IOP Conf. Series: Materials Science and Engineering **301** (2018) 012018 doi:10.1088/1757-899X/301/1/012018

Experimental Study on 3D Chi - Hap Scaffolds for Thyroid Cartilage Repairing

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Abstract. Due to the limitation of self-repairing capability for cartilage injury, the construction of tissue engineering in vitro has been an ideal treatment to repair tissue injury. In this paper, hydroxyapatite (Hap) and chitosan (Chi) were selected to fabricate the scaffold through low temperature deposition manufacturing (LDM) technique. The scaffold was characterized with interconnected structure and high porosity, as well as lower toxicity to cells (TDC-5-EGPE). Animal experiment was performed, Twelve white New Zealand rabbits were randomly divided into two groups, the side of the thyroid cartilage was removed, Chi-HAP composite scaffold was implanted into the cartilage defect as the experimental group A. Group B was treated for thyroid cartilage defects without any treatment. After 10 weeks, hematoxylin-eosin（HE） staining and S-O staining were carried out on the injured tissues. The result showed that newborn chondrocytes were found in repaired areas for group A, and there are no new cells found for group B. Therefore, Chi-HAP composite scaffolds formed by LDM possess biological activity for repairing injury cartilage.

1. Introduction

The larynx possesses exquisite and complex structure as an important organ for the human body, and including arytenoid cartilage, thyroid cartilage, ring cartilage, epiglottis cartilage, hyoid bone [1]. Lesions and congenital malformations of laryngeal cartilage defects are common injuries for ENT, and self-regeneration and self-repair ability for cartilage is limited, which effect serious psychological and physical harm to patients [2].

In recent years, tissue engineering for reconstructing laryngeal cartilage has brought new hope. Autologous tissue, allogeneic, artificial materials and tissue engineering technology were utilized to repair and reconstruct laryngeal cartilage damage [3]. At present, natural macromolecule biomaterials, synthetic polymer materials, bioceramic materials and composites have been the primary materials for tissue engineering scaffolds [4-8]. Natural biological materials possess excellent adhesion for cells, hydrophilicity and compatibility, which include gelatin, sodium alginate, collagen, chitosan, and fibrin gels. Kota Uematsu et al. [9] fabricated three-dimensional porous scaffolds with PLGA and then transplanted the scaffolds into knee joint injury of rabbits. After 12 weeks, the results showed the lesion was filled with smooth and shiny white tissue and connected well with the surrounding tissue. Histochemical analysis indicated cartilage tissue was formed. However, the shortcoming for synthetic polymers include weak adhesion of cells, and the limitation of immune responses and hydrophilicity [10]. Composite materials offset the lack of above materials. Hu Wanqing et al. [11] obtained biological Col-Hap (I collagen and hydroxyapatite) scaffolds with rhBMP-2 via foam gel injection method, and the biological scaffolds were used to repair thyroid cartilage injury. The results exhibited that rhBMP-2, type I collagen and hydroxyapatite composites had good biocompatibility and could induce bone formation and provide a new therapeutic method for cartilage injury repair. Meanwhile,

the desired physical properties of scaffolds are dependent on the design and manufacture. Traditional methods for preparing biological scaffolds include solvent casting, particle leaching [12]. However, the geometrical shape and porosity of scaffolds fabricated via these techniques are generally difficult to control, and a few of the scaffolds tend to lack interconnected channels, which limit their widespread application in tissue engineering [13]. The low-temperature deposition manufacturing technology (LDM) was proposed originally at the beginning of this century [14, 15]. The LDM technology utilizes rapid prototyping technology to fabricate the desired model in low-temperature forming chamber, scaffolds are obtained by thermally induced phase separation [16]. In this study, chitosan-hydroxyapatite scaffolds (Chi-HAP) were fabricated via LDM, the microstructure and porosity of scaffolds were examined. The scaffolds fabricated via this technique possess a well-formed porous structure, which provides large surface areas for cell attachment and sufficient space for cell growth and proliferation. Cytotoxicity of Chi-Hap scaffolds was test by co-culturing chondrocytes and scaffolds, and in vivo tests on thyroid cartilage injuries exhibited satisfactory effects, implying that these scaffolds have potential applications for the treatment of thyroid cartilage injuries

2. Materials and Methods

2.1. Materials and Animals

Chitosan powder was obtained from Sinopharm Chemical Reagent Co. Ltd.(Shanghai, China). Nanoscale hydroxyapatite powder was purchased from Shanghai Aladdin Bio-Chen Technology Co., LTD. (Shanghai, China) was used in this study. Acetic acid was supplied by Guangzhou Pan yu Li qiang Hua gong (Guangzhou, China), and Ethyl alcohol was obtained from Hangzhou Gao jing fine Chemical Co., Ltd. (Hangzhou, China). All materials were used as received without further purification. The New Zealand white rabbits were obtained from the Zhejiang University Experimental Center, the average weight of 2.25kg for the rabbits.

2.2. Fabrication of Chi-Hap scaffolds

Chitosan (4.5 g) and hydroxyapatite powder (4.5 g) were dissolved into 100 ml distilled water containing 0.2 M of acetic acid. The mixture was stirred at room temperature for 2-3 h to obtain a homogeneous solution using double-headed magnetic stirrer (HJ-2, Beijing Songyuan Huaxing Technology Development Co., Ltd.). Structure of 3D solid model was designed using 3Dmax software, and according to the requirements and the pre-designed 3D model for the scaffolds, layering was carried out to obtain the data files by the LDM. After a large number of experiments, the satisfactory biological scaffolds would be obtained while the scanning speed was 12 mm / s, the extrusion speed was 0.2 mm/s, and the molding chamber temperature was -20 degrees. Therefore, the prepared slurry was injected into the equipment cartridge, and designed parameters of control software was adjusted to be the pre-set parameters. The slurry rapidly solidified after extrusion from the nozzle and adhered to each other in the chamber at low temperatures. Finally, Chi-Hap scaffolds were removed and then dried in a freeze dryer (LGJ-12B, Beijing Songyuan Huaxing Technology Development Co., Ltd.) for 24 hours. Rapid prototyping process shown in Figure 1.

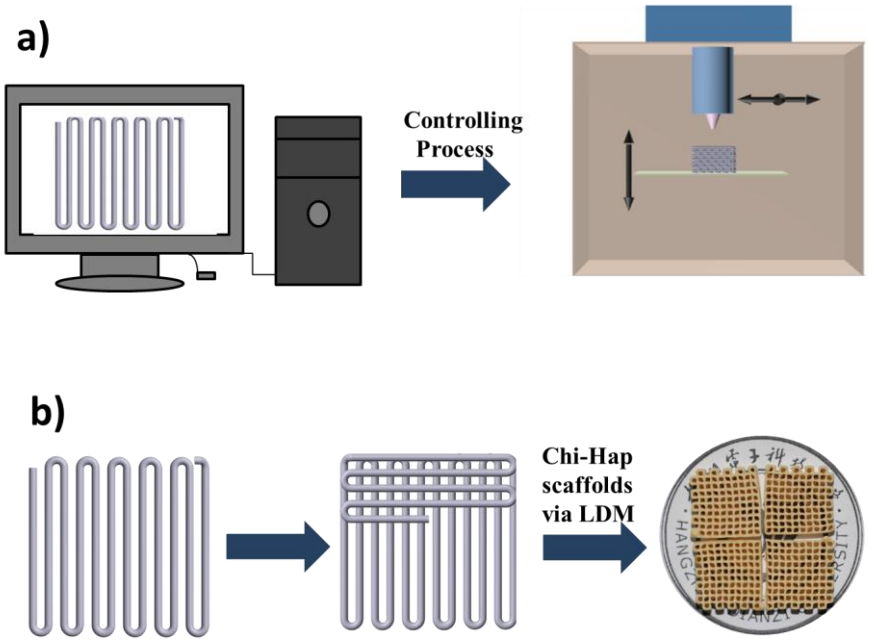

Figure 1 The principle of rapid prototyping. (a) The fabricated process for biomaterial scaffolds; (b) Layering of scaffold was produced according to the pre-designed 3D model.

2.3. Characteristics of Biological Scaffolds

The morphological of the scaffolds fabricated via LDM was observed by field emission scanning electron microscope (JSM-7800F, JEOL, Japan), the Chi-HAP composite scaffold was sprayed and the cross-section and vertical microstructure of composite scaffolds were observed.

2.4. Scaffold Porosity Measurement

In this experiment, the porosity of the scaffolds were evaluated by gravimetric method. The three dried scaffolds chosen respectively were measured and marked as M_0, and then were immersed in the minimus ranges containing alcohol solution, and the quality of measuring cylinder with scaffolds and alcohol solution was determined as M_1. Finally, after removing the scaffold from the measuring cylinder, the quality of cylinder merely containing alcohol solution was recorded as M_2. The porosity ε was calculated based on the formula (1).

$$\varepsilon=(M1-M2-M0)/(M1-M2)\times 100\% \quad (1)$$

2.5. Cell Compatibility Test

In this paper, chondrocytes (ATDC-5-EGFP) were selected as seed cells. The Chi-Hap scaffolds were sterilized by UV irradiation for 48h. As proliferation of chondrocytes being 80-90%, trypsin-EDTA was used to digest the cells into single suspension cells. After centrifugating at 1000r / min for 5min, the cells were suspended and seeded on the Chi-Hap scaffolds, and the scaffolds with cells were placed in a thermostatic chamber to co-culture.

2.6. In Vivo Study with Expermental Animals

12 New Zealand white rabbits were used in this study. Six randomly selected rabbits were assigned to each study group. Prior to surgery, 1 ml of 4% pentobarbital sodium (Shanghai Ha Ling Biotechnology Co., Ltd.) was injected into the rabbit. The necks of rabbits were shaved, as shown in figure 2 (a), and prepped with 70% ethanol prior to the surgery. Cartilage gap approximately 2 ×6 mm was made above the one side of laryngeal cartilage (Figure 2 (b)). Skin and musculature were then dissected the cartilage injury was exposed. Chi-Hap scaffolds were transplanted into the defective site with the medical glue (Zhang jia gang Shuang liu Biotechnology Co., Ltd.) (Figure 2. (c))). Finally,

the incision was stitched with the medical line (Figure 2 (f)). And then spray penicillin (North China Pharmaceutical Co., Ltd.) was used to prevent wound infection. After surgery, 1mg Gentamicin (Shanxi Bikang Pharmaceutical Co., Ltd.) was injected for 3 days. As shown in Figure 2.

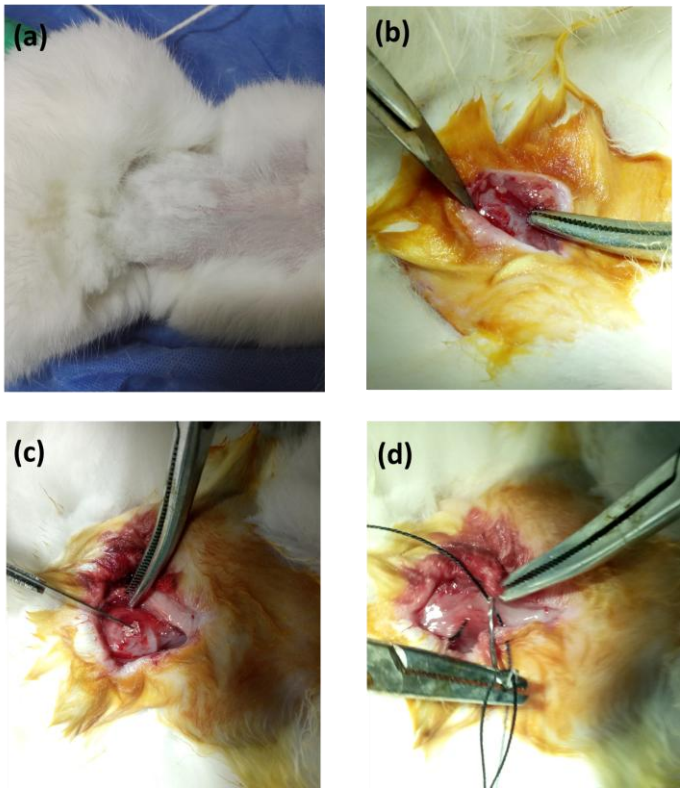

Figure 2 The surgical procedure of repairing thyroid cartilage of rabbit injury. (a) The forelimb of rabbits before surgery; (b) About 2×6mm of cartilage slice on unilateral thyroid cartilage plate was cut; (c) Chi-HAP scaffold was implanted in the injury part; (d)Sew subcutaneous tissue and skin of rabbit.

2.7. Observation and Examination after Surgery

The status of Animals was observed after surgery for 2 h. Histological observation was performed after exacting rabbits at10th week. After the rabbits were sacrificed, the throat and its surrounding tissues were removed and placed in 10% formaldehyde solution for 24 hours, and then carried out decalcification with 10% nitric acid for 2 days and washed with 70%, 80%, 90%, 95% anhydrous ethanol for dehydration. Samples were transparent using xylene .Finally Samples were embedded. The samples were performed frozen sections, and thickness form 3 to 5μm was obtained. Newborn chondrocytes were verified via Hematoxylin -eosin (HE) staining and S-O staining.

3. Results

3.1. Morphological Characterization of The Chi-HAP Scaffolds

Figure 3 shows the microscopic morphology of Chi-HAP composite scaffolds by scanning electron microscope (JSM-7800F, JEOL, Japan). The skeleton structure based on the chitosan was packed and covered with the deposition of Hap, which partly improves the holistic mechanical strength of bio-scaffolds, and offset the deficiency of rapid degradation of Chi[17]. Figure 3. (a) and figure 3.(b) exhibited scaffold possessed highly porous and loose interconnected structure, and figure 3. (c) showed the scaffolds structures were layered stacked in a row, which excellently supported growth of cells and infiltration of regenerated tissue, and are advantageous to circulation of extracellular matrix.

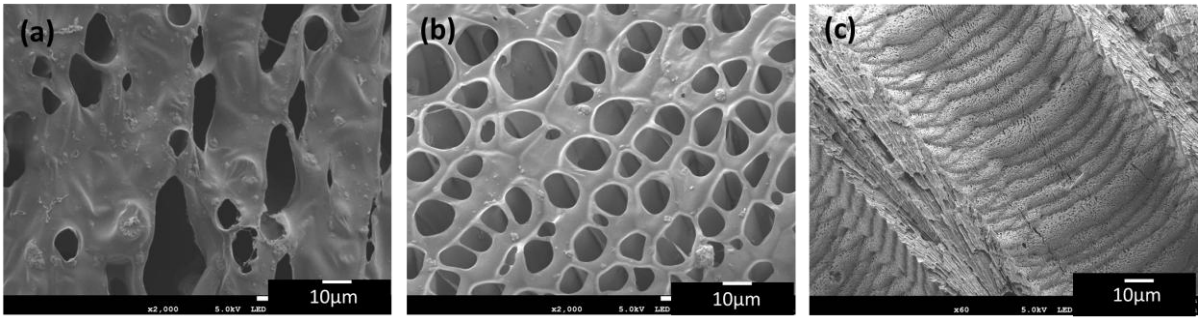

Figure 3 SEM images of Chi-HAP scaffolds; (a) are the cross section of the scaffolds; (b), (d) are the vertical section of the scaffolds.

3.2. Porosity of Chi-Hap Scaffolds

Porosity is an important indicator for the biomedical scaffolds. The scaffolds with higher porosity provide sufficient surfaces for cells attachment and proliferation, and supply competent space for removal of metabolites. Generally, the porosity of > 85% is preferable for the tissue engineering[18], the porosity of chi-Hap showed in Table 1.

Table 1 The porosity for Chi-Hap scaffold

Material Ratio	**Porosity**			
	A	***B***	***C***	***Mean porosity***
1:1	91.16%	90.86%	91.04%	91.02%

The result suggested the average porosity of Chi-Hap scaffolds was 91.02%, which generally higher than scaffolds fabricated via other preparation techniques. Due to the advantage of LDM, the scaffolds possess excellent interconnection and the high porosity, which satisfies the requirements of tissue engineering, and can provide sufficient space for chondrocyte proliferation and growth[19]. The scaffold with high porosity can maintain fluent circulation of the body fluid and blood after the implantation of scaffolds in vivo.

3.3. Biocompatibility

Excellent biocompatibility is necessary indicator for applying biological scaffolds clinically. Cartilage cells in mice (ATDC-5-EGPE) were seeded on sterilized scaffolds to evaluate cytocompatibility of Chi-HAP scaffolds, and the scaffolds with cells were co-cultured in DMEM/F-12 medium supplement with 10% fetal bovine serum and 1% penicillin/streptomycin solution at 37 ℃ in a humidified atmosphere of 5% CO2 incubator (Thermo, USA). The cell proliferation on scaffolds was examined via inverted phase contrast microscope. The microscopic observation is shown in Figure 4.

Figure 4.(A) indicated the morphologies of cells for 1 day. Visible chondrocytes demonstrated round or eccentric spindle shape , and the cellula spreaded and attached on the composite scaffolds scatteredly, furthermore, bits of dead cells were observed(see Figure 4(A).(a)), which was not significantly different from that of the non-treated control(see Figure 4(A).(b)). After co-culture of the chondrocytes and the scaffolds for 3 days, the cells were densely distributed on the scaffold and appear phenomenon for mutual connecting (see Figure 4.(B).(c)). The control group showed viable cells were remarkably increase (see Figure 4.(B).(d)), and there was no significant difference for cells status between the two groups(Figure 4(B)). With the prolong culturing, cells accumulations and more cellular layers were observed, and the viable cellular distributed and attached compactly over the composite scaffolds (Figure 4.(C).(e)), and Figure4.(C).(f) showed same characteristic morphologies of the cellula. The results showed that the Chi- Hap composite scaffolds are nontoxic.

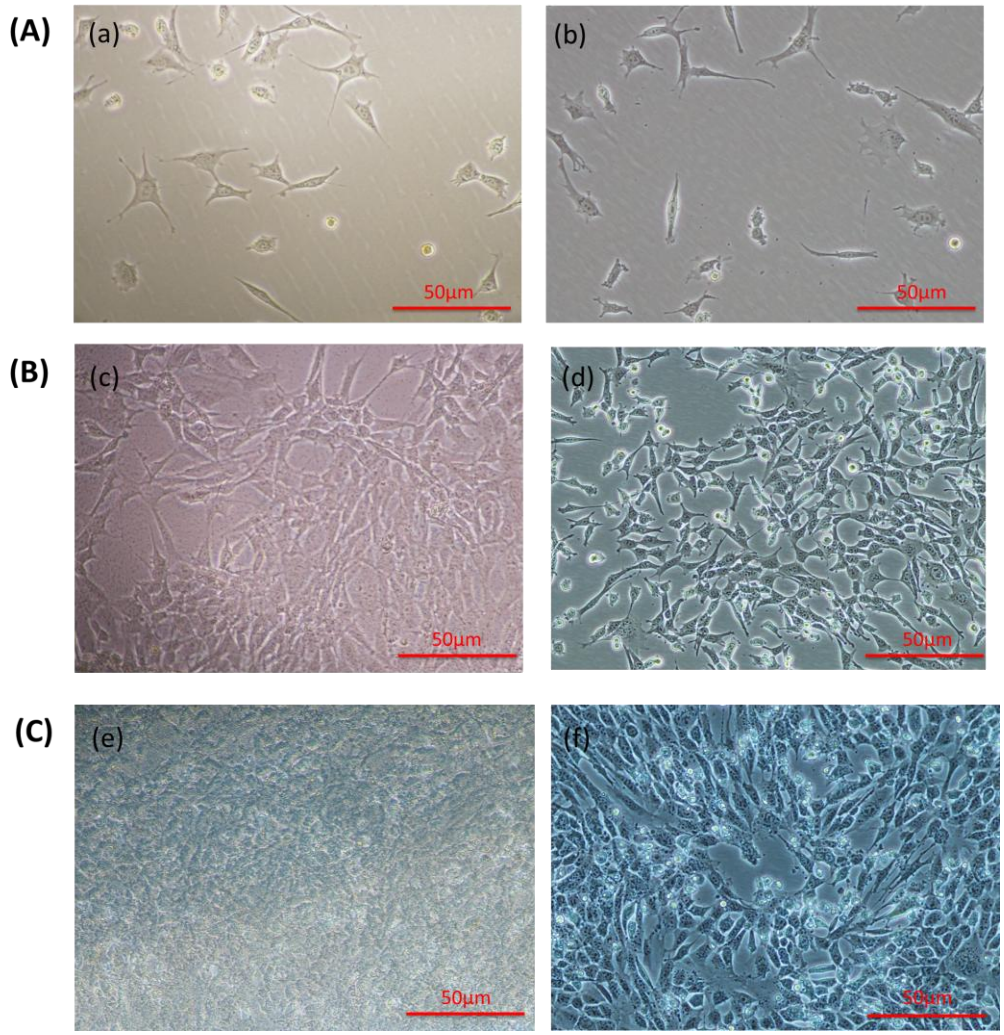

Figure 4 Inverted phase contrast microscope of cell morphologies (×50 um) of ATDC-5-EGPE cells for 1d (A), 3d (B), 5d (C). In (a), (c), (e), cells incubated in the Chi-HAP composite scaffolds with the mass ratio of 1:1, (b), (d), (f) were the no-treated control group, the cells incubated in medium supplement with 10% fetal bovine serum.

3.4. Animal Experiment after Surgery

The rabbits were generally in good condition after Surgery. After 2 hours, the animals started drinking water and sounding normally. The animals were fed on the next day and make normal diets. The animals were recovered well for a week, the injured neck appeared no obvious bleeding, swelling, suppuration and infection. Chi-Hap exhibited no significant immune rejection on the animals.

3.5. Histological Staining

3.5.1. HE staining. After 10 weeks, HE staining was performed on injured laryngeal cartilage samples obtained from executed rabbits. The results for staining are shown in Figure 5. Amounts of newborn chondrocytes (black arrows) appeared in repaired area for the experimental group A, and the newborn cartilage cells was disordered and smaller than the original cells, and there were bits of erythrocytes (white arrows) and inflammatory cells (red arrows) around the newborn cartilage cells(Figure 5(a)). However, there were a large number of red blood cells (white arrows) and inflammatory cells (red arrows) around the injured area, but no newborn chondrocytes were observed in group B (Figure 5(b)).

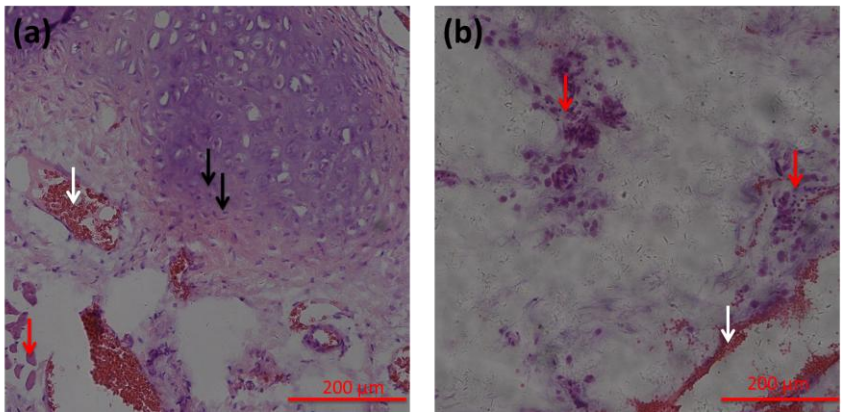

Figure 5 HE staining for tissue samples **at** 10th weeks after surgery.(a) injured areas were implanted with Chi-HAP scaffolds（group A）, (b)Group B was as control group, and the injured areas were filled with no scaffolds

3.5.2. S-O staining. Figure 6 showed the result of S-O staining. The repaired area of group A presented newborn chondrocytes with irregular arrangement (black arrows), and the cells were around by blood cells (red *arrows*), as shown in Figure 6 (a). Figure6 (b) shows that the defect was edema, but there was no new chondrocytes for group B. The results show that Chi-Hap scaffolds are potential biological scaffolds for repairing injured cartilage.

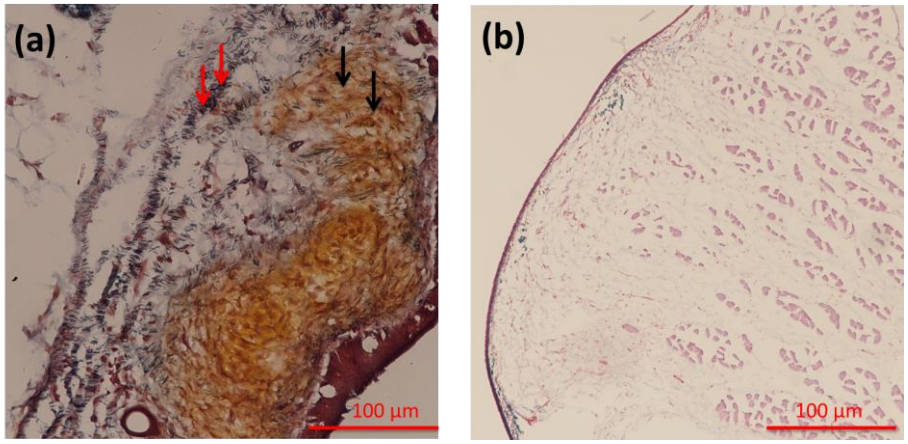

Figure 6 S-O staining for tissue samples at 10 weeks after operation.(a) Sample implant with Chi-HAP (group A); (b) **Sample** implant with no scaffolds(group B)

4. Discussion

Ideal Scaffolds possess three-dimensional structure, high porosity and good biocompatibility, which satisfied the supply of nutrients and excretion of metabolites. At the same time, excellent biological scaffolds should have good mechanical properties. The biochemical and physical properties of biological scaffold play a certain impact on the cells adhesion and tissue growth, which was affected by material selection and preparation process directly. The scaffolds prepared by conventional pressure drying methods exhibited porous structure [20], however, mutual connected channels are hard to obtained[21]. In this paper, the scaffolds fabricated via LDM achieve interconnected channel, which makes up for the shortcomings of the traditional craft. In addition, due to the volatilization of the solvent in the manufacturing process, the scaffolds possess suitable space for the growth of tissue.

With good bioactivity and degradability, chitosan is one of the most popular polymer materials in tissue engineering field. Due to the chitosan bio-scaffolds lack strong mechanical properties and biological signals to promote cell proliferation and differentiation [22], chitosan is always used compounding with other materials. HAP is a natural mineralization with good biocompatibility and

easy-molding. Moreover, a large number of experiments have shown HAP scaffolds implanted in the body appear apatite-like structure with the time prolong, which attract material scientists and tissue engineers attention [23]. Chitosan-hydroxyapatite composites play a significant role in the study of bone injury repair. Sun FF et al. [20] prepare Chi-HAP porous 3D scaffolds to repair rabbit bone injury using pressure drying method. After scaffold implantation for four weeks, the study found that a large number of active neonatal cartilage cells appeared at the interface of the fracture. Therefore, Chi-HAP composite scaffolds prepared by LDM are more favorable for tissue growth. Moreover, Chi-HAP composite scaffold overcomes the shortcomings for fast chitosan degradation, and provide a reasonable support in the cell and tissue growth process.

5. Conclusion

In this paper, Chi-HAP scaffolds were fabricated via the LDM. The study on characteristics of biological scaffolds showed that the Chi-HAP had cross-sectional and interconnected porous three-dimensional structures, which provides a place for the growth of new bone-like tissues and a reorganized channel for the transport of metabolic wastes and nutrients. The porosity and compatibility experiments demonstrated that Chi-HAP scaffolds had high porosity and excellent biocompatibility for seed cells. In animal experiments, with the degradation of Chi and HAP, internal pore size of the scaffolds implanted in the injury site will gradually increase, which was more conducive to the growth of cartilage-like tissue into. After 10 weeks, rabbits were sacrificed and HE and S-O staining was performed on the laryngeal tissues. The results showed that the injury site filling with the Chi-HAP appeared some newborn and disorder cartilage cells. Therefore, Chi-HAP scaffolds possess better bone-inducing activity for repairing cartilage tissue.

6. Acknowledgment

Thanks for help from Prof.Lin Zhihong of Department of Otorhinolaryngology in Second Affiliated Hospital of Zhejiang University, and Prof. Fang Ma-rong of Zhejiang University Medical College.

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