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In vitro cytocompatibility evaluation of alginate dialdehyde for biological tissue fixation

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ABSTRACT

Biological tissues must be chemically fixed before they can be implanted in humans, due to the immediate degradation and presence of antigenicity of naturally derived tissues. To provide a crosslinking reagent which is cytocompatible and may prepare biocompatible fixed tissues, a novel crosslinking agent, alginate dialdehyde (ADA), was employed to fix biological tissues by our group. The study was to evaluate the cytocompatibility of ADA for biological tissue fixation. Glutaraldehyde and genipin counterparts were used as controls. The result suggested that the cytotoxicity of ADA was significantly lower than that of glutaraldehyde and genipin. Additionally, in the evaluation of cytotoxicity of fixed tissue itself and the residues, as well as the cell adhesion property, ADA-fixed tissue was significantly superior to its glutaraldehyde counterpart and comparable to its genipin counterpart. The results obtained in this study demonstrate that ADA is a cytocompatible crosslinking reagent for biological tissue fixation.

1. Introduction

Naturally derived biological tissues have been used extensively to fabricate various bioprostheses such as heart valves (Zhai et al., 2006), vascular grafts (Yu, Liu, Xu, & Wan, 2010), ligament substitutes (Sung, Shih, & Hsu, 1996) and pericardial patches (Jayakrishnan & Jameela, 1996), because that they can offer better constructions for adhesion and growth of cells than synthetic materials and possess mechanical properties similar to those of native tissues. However, due to the immediate degradation and presence of antigenicity, these naturally derived tissues must be chemically fixed before they can be implanted in humans (Schmidt & Baier, 2000). In recent years, various synthetic crosslinking reagents including formaldehyde, glutaraldehyde (Cheung, Perelman, Ko, & Nimni, 1985), polyepoxy compound (Sung et al., 1996) and cyanimide, 1-ethyl-3(-3 dimethyl aminopropyl) carbodiimide hydrochloride (EDC) (Lee, Edwards, & Pereira, 1996), have been widely used in fixing biological tissues, however, these synthetic crosslinking reagents are all highly (or relatively highly) cytotoxic (Nishi, Nakajima, & Ikada, 1995; Yu, Wan, & Chen, 2008). Therefore, to overcome the cytotoxic effect of the aforementioned synthetic crosslinking reagents, a novel crosslinking agent, alginate dialdehyde (ADA), was developed to fix biological tissues by our group.

Alginate (ALG), an important naturally occurring carbohydrate polymer derived from brown algae, has been widely used in a variety of biomedical applications, mainly due to its high biocompatibility and nontoxicity (Goh, Heng, & Chan, 2011; Isiklan, Inal, Kursun, & Ercan, 2010; Knill et al., 2004; Li, Ramay, Hauch, Xiao, & Zhang, 2005; Wang et al., 2010; Yang, Xie, & He, 2011). In recent years, it was reported that alginate can be oxidized with periodate to produce entity with multiple functional aldehyde groups (aldehyde alginate, ADA) (Bouhadir et al., 2001). As with glutaraldehyde, there exist multiple functional aldehyde groups in the chemical structure of ADA, which can produce crosslinking within biological tissues. In our previous study, the feasibility of using ADA as a crosslinking agent in fixing biological tissues was evaluated. Glutaraldehyde and polyepoxy compound were used as controls. It was found that ADA was in possession of the fixation index and mechanical strength comparable to glutaraldehyde and significantly superior to polyepoxy. Histological examination of the tissues after the ADA fixation process also showed intact total framework. This indicated that ADA is an effective crosslinking agent in the fixation of biological tissues (Xu, Li, Yu, Gu, & Zhang, 2012).

The present study was conducted to further evaluate and analysis the cytocompatibility of ADA for biological tissue fixation in vitro. In the study, fresh porcine aortas procured from a slaughterhouse were used as raw materials. Glutaraldehyde (GA) and genipin (GP)-fixed counterparts were used as controls. Glutaraldehyde is the most commonly used crosslinking reagent for tissue fixation (Jayakrishnan & Jameela, 1996) and genipin is the most popularly

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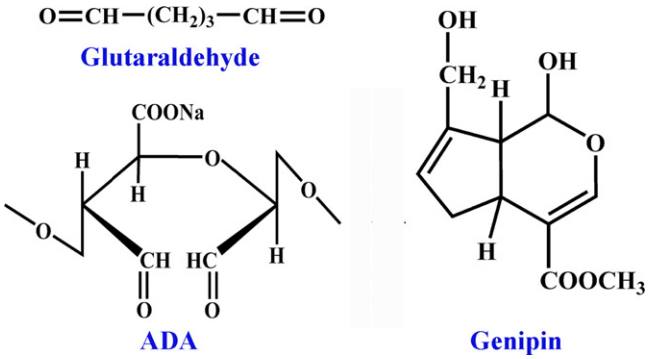

Fig. 1. The chemical structure of glutaraldehyde, genipin and alginate dialdehyde (ADA) in the study.

investigated low-cytotoxicity crosslinking reagent in recent years (Huang, Sung, Tsai, & Huang, 1998). The chemical structures of these crosslinking reagents are shown in Fig. 1.

2. Materials and methods

2.1. Materials

Sodium alginate (viscosity: 495 cps at 25 °C) was obtained from Qingdao Jingyan Biotechnology Co. LTD (China). Glutaraldehyde and diphenyl tetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Genipin was purchased from Linchuanzhixin Biotechnology Co. LTD (China). Triton X-100 was obtained from Amresco Co. (USA). DNaseI and RNseA were obtained from Aladdin Co. (Shanghai, China). Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT, USA). DMEM, trypsin, penicillin and streptomycin were purchased from Gibcobrl (Grand Island, NY, USA). Sodium periodate and other reagents (analytical grade or equivalent grade) were obtained from Kelong Co. (Chengdu, China). Distilled water was employed throughout.

2.2. Preparation and assessment of ADA

ADA was prepared according to our previously reported method (Vieira, Cestari, Airoldi, & Loh, 2008; Xu et al., 2012). The sodium alginate (5 g) was dissolved in 200 ml distilled water and 50 ml pure ethanol by prolonged magnetic stirring in a beaker. And then 5.7 g of sodium periodate was added to the solution in the dark at room temperature to obtain the product. After 24 h, the reaction was stopped by 10 ml of ethylene glycol under dark for 2 h. 5 g of sodium chloride was then added to the solution, followed by precipitation with 800 ml of ethyl alcohol. The precipitates, collected by a centrifuge, were then dissolved in about 100 ml distilled water again and re-precipitated by the addition of 600 ml ethanol. This procedure was repeated three times. Furthermore, the product solution was dialyzed using dialysis tube (MWCO, 3500) against distilled water with several changes of water until it was free from periodate (Balakrishnan, Lesieur, Labarre, & Jayakrishnan, 2005). The dialyzate was then lyophilized to obtain the product.

2.3. Decellularization and crosslinking process

In the study, fresh porcine aortas obtained from a local abattoir were used as raw materials. The procured aortas were brought to the laboratory in sterile phosphate buffered saline (PBS). Upon return, the excess blood on the tissues was immediately removed by rinsed with fresh saline and the adherent fat was also carefully trimmed from the aortas surface with a scalpel. The warm ischemic time was less than 6 h from the time of tissue retrieval to decellularization (Yu et al., 2010). Subsequently, the decellularization process was conducted as the methods developed by Liang et al. with slight modification (Liang, Chang, Hsu, Lee, & Sung, 2004). The fresh porcine aortas first were treated by 0.1% trypsin and 0.02% EDTA solution at 37 °C for 4 h, and then washed with sterile PBS, followed by treatment in a hypotonic tris solution with 1% Triton X-100 for 48 h. Finally, the tissues were further incubated with RNaseA (0.02 mg/ml) and DNaseI (0.2 mg/ml) at 37 °C for 4 h with agitating to remove cellular components. To observe the effect of decellularization process, the decellularized sample was fixed in a 3% glutaraldehyde solution for SEM observation (JSM-7500F, JEOL) and in a 10% formalin solution for Hematoxylin and Eosin Y (H&E) staining.

After washing with sterile phosphate-buffered saline solution, the decellularized aortas were fixed in a 15% ADA solution, which was buffered with phosphate-buffered saline (pH 7.4). Meanwhile, the samples fixed with 0.625% glutaraldehyde solution (buffered with phosphate-buffered saline, pH 7.4) (Xu et al., 2012) and 0.625% genipin solution (buffered with citric acid/sodium citrate buffered saline, pH 4.0) (Gu et al., 2011) were also used as controls. The samples of each group were all fixed at 37 °C for 72 h under continuous shaking.

2.4. Cell culture

The mouse-derived established cell line of L929 fibroblasts was utilized in this study, which was purchased from West China Hospital, Sichuan University (China). The cells were cultured in high Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin and incubated at 37 °C in a humidified atmosphere (5% CO_2 in 95% air). When reached the stage of confluence, cells were passaged at a 1:2 split ratio following trypsinization with 0.25% trypsin. Cells from passage 3 were used in our experiments. During cell-culture period, the culture mediums were changed every 2 days.

2.5. Cytotoxicity of the crosslinking reagents

When the cells reached the stage of confluence, they were harvested by trypsinization, followed by the addition of fresh culture medium to create cell suspension. The cells were seeded into 96-well plate at a density of 2×10^3 cells/well and cultured in DMEM medium. After 1 day, the medium in wells was respectively replaced with medium supplemented with glutaraldehyde in a serial concentration of 0.0156, 0.156, 0.315, 0.625, 1.25 mg/ml (Zhai et al., 2006), genipin in a serial concentration of 25, 50, 125, 250, 375 mg/ml (Sung, Huang, & Huang, 1998), or ADA in a serial concentration of 125, 250, 375, 500, 1000 mg/ml ($n = 6$). The cell culture was performed at 37 °C in humidified 95% air/5% CO_2. The viable cell number cultured in each well was determined indirectly by MTT assay at 2, 4, and 6 days. Details of the methodology used in the MTT assay were previously described (Yu et al., 2008). Briefly, at the end of incubation period, 20 μl of MTT solution (5 mg/ml in phosphate buffered saline) was added to each well and then the plate was incubated at 37 °C to form the insoluble formazan crystals. After 4 h, the culture medium was aspirated and 200 μl of DMSO was added to each well, followed by constant shaking at room temperature to dissolve the dark blue crystals, the product of deoxidized MTT yielded by mitochondrial dehydrogenases of viable cells. After 10 min, the optical density (OD) was measured at 492 nm using a Microplate Reader (Model550, Bio Rad Corp.).

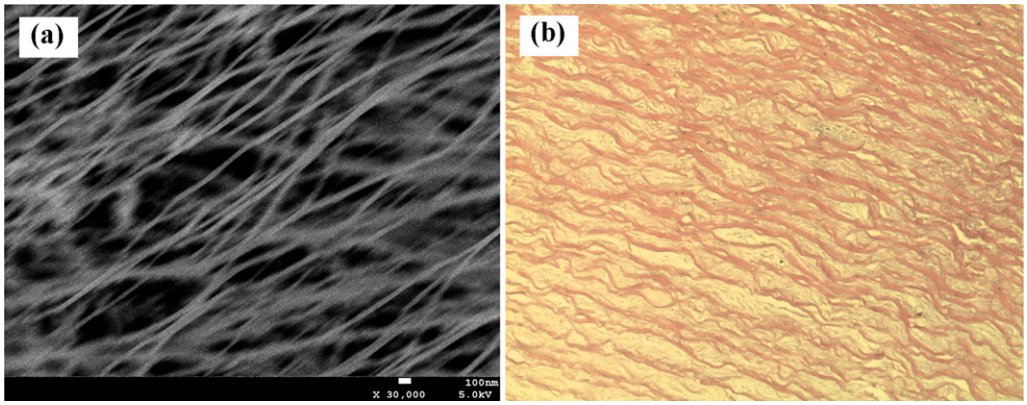

Fig. 2. Photomicrographs of SEM (a) and H&E staining (b) of the decelluarized tissues.

2.6. Cytotoxicity of the fixed tissues

A drawback of the chemically fixed biological tissues is the potential cytotoxicity, which may be from fixed tissues itself and its residues such as the remaining cross-linking reagent and/or the particles from abrasion or hydrolysis of the tissues (Nishi et al., 1995). Therefore, the cytotoxicity of the fixed tissues was evaluated by both the direct contact assay and indirect extraction assay. The direct contact assay (light microscopic observation) was applied to observe the culture of cells in the vicinity of the fixed-tissues, while the indirect extraction assay (MTT assay) was used to quantitative assess the proliferative activity of cells after exposure to extraction liquid of the fixed-tissues.

In the direct contact cytotoxicity study of the fixed-tissues, a sterilized test sample (5 mm × 5 mm) cut from each studied group was placed to the center of each well in a 24-well plate and rinsed with 500 μl cell culture medium for 4 h ($n = 3$). Subsequently, L929 fibroblasts at 5×10^4 cells/well were seeded evenly in the well. The cell culture was maintained in a humidified incubator at 37 °C with 5% CO_2 in air. The cells cultured in the vicinity of the fixed-tissues were photographed using an inverted light microscope (Olympus Corporation, Japan) daily after cell seeding. During this period, the growth medium was changed every 2 days (Sung et al., 1998).

In the indirect cytotoxicity study of the fixed-tissues, the sterilized tissues ($n = 6$) were first placed in the saline for 24 h at 37 °C in 5% CO_2 for extraction liquid. Subsequently, the cells were seeded into 96-well plate at a density of 2×10^3 cells/well in 100 μl DMEM medium, followed by an addition of 100 μl extraction liquid of the related fixed-tissues. The cell culture was maintained in a humidified incubator at 37 °C with 5% CO_2 in air. The viable cell numbers cultured in each well were determined indirectly by MTT assay at 1, 3, 5 and 7 days. During this period, the growth medium was changed every 2 days (Xu et al., 2012).

2.7. Cell adhesion of fixed tissues

To study the effects of fixed-tissues on cell adhesion, all test samples (7 mm × 7 mm) coated with type I collagen were put in a 24-well plate and rinsed with 500 μl cell culture medium for 4 h ($n = 3$). Subsequently, 100 μl cell suspensions at a density of 5×10^5 cells/ml were used to seed the samples kept in 24 well plates. To allow the cells to adhere to the tissues, the plate were incubated at 37 °C for 4 h and then followed by an additional 400 μl cell culture medium to each well. The cell culture was incubated at 37 °C in a 5% CO_2 and 95% air atmosphere. During this period, the culture mediums were changed every 2 days. At the 7th day post seeding, the test tissues were retrieved from the wells and fixed in a 10% formalin solution. To observe the cells adhered on the surface, the retrieved test samples were stained with Hematoxylin and Eosin Y (H&E) to color the cell nuclei blue. The stained samples were examined using light microscopy (Yu et al., 2010).

2.8. Statistical analysis

Statistical analysis was performed with SPSS (v13.0). Quantitative data are presented as a mean value with its standard deviation indicated (mean ± SD), and statistical significance was set at $p < 0.05$.

3. Result and discussion

3.1. Decellularization and crosslinking of the tissues

As shown in Fig. 2(a) and (b), the morphology of the porcine aortas demonstrated an intact total framework and excellent porous micro architecture after the decellularization treatment. It was obvious that this decellularization processes could result in porous scaffolds without apparent disruptions of histoarchitecture, which had prominent influence on cell intrusion, proliferation, and function in tissue engineering. Additionally, there was no cellular components observed in the decelluarized vascular, which indicated the successful decellularization process through the thickness of the vessels. Residual cellular components in natural biomaterials may cause undesired effects, such as immunological recognition (Schmidt & Baier, 2000). This complete removal of original cell residual substance or cellular components in biological tissue scaffolds can reduce the antigenicity derived from cell, and furthermore diminished the immune response elicited to these natural biomaterials for use in cell seeding and tissue engineering applications (Murayama, Satoh, Oka, Imanishi, & Noishikl, 1988).

Fig. 3 gives a photograph of fresh, glutaraldehyde-fixed, genipin-fixed and ADA-fixed porcine aortas respectively. After fixation, it was found that the biological tissue fixed with genipin became dark bluish while the color of both glutaraldehyde-fixed and ADA-fixed tissue turned yellow. This difference was mainly caused by the different crosslinking mechanism of various crosslinking reagents. The crosslinking mechanism of genipin react with biological tissues is not thoroughly understood, however, the reason for the dark-bluish color observed with the genipin-fixed tissue may be the reaction of genipin with the amino acid residues biological tissues. It was reported that genipin can spontaneously react with amino acids to form dark blue pigments (Huang et al., 1998). Whereas, presumed crosslinking mechanism of ADA with biological tissues may be similar to glutaraldehyde, which was based on the formation of Schiff bases by the reaction of aldehyde functional groups with amino groups (Xu et al., 2012). The relatively deeper yellow

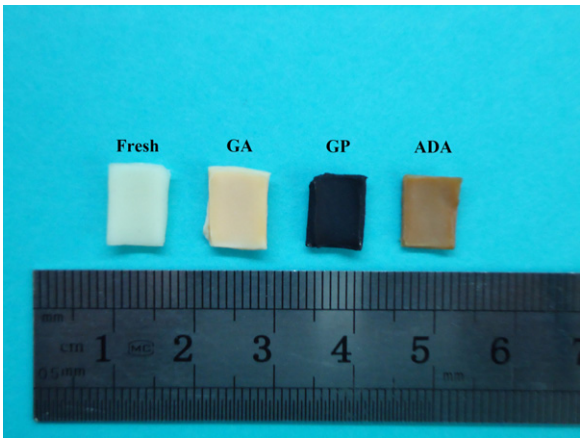

Fig. 3. Photographs of fresh, glutaraldehyde (GA)-fixed, genipin (GP)-fixed and ADA-fixed tissues.

of ADA-fixed tissues than that of glutaraldehyde may be a result of higher concentration of aldehyde groups.

3.2. Cytotoxicity of the crosslinking reagents

Fig. 4 showed the optical density (OD) value of the L929 fibroblasts cultured in the medium drugged with varying concentrations of glutaraldehyde, genipin or ADA acquired in the MTT assay after 6 days of culture, respectively. The optical density value in the MTT assay reflected the number of the viable cells in culture medium. A larger optical density value compared with the negative control indicated the higher viability of the cells in the culture medium, that was to say, the cytotoxicity of the test sample is much lower or did not exist (Liu et al., 2009).

As shown in Fig. 4(a) and (b), the optical density value decreased with an increase in concentration of glutaraldehyde or genipin drugged in the growth medium. Glutaraldehyde obviously inhibits cell proliferation even at the concentration of 0.625 μg/ml, while genipin exhibited an obvious inhibition effect on cell proliferation at the concentration of 50 μg/ml. This result indicated that the glutaraldehyde exhibited significantly higher cytotoxicity than did genipin. Sung et al. conducted an in vitro study to investigate the cytotoxicity of genipin used for crosslinking biological tissues (Sung et al., 1998). They found that genipin was significantly less cytotoxic than glutaraldehyde. The results of our cytotoxicity study also confirmed their finding.

Fig. 4(c) showed a faster proliferation of L929 fibroblasts in medium supplemented with 125–250 μg/ml ADA than in the control group. Meanwhile, the ADA did not show any inhibition effect on cell proliferation as compared with the control at the concentration of 375 μg/ml. When the concentration of ADA was more than 500 μg/ml, there was a significant decrease in the optical density value of the L929 fibroblasts, indicating an obvious inhibition of cell proliferation. This result indicated that ADA even had a stimulatory effect on cell proliferation at a low concentration and the cytotoxic effect of ADA was 10 times lower than that of genipin or nearly 1000 times lower than that of glutaraldehyde. The low cytotoxicity of ADA itself on L929 fibroblasts indicated that ADA may be a more cytocompatible crosslinking reagent than glutaraldehyde and genipin.

3.3. Cytotoxicity of the fixed tissues

The cytotoxicity of the fixed tissues in this study was evaluated by both the direct contact assay and indirect extraction assay. Fig. 5 gives photomicrographs of L929 fibroblasts directly cultured in the vicinity of the fixed tissues, which provided a visual comparison of cell quantity and cell morphology. As shown in Fig. 5(a) and (b), remarkably few cells were observed in the vicinity of the glutaraldehyde-fixed tissue and the morphology of these cells was rounded, which was the characteristic of cell death. In Fig. 5(c)–(f), a distinct increase in cell quantity and less noticeable cell distortion was observed surrounding the fixed tissues. Furthermore, no noticeable difference was observed between genipin-fixed and ADA-fixed tissue both in the quantity and morphology of the cells. Similar cytotoxicity result for genipin was reported by Sung et al. in the literature (Sung et al., 1998). The indirect contact assay results demonstrated that the cytotoxic effect was extremely marked when cells were directly contacted with glutaraldehyde-fixed tissue while the genipin-fixed and ADA-fixed tissue elicited comparatively less cytotoxic response to the surrounding cells.

Fig. 6 illustrates the optical density value obtained in the MTT assay for the L929 fibroblasts cultured in the extraction liquid of the fixed tissues. The optical density value of the test sample demonstrated the effect of fixed tissues on cell proliferation, and then reflected the cytotoxicity of fixed tissues. As shown in Fig. 6, the optical density value of glutaraldehyde-fixed samples declined markedly compared to the negative control, meaning an obvious inhibition of glutaraldehyde-fixed tissues for cell proliferation. In contrast, the optical density value for the L929 fibroblasts cultured in the extraction liquid of genipin-fixed and ADA-fixed tissues was significantly higher than that of the negative control, indicating a stimulatory effect on cell proliferation. All these results in the MTT assay were consistent with the previous results on the light microscopic observation. However, this comparable result for genipin-fixed and ADA-fixed tissues was different from the result obtained in the aforementioned cytotoxicity of the crosslinking reagents, which may be contributed to the higher concentration of functional groups used in the fixation process.

It was reported that the potential cytotoxicity of the chemically fixed biological tissues may be from crosslinking reagent, fixed

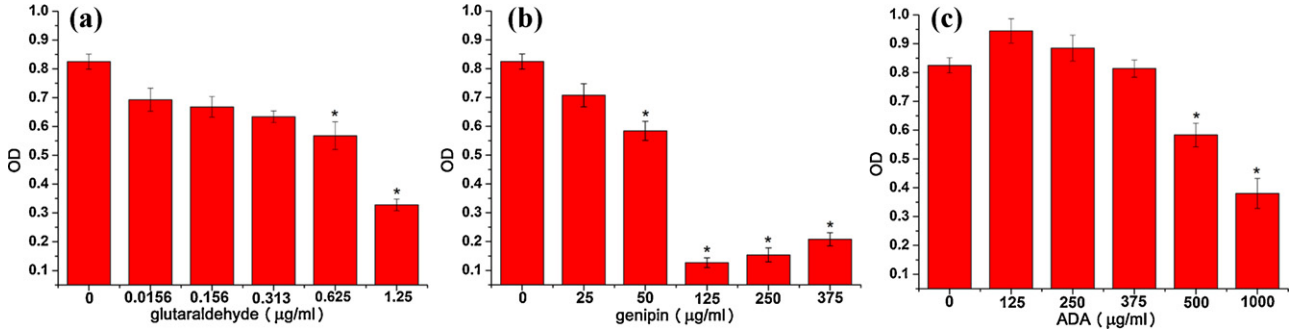

Fig. 4. The optical density of L929 fibroblasts cultured in the medium drugged with varying concentrations of glutaraldehyde (a), genipin (b) or ADA (c) obtained in the MTT assay.

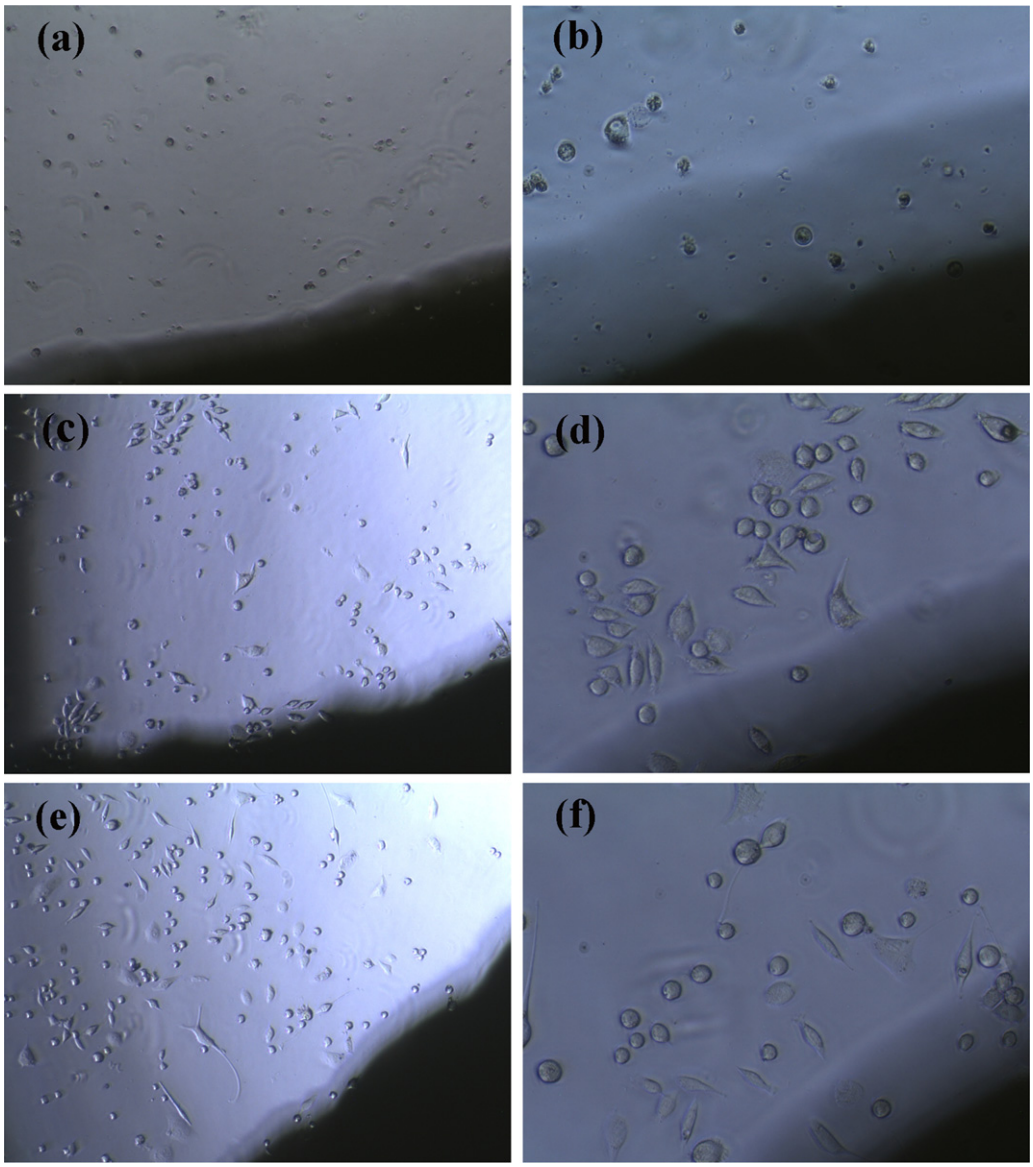

Fig. 5. Photomicrographs of L929 fibroblasts cultured in the vicinity of the fixed tissues for 4 days. (a) Glutaraldehyde-fixed tissue, 40× magnification, (b) glutaraldehyde-fixed tissue, 100× magnification, (c) genipin-fixed tissue, 40× magnification, (d) genipin-fixed tissue, 100× magnification, (e) ADA-fixed tissue, 40× magnification, and (f) ADA-fixed tissue, 100× magnification.

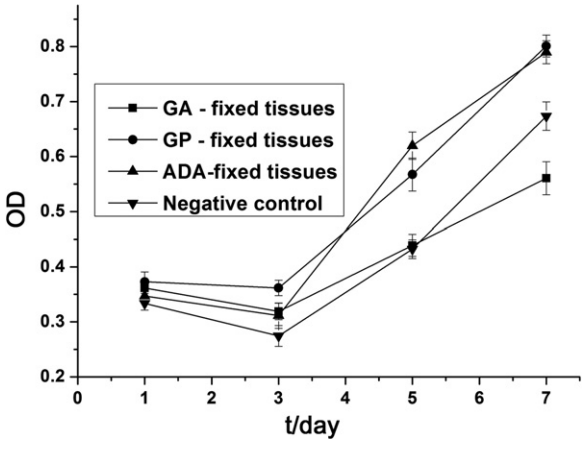

Fig. 6. Proliferation of the L929 fibroblasts cultured in the extraction liquid of the fixed tissues. GA: glutaraldehyde; GP: genipin.

tissue itself, as well as the continuous leaching-out of the residues such as unreacted crosslinking agents and/or the degraded particles from abrasion or hydrolysis of the tissues (Nishi et al., 1995). Taking these factors into account, the results in our cytotoxicity study suggested that crosslinking with ADA could provide a well cytocompatible biological scaffold without inhibition in cell proliferation.

3.4. Cell adhesion of fixed-tissues

Fig. 7 presented the a photomicrograph of Hematoxylin and Eosin staining for L929 fibroblasts cultured on the surface of fixed tissues for 7 days (arrow indicates fibroblasts seeded on acellular tissues). The type I collagen was coated on the surfaces of fixed tissues in order to facilitate the adhesion of cells (Yu et al., 2008). As shown in the figure, no cell was observed on the surface of the glutaraldehyde-fixed tissue, while cells seeded on the surface of the genipin-fixed tissue were observed to adhere to the surface and form a significantly confluent monolayer. Compared with

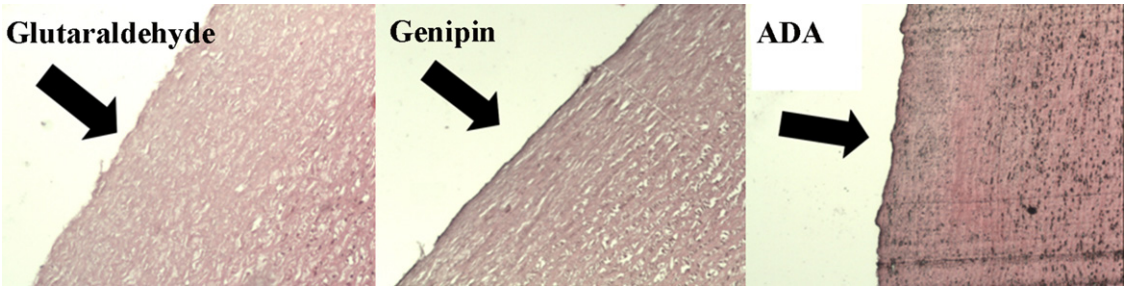

Fig. 7. HE staining for fibroblasts cultured on the surface of fixed tissues (arrow indicates fibroblasts seeded on acellular biomaterials).

the genipin-fixed biological vascular scaffold counterpart, a significantly confluent monolayer was also found on the surface of the ADA-fixed tissue. Moreover, of interest is that these fibroblasts cultured on the surface of ADA-fixed tissue seemed to have invaded into the scaffold, which would be investigated thoroughly in our further study.

In this cell adhesion study of test fixed-tissues, it was observed grossly that the cells seeded on the surface of the glutaraldehyde-fixed tissue were not able to adhere to the surface. In contrast, a significantly confluent cell monolayer was observed on the surface of the both genipin-fixed and ADA-fixed tissues, which implied that the cells seeded on the genipin-fixed tissue and ADA-fixed tissue could not only adhere but also proliferate. These findings further demonstrated that the cellular compatibility of the ADA-fixed tissue was comparable to its genipin-fixed counterpart and significantly superior to its glutaraldehyde-fixed counterpart, which was also consistent with the previous result obtained in the cytotoxicity of the fixed tissues.

Besides appropriate chemical and physical properties, the most important requirement for a chemically fixed biological material used for bioprosthesis is its biocompatibility. Cytocompatibility study of a material is the initial step to evaluate its biocompatibility and the fast effective method to choose the appropriate material. Our previous feasibility study of using ADA as a crosslinking agent in fixing biological tissues proved that the crosslinking characteristics of the ADA were compared to those of glutaraldehyde and polyepoxy.

In this experiment, the cytocompatibility of ADA as a crosslinking agent in fixing biological tissues was studied by evaluating the cytotoxicity of crosslinking reagent, the cytotoxicity of fixed tissue itself and the cell adhesion effect of the fixed tissue. The results obtained in the aforementioned studies all suggested that the cytocompatibility of ADA was significantly superior to its glutaraldehyde counterpart and comparable to its genipin counterpart. Genipin is the most popularly investigated low-cytotoxicity crosslinking reagent in recent years, mainly for its good biocompatibility used in the biomedical applications (Huang et al., 1998). On the other hand, glutaraldehyde is the most commonly crosslinking reagent for tissue fixation in clinical practice since late 1960s, which is mainly due to its excellent crosslinking characteristics (Jayakrishnan & Jameela, 1996). However, the high cytotoxicity of glutaraldehyde has been animadverted all along, which was reported to result from its aldehyde groups interacting with the cells (Gendler, Gendler, & Nimni, 1984). Of interest was that ADA may fix tissues in the same way as glutaraldehyde, by using its aldehyde functional groups reacting with the free amino groups within biological tissues. In our study, ADA demonstrated a very good cytocompatibility, in spite of its aldehyde groups.

This difference in cytocompatibility between the glutaraldehyde and ADA may be caused by their differences in source and chemical structure. As mentioned above, glutaraldehyde is typically synthetic crosslinking reagent while ADA is derived from alginate, a naturally occurring polysaccharide proved to be biocompatible. Moreover, the molecular weight of ADA (about 10,000) is much larger than that of glutaraldehyde (about 100), which might make the diffusion of ADA into the cells more difficult than glutaraldehyde. Therefore, ADA is more difficult to react with proteins or polysaccharides on and inside the cells, and thus result to less cell death than glutaraldehyde. In summary, the results obtained in our study suggest that ADA is a cytocompatible crosslinking reagent for biological tissue fixation. Moreover, our further study will pay attention to the biocompatibility the ADA-fixed tissues in vivo.

4. Conclusion

In summary, this study demonstrated the cytocompatibility to use ADA as a novel crosslinking reagent in the fixation of biological tissues. The result suggested that the cytotoxicity of ADA crosslinking reagent was significantly lower than that of glutaraldehyde and genipin. Additionally, in the cytotoxicity of fixed tissue itself and the residues from the fixed tissues, as well as the cell adhesion property, ADA-fixed tissue was significantly superior to its glutaraldehyde counterpart and comparable to its genipin counterpart. The results obtained in this study in vitro demonstrate that ADA is a cytocompatible crosslinking reagent for biological tissue fixation. Moreover, our further study will pay attention to the biocompatibility the ADA-fixed tissues in vivo.

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