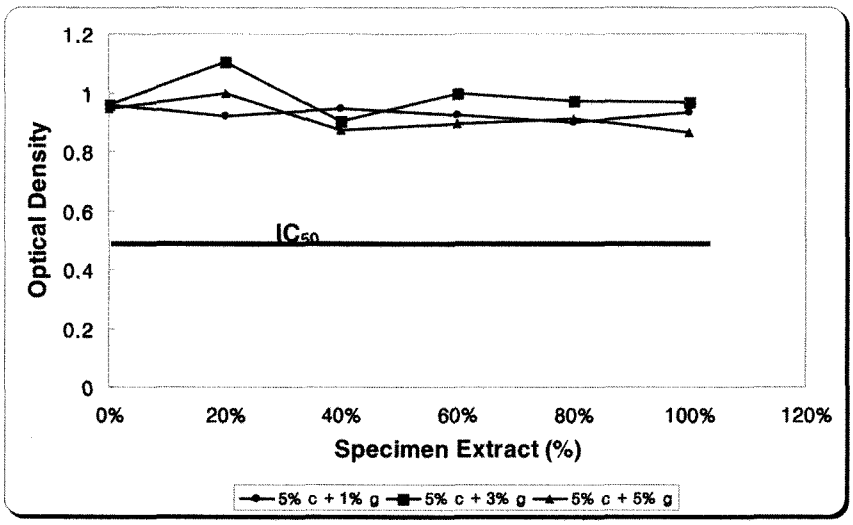

Fig. 7 Results of MTT cytotoxicity test for 5 % chitosan patch scaffolds, c: chitosan, g: glycerol.

new artificial eardrum. The results of MTT cytotoxicity test showed that CPSs had no cytotoxicity. The thickness of CPSs was about 0.03-0.04 mm. Tensile strength and elongation of CPSs depended on the concentration of chitosan and glycerol. CPSs showed suitable tensile strength and high elongation when 3% glycerol was added to 3% chitosan and recommended as the artificial eardrum. The result of SEM analysis showed that surface structure of CPSs were very smooth and uniform without pores at x 1000. It was considered that CPS could be used as a scaffold for eardrum regeneration.

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