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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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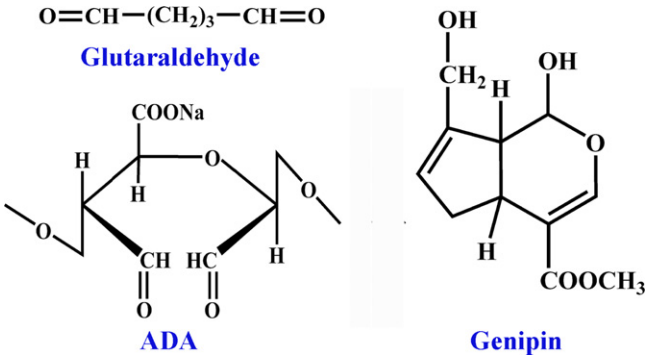

Fig. 1. The chemical structure of glutaraldehyde, genipin and alginate dialdehyde (ADA) in the study.

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.).