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Fabrication and evaluation of chitosan–gelatin based buckling implant for retinal detachment surgery

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Abstract The traditional nonabsorbable silicone buckling implant (buckle) may cause some long-term complications when it is used in the retinal detachment surgery. In this study we fabricated a chitosan–gelatin based buckling implant to replace the traditional one. We evaluated its biocompatibility with human scleral fibroblasts (HSF) in vitro and its cytotoxicity with L929 cell. To evaluate elasticity and hardness of the blends buckle, the mechanical properties of the chitosan–gelatin buckle were compared with the traditional silicone buckle. The light and electron microscopy coupled with immunocytochemistry demonstrated that chitosan–gelatin blends supported the survival and growth of primarily cultured HSF without significant cytotoxic effects. MTT analysis and cell cycle analysis indicated that chitosan–gelatin blends promoted the proliferation of HSF. A preliminary in vivo implantation test indicated that chitosan–gelatin buckling implant were compatible with the surrounding tissue. The results collectively demonstrated that chitosan–gelatin blends could be a candidate biodegradable material for scleral buckling surgery.

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1 Introduction

Retinal detachment is a common serious ocular disease that can cause severe visual dysfunction. The most efficient treatment is through surgery in which scleral buckling is one of the main operations. Buckling implants (buckle) must be used in the surgery to provide mechanical support until the broken retina has reattached with choroids. An ideal scleral buckle should biodegrade after the scar between the broken retina and choroid was formed. Silicone buckles have been used in clinical operation for decades but can cause many long-term complications such as intrusion of buckling implants [1, 2], refractive errors [3, 4], chronic infection [5, 6] and buckle extrusion [7, 8] due to the nonabsorbability. An absorbable buckle will obviously reduce these complications. Some novel absorbable scleral buckling materials such as polylactide [9] and collagen-glycosaminoglycan [10] have been reported in recent researches. However, the application of these materials was limited by considerable infections after implantation and an undesirable degradation [9, 10].

As the fully or partially deacetylated form of chitin, chitosan (CS) is the second most abundant polysaccharide in nature next to cellulose [11]. Chitosan possesses favorable physico-chemical and biological properties, providing a range of applications in biomedicine such as bandage material, skin grafting template, hemostatic agent, hemodialysis membrane, and drug delivery vehicle [12–15]. As a protein derived from collagen, gelatin does not express antigenicity in physiological conditions. Since gelatin's physical and chemical properties can be easily modulated

and it is cheaper than collagen [16], gelatin has been widely applied in tissue engineering such as artificial skin, neuronal regeneration and bone grafts [17–19]. Recently, much attention has been paid to utilize chitosan–gelatin complexes in biomedical applications, such as construction of cages for spinal operations [20] and reconstruction of artificial cornea [21].

In this study, we added chitosan to improve the stiffness of the blends and adjusted the elasticity by changing the amount of gelatin. From different chitosan/gelatin ratio blends, a buckle possessing most similar mechanical properties to the silicone buckle was selected for the subsequent testing. We cultured HSF on chitosan–gelatin membrane to examine the in vitro biocompatibility, and also cultured HSF in the chitosan–gelatin extract to evaluate the cell proliferation. To the best of our knowledge, this is the first study which evaluates biocompatibility between chitosan–gelatin blends (with no crosslinking agent) and HSF in vitro. And we also took the preliminary in vivo implantation test which showed good biocompatibility with the surrounding tissue. In brief, the present results could provide a candidate biomaterial for scleral buckling surgery.

2 Materials and methods

2.1 Preparation of chitosan–gelatin buckles and membranes

Chitosan powder (Nantong Xincheng Biochemical Company, Jiangsu, China), with a degree of deacetylation of 92.3% and an average molecular weight (Mw) of 2.8×10^4 kDa was dissolved in 2% (v/v) acetic acid and stirred. Gelatin was dissolved in distilled water at 50°C and stirred, and then the solution was added into chitosan solution. The blend solution was subsequently heated and agitated to form a viscous solution, with which the blends buckles or membranes were prepared by a casting method. The buckles or membranes were ultimately immersed in 20% (m/v) and 4% (m/v) NaOH aqueous solution respectively for 12 h to neutralize the remaining acetic acid, followed by washes with distilled water until neutral pH was obtained. We prepared several chitosan–gelatin buckles with CS/G ratio of 25/75, 40/60, 50/50, 60/40 and 75/25 by this method.

2.2 Characterization of chitosan–gelatin buckle

To select a chitosan–gelatin buckle with most similar mechanical properties to the silicone buckle, we tested the blends buckle and the traditional silicone buckle for the compression strength generating 30% depression and their recovery rate of elastic deformation respectively. The test was performed at room temperature.

Fourier transform infrared (FTIR) spectra of chitosan–gelatin buckle, pure chitosan and pure gelatin were recorded with a spectrometer (Nicolet 5700, Madison, WI).

The pre- and post-implantation chitosan–gelatin buckles were coated with gold in a JFC-1100 unit (Jeol Inc., Japan), and then examined under a scanning electron microscope (JEM-T300, Jeol Inc.).

2.3 Tissue and cell culture

HSF were obtained from tissue explants of patients who underwent enucleation or evisceration of eyeball after informed written consent, according to the tenets of the Declaration of Helsinki and the hospital ethics committee's approval. The surrounding connective tissue was dissected from the sclera carefully, and the sclera was then washed with phosphate buffer saline (PBS, pH 7.2) and sheared into 0.01 mm^3 tissue fragments which were planted into 75 cm^2 tissue flasks (Corning, USA). The clusters were incubated in DMEM containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin and streptomycin, in a 37°C humidified incubator with 5% CO_2. Half of the medium was replaced every 2 days.

Primarily cultured HSF were seeded onto the coverslips or chitosan–gelatin membranes which had all been placed onto a 24-well culture plate, followed by soaking in DMEM supplemented with 10% FBS. Another portion of primarily cultured HSF or L929 mouse fibroblasts (ATCC, USA) was seeded in a 96-well plate at a cell density of 0.5×10^4/well and the DMEM supplemented with 10% FBS or chitosan–gelatin extract were added into the plate, respectively.

2.4 Scanning electron microscopy

After 3- and 7-day culture, HSF on chitosan–gelatin membranes was washed twice with PBS and fixed in 4% glutaraldehyde. They were then post-fixed with 1% OsO_4, dehydrated stepwise in increasing concentrations of ethanol, and dried in a critical point drier (Hitachi, Tokyo, Japan), followed by coating with gold in a JFC-1100 unit (Jeol Inc., Japan) and observation under a scanning electron microscope (JEM-T300, Jeol Inc., Japan).

2.5 Immunocytochemistry

After 7-day culture on the chitosan–gelatin membranes or in the chitosan–gelatin extract, the samples was washed three times with PBS and fixed with freshly prepared 4% paraformaldehyde solution in 0.01 M PBS for 30 min at room temperature. The fixed samples were incubated for

1 h in a solution containing 10% goat serum, 3% bovine serum albumin and 0.1% Triton-X 100 at room temperature to block nonspecific binding. Then, they were allowed to incubate with primary antibodies: mouse monoclonal anti-Vimentin (1:50 dilution, Santa Cruz), mouse monoclonal anti-Collagen Type I (1:200 dilution, Millipore) overnight at 4°C in a humidified chamber. After being washed three times with PBS, the samples were further reacted with second antibodies: Alexa Fluor 488 goat anti mouse (1:200 dilution, Invitrogen), TRITC-labeled goat anti-mouse IgG (1:200 dilution, Santa Cruz) for 2 h at 37°C. The samples were washed three times with PBS, then incubated with 5 μg/ml Hoechst 33342 (Sigma) at room temperature for 15 min, mounted in fluorescent mounting medium. Finally, the cell samples were viewed under a confocal laser scanning microscope.

2.6 Cytotoxicity studies

According to ISO-10993, a modified MTT test, in which the yellow MTT is reduced to a purple formazan by mitochondrial dehydrogenase in cells, was performed to determine the in vitro cytotoxicity of the chitosan–gelatin extract on the cultured L929 cells derived from an immortalized mouse fibroblast cell line. After 1-, 2-, 3-day incubation in chitosan–gelatin extract or DMEM supplemented with 10% FBS, the viability of L929 was assessed. Briefly, L929 were washed three times with DMEM. The culture medium in each well of the plate was replaced with 100 μl DMEM and 25 μl MTT (5 mg/ml in PBS). After 4 h incubation at 37°C, the reaction solution was carefully removed from each well and 100 μl dimethyl sulfoxide was added. The plates were gently agitated until the formazan precipitate was dissolved, followed by measurement of OD values by spectrophotometry at 500 nm with an ElX-800 Microelisa reader (Bio-Tek Inc., USA).

2.7 Proliferation tests of HSF

MTT assay coupled with cell cycle analysis was performed to assess the proliferation of HSF. After 1-, 2-, 3-, 5-, 7- and 10-day culture, the viability of HSF cultured in DMEM supplemented with 10% FBS or chitosan–gelatin extract were assessed as above described. As to the cell cycle test, after 1-, 2-, 3-, 5- and 7-day culture, the HSF were collected by trypsinization (10^6 per tube) and washed twice (5 min per time) with PBS via centrifugation at 10,000 rpm. The cells were suspended in PBS, fixed with 70% ethanol (v/v) at −20°C. Samples were washed with PBS and stained with PI/RNase Staining Buffer (BD PharMingen) for 30 min in dark at 4°C. The number of cells at different phases of the cell cycle was analyzed using a flow cytometer (BD FACScalibur, BD Bioscience, USA).

2.8 In vivo biocompatibility study

All procedures used in the vivo biocompatibility study were in full accordance with the animal ethic rules indicated in the European Directive 86/609/EEC. And all efforts were made to minimize animal suffering and to reduce the number of animals used.

15 adult male white rabbits weighing 2.5–3.0 kg were used to evaluate the in vivo biocompatibility of the chitosan–gelatin buckle. The rabbits were anaesthetized by intravenous injection of 3% (m/v) sodium pentobarbital (50 mg/kg) into the marginal ear vein. All the surgical procedure imitated the clinical scleral buckling surgery. In every rabbit, chitosan–gelatin buckle was implanted in one eye while silicone buckle was implanted in another eye as a control. Three rabbits were killed at different time point: 1, 2, 4, 8 and 12 weeks after implantation. Then the buckles with the surrounding tissue were retrieved and fixed in 4% (v/v) paraformaldehyde overnight at 4°C, embedded, and cut on a cryostat into 10-μm-thick sections that were perpendicular to the longest axis of buckles, followed by haematoxylin and eosin (H&E) staining and observation under light microscopy.

2.9 Statistical analysis

At least three repetitive tests were performed, and all data were expressed with means ± SEM. A one-way ANOVA with the Stata 6.0 software package (Stata Corp., College Station, TX) was used to conduct statistical analysis, and statistical significance was accepted at the probability level $P < 0.05$.

3 Results

3.1 Characterization of chitosan–gelatin buckle

The compression strength generating 30% depression of different CS/G ratio buckles was showed in Table 1. The results indicated that the compression strength rose when the chitosan proportion was elevated. According to the Table 1, the compression strength of the buckle with CS/G ratio of 25/75 (1.27 ± 0.03 N) was most similar to that of the silicone buckle (1.13 ± 0.03 N). We also found that different chitosan–gelatin buckle and silicone buckle all returned to an initial form as soon as we moved the counterweight, and their recovery rates of elastic deformation were all within 1 s. The buckle with CS/G ratio of 25/75 was chosen to complete the subsequent tests.

Table 1 The compression strength of different CS/G ratio buckles comparing with the silicone buckle, in which the data of the buckle with CS/G ratio of 25/75 was found most similar to that of the silicone buckle

Buckle	Compression strength (N)
Silicone buckle	1.13 ± 0.03
CS/G: 25/75	1.27 ± 0.03
CS/G: 40/60	1.38 ± 0.03
CS/G: 50/50	1.43 ± 0.03
CS/G: 60/40	1.51 ± 0.02
CS/G: 75/25	1.62 ± 0.03

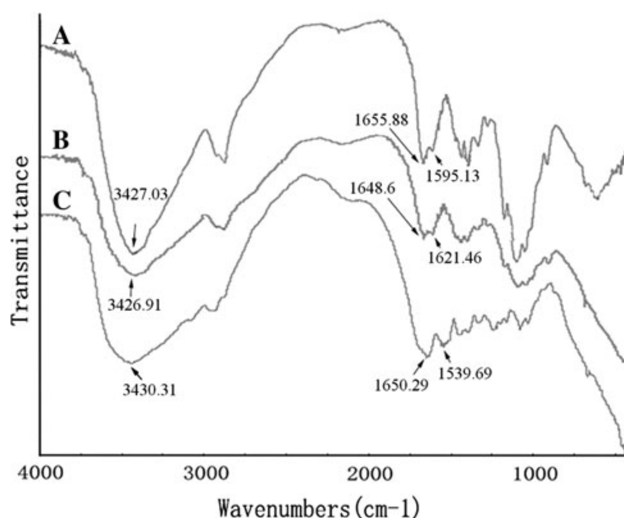

Fig. 1 The FTIR spectra of the pure chitosan (*a*), pure gelatin (*c*) and the chitosan–gelatin blends (*b*)

Figure 1 showed FTIR spectra of the chitosan, gelatin and the blends respectively. In Fig. 1a, the characteristic absorptions of the chitosan were displayed at 1655.88 cm^{-1}, 1595.13 cm^{-1} attributable to amide bands I, II and at 3427.03 cm^{-1} attributable to hydroxyl band. In Fig. 1c, the characteristic absorptions of the gelatin of it's amino band were at 1539.69 cm^{-1}, carbonyl peak at 1650.29 cm^{-1} and hydroxyl band at 3430.31 cm^{-1}. As to the blends, showed in Fig. 1b, the characteristic bands were appeared at 3426.91 cm^{-1} for OH, and at 1648.60 cm^{-1}, 1621.46 cm^{-1} for amide bands respectively. This result can not indicate the formation of new groups in chitosan–gelatin blends obviously owing to their same main functional groups, which is consistent with the reported results [22, 23].

A scanning electron microscope was conducted to compare the interior microstructure of pre- and post-implantation (10 weeks) chitosan–gelatin buckle. We found that the transverse section of post-implantation buckle was composed of an interconnected porous microstructure comparing a compact surface of pre-implantation buckle (Fig. 2).

3.2 Scanning electron microscopy

After 3-day culture on the chitosan–gelatin membranes, HSF showed a flat or slender spindle-shaped morphology with a low density (Fig. 3a), comparing with that of higher density after 7-day culture (Fig. 3b). On the other hand, after 7-day culture, HSF took compact arrangements of side-by-side configuration featuring like a whirlpool. This result indicated chitosan–gelatin blends supported the adhesion and growth of HSF.

3.3 Immunocytochemistry

Vimentin is a common cell marker of fibroblasts. Anti-vimentin immunocytochemistry of HSF after 7-day culture showed a number of vimentin positive (green fluorescence) cells with spindle-shaped morphology spread on the coverslips (Fig. 4A) and chitosan–gelatin membranes (Fig. 4B). Moreover, morphology of the cell on the chitosan–gelatin membranes was noted to be similar to that on the coverslips. Interestingly, the immunocytochemistry of HSF on chitosan–gelatin membranes showed a mild green or violet background, which may due to the membrane's absorption of fluorescence.

The results of anti-Collagen Type I immunocytochemistry showed that no significant differences about morphology and fluorescence were observed between HSF cultured in chitosan–gelatin extract (Fig. 5B) and that in DMEM supplemented with 10% FBS (Fig. 5A) after 7-day culture.

3.4 Cytotoxicity studies

The MTT result for L929 cell (Fig. 6) showed that the cell viability of L929 cultured in chitosan–gelatin extract was not significantly different from that in DMEM supplemented with 10% FBS after 24, 48 and 72 h, which indicated chitosan–gelatin had no obvious cytotoxic effect.

3.5 Proliferation tests of HSF differential absorption ratio

To investigate whether chitosan–gelatin extract have an effect on the proliferation of HSF, we performed MTT tests and cell cycle analysis. The MTT results showed that the cell viability of HSF cultured in chitosan–gelatin extract was not significantly different from that in DMEM supplemented with 10% FBS after 1-, 2-, 3-, 5- and 7-day culture, but higher than the latter after 10-day (Fig. 7a). The results of cell cycle analysis showed that the proliferation index (PI) of HSF cultured in chitosan–gelatin extract was not significantly different from that in DMEM

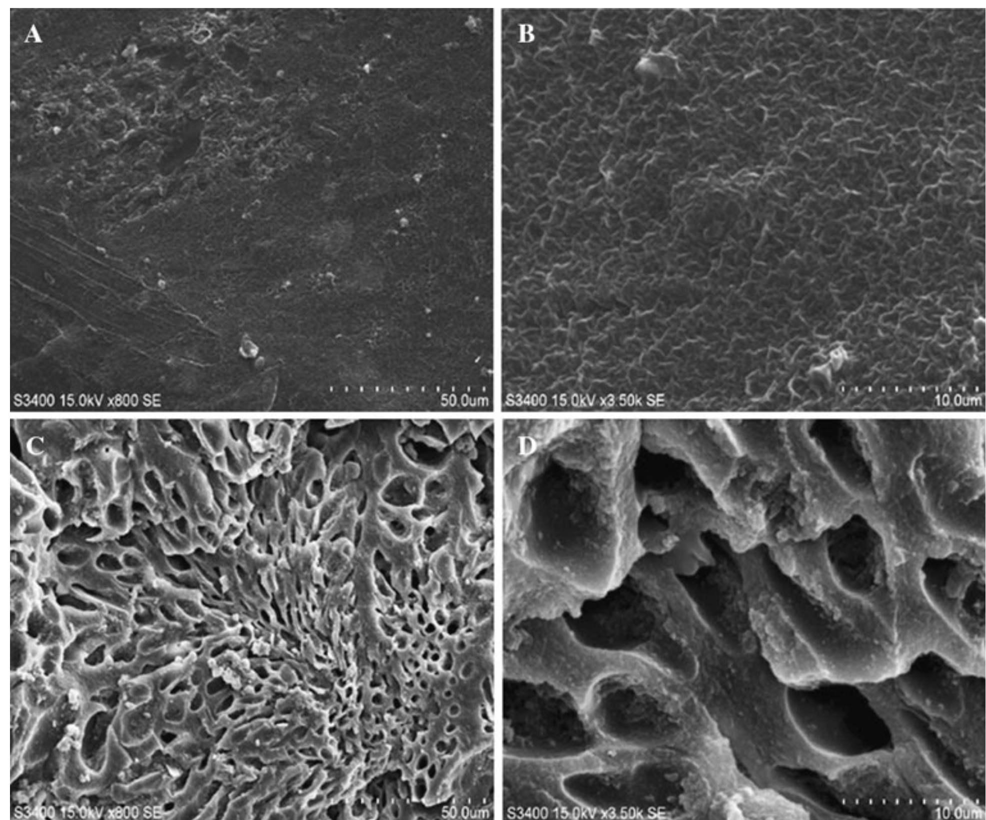

Fig. 2 Scanning electron micrographs (SEM) of the transverse section of pre-implantation chitosan–gelatin buckle (**a**, **b**) and post-implantation chitosan–gelatin buckle (**c**, **d**). (**b**), (**d**) is the higher magnification of (**a**) and (**b**). Scale bars: 50 or 10 μm

supplemented with 10% FBS after 1-, 2-day culture, but higher than the latter after 3-day (Fig. 7b).

3.6 In vivo biocompatibility study

At 1 week after operation, an acute inflammatory response was observed with a rapid accumulation of cells resembling neutrophils and macrophages at the site between the buckle and the sclera. At 2 week after operation, there were more lymphocytes around the buckle. At 10 week after operation, a capsule was found around the buckle, and there were still more inflammatory cells between the chitosan–gelatin buckle and the sclera (Fig. 8a) while relatively less inflammatory cells were found in sclera surrounding the silicone buckle (Fig. 8b). But the silicone buckle can not seen in Fig. 8 due to the technological reason. We also found the chitosan–gelatin buckle became thinner and the pores inside the buckle were bigger (Fig. 8a), which indicated that the buckle started to biodegrade partially.

4 Discussion

Scleral buckling surgery is used to treat retinal detachment by creating an indentation with the compression of a buckle [9]. This indentation is maintained by suturing the buckle in place episclerally or intrasclerally, so the buckle needs good toughness to provide mechanical support until the broken retina has reattached with choroids [10]. Having good elasticity and moderate hardness, the silicone buckle has been extensively used in clinical operation for decades. Despite the complications due to the biocompatibility and nonabsorbability, the silicone buckle is still main material for scleral buckling surgery because of its favorable mechanical properties.

In this study, we added chitosan to improve the stiffness of the blends and adjusted the elasticity by changing the amount of gelatin. In this way, we prepared five types of chitosan–gelatin buckle with different CS/G ratio, and found the buckle (CS/G: 25/75) possessed most suitable mechanical properties in comparison with the silicone buckle, which ensured creating an effective indentation of the surgery. We also hoped that a buckle with high proportion of gelatin could possess good biodegradability. According to the SEM result, the chitosan–gelatin buckle started to biodegrade partially after 10 weeks. An interconnected pore structure of the post-implantation biomaterial can increase the vascularization and nutrient exchange between the surrounding tissues, and accelerate the material degradation.

In the reported literature, chitosan–gelatin blends have usually been prepared with a chemical crosslinker such as

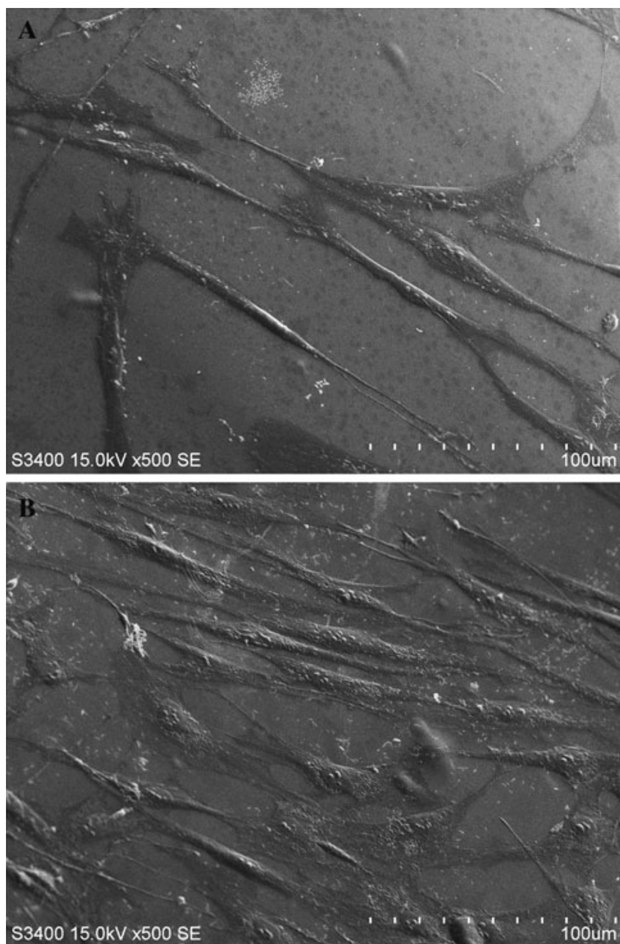

Fig. 3 Scanning electron micrographs (SEM) of HSF cultured on chitosan–gelatin membranes, in which HSF showed a low density after 3-day culture (**a**) comparing with that of higher density after 7-day culture (**b**). Scale bar: 100 μm

glutaraldehyde [24] and carbodiimides [25] or through a physical crosslinking method such as dehydrothermal treatment (DHT) [26] and UV-irradiation [27]. However, the main limitation of chemical crosslinker arises from the cytotoxic effect of the residual crosslinker during in vivo biodegradation. And it takes a long time for the physical crosslinking method to achieve a high crosslinking density. So we prepared chitosan–gelatin buckle by a blending method without any crosslinker, and the FTIR result showed the blends did not modify the chemical properties of chitosan and gelatin significantly.

L929 is easy to prepare and culture, which can provide more reproducible results, and is routinely used for cytotoxicity studies. The MTT assay is also a standard assay to evaluate the cytotoxicity of biomaterials. Therefore, according to ISO-10993, MTT test was performed to determine the in vitro cytotoxicity of the chitosan–gelatin extract on L929 cells. The result indicated that chitosan–gelatin was not obviously cytotoxic.

Human scleral fibroblast (HSF) is the primary structural and functional cell of sclera, while sclera is the main tissue in contact with the scleral buckle. Some serious long-term complications such as intrusion or extrusion of the buckle arise from the thinning sclera in which HSF may play a crucial role. So we focused on evaluating the in vitro biocompatibility between HSF and chitosan–gelatin. In order to examine the adhesion and growth of HSF on the chitosan–gelatin membranes, scanning electron microscopy was conducted. And the result indicated that HSF cultured on the chitosan–gelatin membranes showed normal morphology and growth, suggesting a good biocompatibility.

Vimentin is a common cell marker of fibroblasts. Anti-vimentin immunocytochemistry indicated that a number of vimentin positive cells cultured on the chitosan–gelatin membranes, identified as HSF, had similar morphology to that on the coverslips. As the most important extracellular matrix of sclera, Collagen Type I secreted by HSF plays an important role in the thinning of sclera [28]. The anti-Collagen Type I immunocytochemistry result indicated that no significant differences existed between HSF cultured in chitosan–gelatin extract and that in DMEM supplemented with 10% FBS, suggesting no inhibitory effects of chitosan–gelatin extract on the biological functions of HSF.

Proliferative status, as well as morphology, is also an important evaluating indicator for biocompatibility. In this study, MTT coupled with proliferation index (PI), referred to as the ratio of ($G_2M + S$) to ($G_2M + G_0G_1 + S$), were used to evaluate the proliferative status of cells. In Thein-Han et al.'s report, their MTT result demonstrated that chitosan–gelatin scaffolds promoted the proliferation of buffalo embryonic stem (ES) cells. And they considered the addition of gelatin facilitating greater cellular activity [29]. Mao et al.'s cell cycle result also showed chitosan–gelatin blends promoted the proliferation of L929 [30]. Our results coincided with the above reports, suggesting that the chitosan–gelatin blends have a good biocompatibility with HSF. We found that there was a delay in the OD value rising of MTT in comparison with the rising of PI. This delay may be attributed to the fact that it took the cells time to proliferate since the elevation of ratio of the S-phase cell.

As in vivo implantation test is most significant evaluating method for biocompatibility, a preliminary in vivo test was determined in this study. No obvious complications such as exposure of the buckle and faulty union of conjunctiva incision were found. And the HE staining result showed that chitosan–gelatin buckle evoked a mild tissue response and then started to biodegrade partially since postoperative 10 week. So we thought the chitosan–gelatin buckle was biocompatible in vivo. But more studies for longer time biocompatibility and biodegradation need to be conducted in the future.

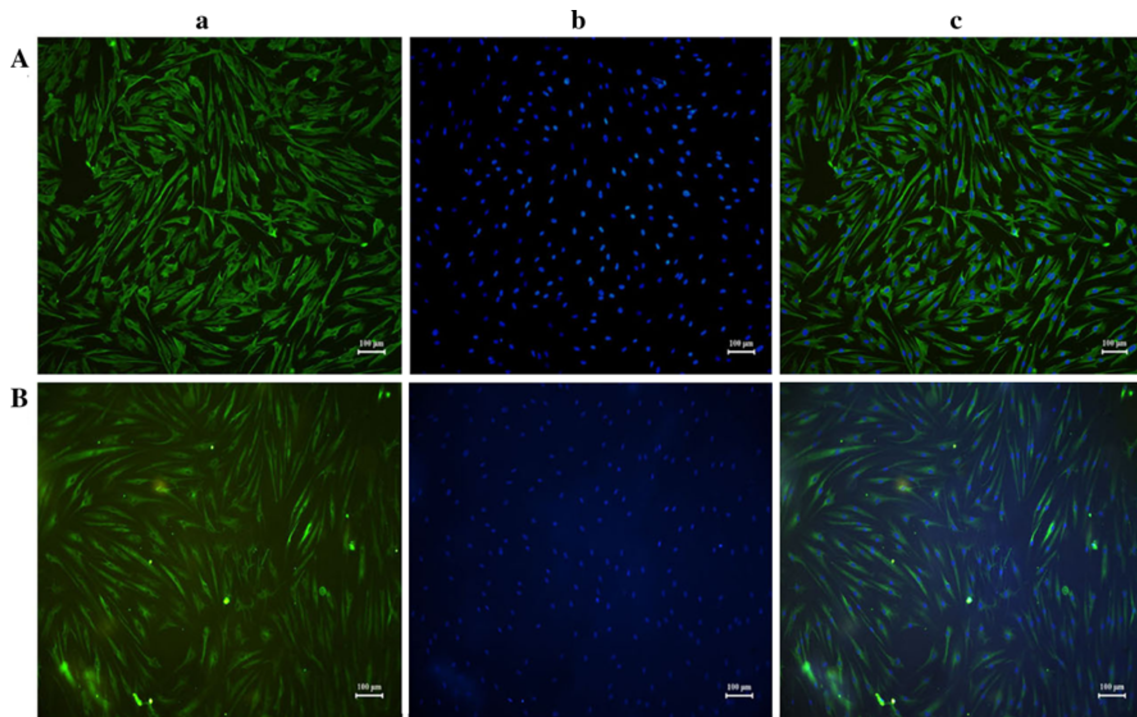

Fig. 4 Immunocytochemistry with anti-vimentin and Hoechst staining for HSF after 7-day culture on the coverslips (**A**) and chitosan–gelatin membranes (**B**), in which vimentin immunopositive cells were seen in (*a*), Hoechst-labeled cell nuclei of cells were seen in (*b*), and (*c*) is the merge of (*a*) and (*b*). Scale bar: 100 µm

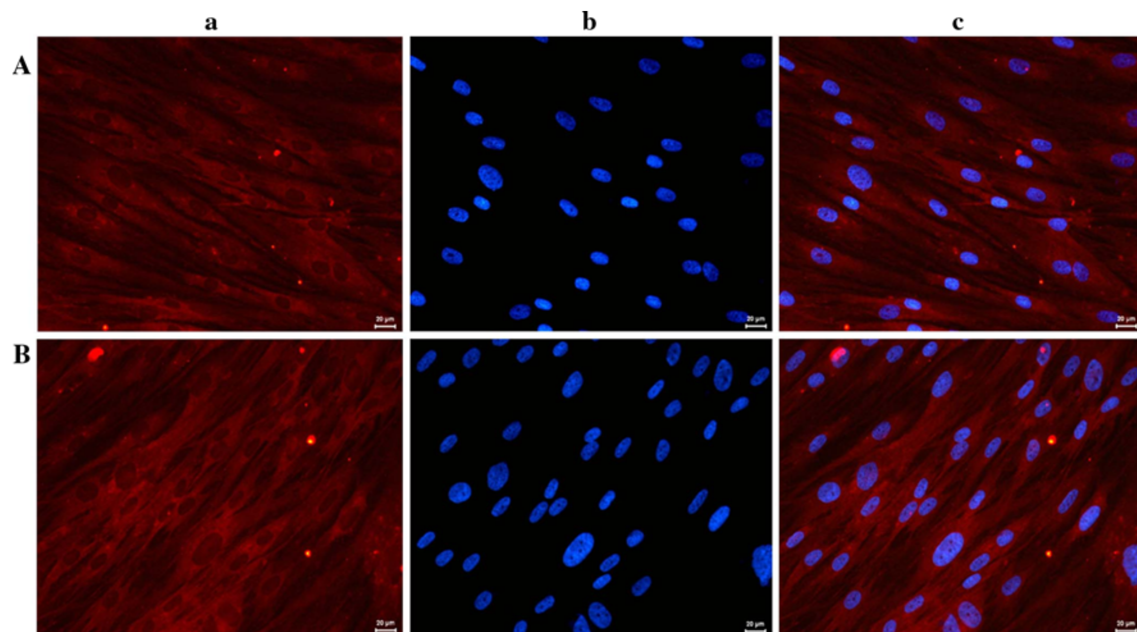

Fig. 5 Immunocytochemistry with anti-Collagen Type I and Hoechst staining for HSF after 7-day culture in the DMEM supplemented with 10% FBS (**A**) and chitosan–gelatin extract (**B**), in which Collagen Type I immunopositive cells were seen in (*a*), Hoechst-labeled cell nuclei of cells were seen in (*b*), and (*c*) is the merge of (*a*) and (*b*). Scale bar: 20 µm

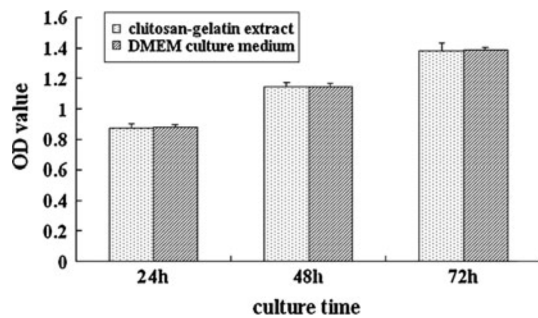

Fig. 6 Cell viability of L929 cells cultured in DMEM supplemented with 10% FBS and chitosan–gelatin extract at different culture time, in which no significant difference was found

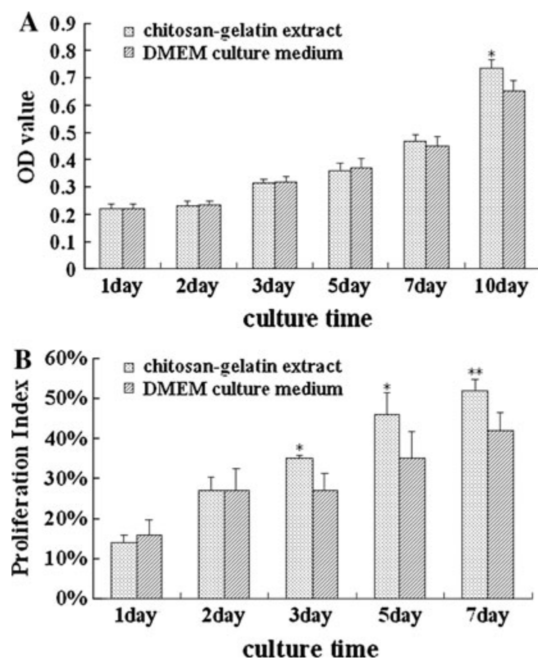

Fig. 7 MTT assay (**a**) and cell's proliferation index (**b**) after HSF cultured in DMEM supplemented with 10% FBS or chitosan–gelatin extract at different culture time. Both * $P < 0.05$ and ** $P < 0.01$ versus the corresponding values in DMEM supplemented with 10% FBS

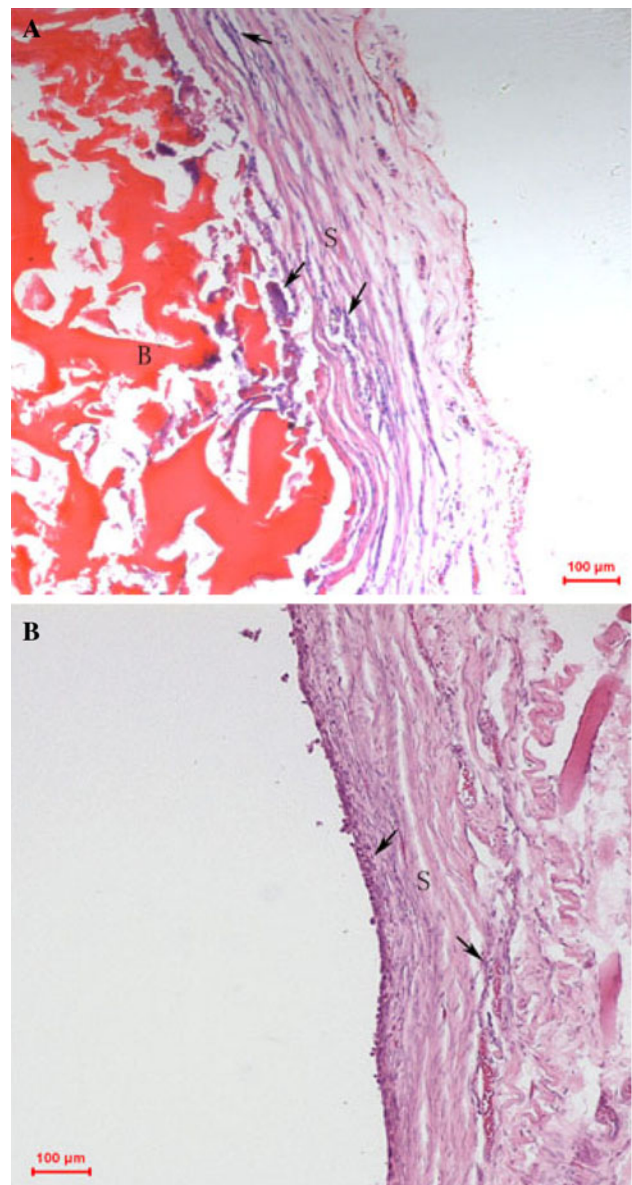

Fig. 8 Hematoxylin and eosin staining of the tissues implanted with chitosan–gelatin buckle (**a**) and silicone buckle (**b**) at 10 week, in which inflammatory cells (*black arrow*) were found between the chitosan–gelatin buckle (*B*) and the sclera (*S*). And the silicone buckle can not be seen in the photomicrograph due to the technological reason. Scale bar: 100 μm

5 Conclusion

In this study, we prepared chitosan–gelatin buckle and membranes by a blending method. The blends buckle was adjusted to possess good mechanical properties, which was suitable for the scleral buckling surgery. We found that chitosan–gelatin supported the adhesion and growth of the cultured HSF and promoted the proliferation of HSF. We also took the preliminary in vivo implantation test which showed good biodegradation and good biocompatibility with the surrounding tissue. These results suggested that chitosan–gelatin blends could be a good biodegradable material for retinal detachment surgery in the future.

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