ORIGINAL ARTICLE

A Comparative Study of the Effects of Different Decellularization Methods and Genipin-Cross-Linking on the Properties of Tracheal Matrices

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Abstract

BACKGROUND: Different decellularization methods can affect the integrity and the biomechanical and biocompatible properties of the tracheal matrix. Natural cross-linking with genipin can be applied to improve those properties. The goals of this study were to evaluate the effects of different decellularization methods on the properties of genipin-cross-linked decellularized tracheal matrices in rabbits.

METHODS: The tracheas of New Zealand rabbits were decellularized by the Triton-X 100-processed method (TPM) and the detergent-enzymatic method (DEM) and were then cross-linked with genipin. Mechanical tests, haematoxylin–eosin staining, Masson trichrome staining, Safranin O staining, DAPI staining, scanning electronic microscopy (SEM), and biocompatibility tests were used to evaluate the treatment. The bioengineered trachea and control trachea were then implanted into allogeneic rabbits for 30 days. The structural and functional analyses were performed after transplantation.

RESULTS: The biomechanical tests demonstrated that the biomechanical properties of the decellularized tracheas decreased and that genipin improved them ($p < 0.05$). The histological staining results revealed that most of the mucosal epithelial cells were removed and that the decellularized trachea had lower immunogenicity than the control group. The analysis of SEM revealed that the decellularized trachea retained the micro- and ultra-structural architectures of the trachea and that the matrices cross-linked with genipin were denser. The biocompatibility evaluation and *in vivo* implantation experiments showed that the decellularized trachea treated with the DEM had better biocompatibility than that treated with the TPM and that immunogenicity in the cross-linked tissues was lower than that in the uncross-linked tissues ($p < 0.05$).

CONCLUSIONS: Compared with the trachea treated with the TPM, the rabbit trachea processed by the DEM had better biocompatibility and lower immunogenicity, and its structural and mechanical characteristics were effectively improved after the genipin treatment, which is suitable for engineering replacement tracheal tissue.

Keywords Tissue engineering · Trachea · Decellularization · Genipin · Biological properties

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1 Introduction

Tracheal diseases derive from a series of factors, such as infection, congenital defects and tumour [1]. The resection of the diseased trachea and an end-to-end anastomosis is the classic surgical procedure for the treatment of tracheal diseases. However, the treatment can not be performed when the tracheal defect length is longer than 1/3 of the trachea in children or 1/2 in adults [2]. Tissue-engineered (TE) tracheal replacement has successfully repaired the defective trachea [3, 4], suggesting that TE transplants provide new therapeutic options for patients. The ideal tracheal replacements have characteristics similar to those of natural trachea, including tightness, non-degradability and non-toxicity [5]. TE involves the interaction among the isolated cells, tissue and extracellular matrix, thereby inducing the formation of new tissues or organs. The bio-material scaffold plays an important role in TE by providing the initial mechanical properties and geometry to support tissue regeneration, so long as there are environmental cues for regulating cell growth and differentiation [6, 7]. The scaffold should possess mechanical strength but not immunogenicity, and the acellular tracheal matrices produced by decellularization are considered excellent scaffolds for TE.

Triton-X 100 has been considerably prominent in tissue engineering and was used for decellularization as early as 1987 by Cortijo [8]. However, the concentration of Triton-X 100 is currently controversial. Johannes et al. used a 3% concentration of Triton, while Wallis et al. used a 2% [9, 10]. Triton-X 100 has often been applied with other reagents, such as sodium deoxycholate, to enhance its effect [11, 12]. In addition, to improve the removal efficiency of DNA, DNase-I has been used to hydrolyse the remaining DNA after epithelial cells are dissolved by Triton-X 100. However, there is no clear standard for the cycle of decellularization. Scholars have studied 2, 6, 7, 8, cycles, and so on [13]. Our experimental team used 2% concentration of Triton and 7-cycle of DNase-I treatment according to previous studies. Epithelial cells and their nuclear materials, which are the main antigens that mediate the immune response, were mostly removed. The tracheal cartilage is preserved, which provides the scaffold for bioengineering. Numerous decellularization methods for preparing the matrices, such as Triton-X 100-processed method (TPM) and detergent-enzymatic method (DEM), have been reported to simultaneously improve the effectiveness of removing the cellular ingredients and preserve of the structural and biomechanical properties of the extracellular matrix [14, 15]. However, even though the acellular tracheal matrices were prepared well, the rapid degradation of the decellularized scaffold still restricted

their clinical applications. Genipin is a natural cross-linking agent, and it forms a cross-linked product with strong stability, low cytotoxicity, good biocompatibility, and low susceptibility to calcify. Genipin enhances the biomechanical properties and prevents degradation by blocking the access of degrading enzymes to the matrices. Thus, the purposes of our investigation are to compare the impact of TPM and DEM on the integrity, biomechanical properties and biocompatible properties of the extracellular tracheal matrices in rabbit, to evaluate whether genipin improves the structural and biomechanical properties of decellularized tracheal matrices, and to find a suitable method for preparing tissue engineering tracheal scaffolds.

2 Materials and methods

New Zealand rabbits ($n = 55$) were selected, weighing 2.5 ± 0.5 kg, and were obtained from the Experimental Animal Center of Yangzhou University. All rabbits were treated in compliance with the rules and regulations of Laboratory Animals formulated by the National Ministry of Science (IACUC no. SYXK2016-0041).

2.1 Study design

All tracheas were harvested from the donor rabbits ($n = 25$) in sterile surroundings according to the standard surgical procedure. The excised tracheas were subsequently stored in cold phosphate-buffered saline (PBS, HyClone, Pittsburgh, PA, USA) that contained 1% antibiotic and antimycotic (Sigma, St. Louis, MO, USA). The separated tracheas were treated with PBS (control group, $n = 5$, Group A₀), TPM ($n = 5$, Group B₀), DEM ($n = 5$, Group C₀), TPM with genipin ($n = 5$, Group D₀), and DEM with genipin ($n = 5$, Group E₀). The New Zealand rabbits ($n = 30$) used for the *in vivo* implantation were randomly divided into six experimental groups (five animals each): Group A-rabbits receiving the tracheas in group A₀; Group B-rabbits receiving the tracheas in group B₀; Group C-rabbits receiving the tracheas in Group C₀; Group D-rabbits receiving the tracheas in Group D₀; Group E-rabbits receiving the tracheas in Group E₀; and Group F-sham rabbits. The decellularization methods are described in the following sections (part 2.1.1 and part 2.1.2).

2.1.1 Triton X-100-processed method

The tracheas were washed three times by sterile PBS and then immersed in sterile distilled water at 4 °C for 48 h, followed by incubation in 2% Triton X-100 [16] (Meilun, Dalian, China) at 37 °C for 12 h. A DNase-I solution (2 kU ml^{-1} ; Sigma, St. Louis, MO, USA) in 1 M NaCl

was used to remove the remaining nucleic acid with constant rotation for 4 h at room temperature.

2.1.2 Detergent-enzymatic method

The tracheas were washed three times by sterile PBS and then immersed in sterile distilled water at 4 °C for 48 h. Afterwards, they were soaked in sodium deoxycholate (Sigma, St. Louis, MO, USA) for 4 h at 37 °C, followed by treatment with DNase-I in 1 M NaCl for 4 h at room temperature [17]. After washing with sterile distilled water three times (5 min each), the tracheas were stored in PBS containing 1% antibiotics and antimycotics at 4 °C overnight. This procedure was performed seven times.

2.1.3 Genipin-cross-linking

The decellularized tracheas processed by the TPM or DEM were incubated with 1% genipin (Merlin, Hangkong, China) at room temperature for 2 h and then washed with distilled water three times. Afterwards, the tracheas were stored in PBS containing 1% antibiotics and antimycotics for analysis.

2.2 Biomechanical tests

A vernier caliper was used to measure the length, thickness, and transverse and longitudinal diameters of the tracheas. The strain characteristics were tested via a tensile testing instrument (Instron 3367, Canton, Norwood, MA, USA). The tissues were fixed on the machine, subjected to a pre-load of 0.1 N and tested at a constant elongation rate of 30 mm/min at 37 °C. The tensile stress and elastic modulus were recorded when the tracheas were subjected to an increasing uniaxial tensile force until rupture. The compressive properties of each sample were recorded by a Perkin DMA Q800 instrument (TA instruments, Inc, New Castle, UK) in real time.

2.3 Histological staining

The native and decellularized tracheas were fixed in 10% neutral formaldehyde (Sigma, St. Louis, MO, USA) for 24 h at room temperature and then washed with distilled water. After gradient alcohol dehydration and paraffin embedding, the samples were sectioned into 4 µm slices. After dewaxing, the slices were stained with haematoxylin and eosin (H&E; KeyGNE, Nanjin, China), the Masson reagent (Leagene, Beijing, China), and the safranin O reagent (Solarbio, Beijing, China) to observe the morphological changes.

2.4 DAPI staining

The removal of cell nuclei in the tracheas was detected by the DAPI (KeyGNE, Nanjin, China) staining assay. Five µm of the frozen sections were stained with the DAPI working solution (0.1 µg/ml) for 15 min at 37 °C. After rinsing with methanol, the slices were soaked in Buffer A and observed with a fluorescence microscope (EVOS, Thermo Fisher, Waltham, MA, USA).

2.5 Scanning electronic microscopy

The tracheas were fixed with 2.5% glutaraldehyde (Aladdin, Shanghai, China) for 24 h. After washing with phosphate buffer three times (15 min each), the samples underwent gradient alcohol dehydration, critical point drying, fixation and metal spraying and were then qualitatively observed by scanning electronic microscopy (SEM; S-4800, Hitachi High-Technologies Corporation, Tokyo, Japan) to identify the matrix structure of the tracheas.

2.6 Cell attachment and proliferation test

The BMSCs were cultured and passaged until the fourth generation, similar to a previous study [18]. Before planting the cells, the trachea was trimmed to $0.5 \times 0.5 \text{ cm}^2$, immersed in 75% medicinal alcohol for 30 min and then placed under UV irradiation for 2 h. A one hundred µL drop of the cell suspension was placed onto the trachea at a density of 3×10^4 cells/piece in a 96-well plate and incubated for 4 h. The plates were then replaced, and 200 µL of 10% foetal bovine serum (FBS, Clark, Salt Lake, UT, USA) in DMEM-F12 medium (HyClone, Pittsburgh, PA, USA) was added to each well. The cells and trachea were co-cultured for 7 days with a medium change every 48 h, and the adhesion and proliferation of the cells were examined within 1, 3, 5, and 7 d. After removing the medium, 100 µL of a CCK-8 working solution (dilution ratio: 1/10, under dark conditions, Biosharp Biotech, Anhui, China) was added on the surface of the trachea and incubated for 2 h; then, 50 µL of the supernatant was pipetted, and the OD₄₅₀ was measured by a microplate reader (BioTek, Winooski, VT, USA).

2.7 In vivo implantation

2.7.1 Surgical procedures

After anaesthesia with 0.2 ml/kg xylazine hydrochloride, the skin on the back of the neck was incised, and the superficial fascia was isolated. The side of the spine was separated, and the tissue was embedded into it, followed by

interrupted suture. Penicillin (50,000/U Qd, Lukang Pharmaceutical, Shandong, China) was injected intramuscularly in the first week after surgery. The experimental animals euthanized on the 30th postoperative day.

2.7.2 Histological staining

The experimental method for processing the harvested plants was described above (part 2.3). The slices were stained with H&E and the CD68 molecule (dilution ratio: 1/200, Abcam, Shanghai, China) after dewaxing. The cells that had a major role in the allograft immune response were marked, and their tissue morphology in different regions was evaluated in the same group and compared with the tissue and cell compositions in the same region in different groups.

2.8 Statistical analysis

The experimental data were analysed with SPSS 19.0 (Chicago, IL, USA). All differences were deemed statistically significant at the level of $p < 0.05$.

3 Results

3.1 Biomechanical properties

After decellularization, the tubular structures of the tissues were kept. The test of stretch mechanical property revealed that there was significant difference in the maximum stress required to break the samples and the tensile strength between the decellularized and native tracheas ($p < 0.05$). However, the parameters of maximum stress and tensile strength in the cross-linked group were higher than those of the uncross-linked group ($p < 0.05$). In the test of compressive mechanical property, compared with those of the native trachea, the stress loading of 50% deformation and the elastic modulus were lower in the decellularized

trachea treated by TPM and DEM; however, the cross-linked tissue had higher values than the uncross-linked tissue ($p < 0.05$). This indicated that genipin improved the biomechanical properties of the tracheas (Table 1).

3.2 Histological staining

Compared with the control group (Figs. 1A, 2A), H&E and Masson trichrome staining revealed that TPM (Figs. 1B, 2B) and DEM (Figs. 1C, 2C) removed more mucosal epithelial cell components. Compared with the control group (Fig. 3A), Safranin O staining demonstrated that the glycosaminoglycan level was slightly decreased in both the TPM (Fig. 3B) and DEM (Fig. 3C) groups. Compared with the uncross-linked trachea, the structure was tighter in the trachea cross-linked by TPM (Figs. 1D, 2D) and DEM (Figs. 1E, 2E).

3.3 DAPI staining

The nuclei of the mucosal epithelial cells and cartilage cells in the native rabbit trachea were stained by DAPI and emitted blue fluorescence (Fig. 4A). TPM (Fig. 4B) and DEM (Fig. 4C) removed the mucosal epithelial cells, while some cartilage cells were still detected. These results indicated that both TPM and DEM effectively removed immunogenicity.

3.4 Scanning electronic microscopy

A great number of cilia were observed on the outer surface of the native trachea (Fig. 5A). The basement membrane was relatively smooth but was slightly disrupted by the TPM (Fig. 5B). The decellularized trachea retained the micro- and ultra-structural architectures of the trachea, including those of the luminal basement membrane, while the cellular components of the mucosa and submucosa were completely removed, and the epithelial cells were removed completely (Fig. 5B–C). After cross-linking by

Table 1 Biomechanical properties of the native and bioengineered tracheas

Characteristics	Native	TPM	DEM	TPM with genipin treatment	DEM with genipin treatment
<i>Stretch mechanical property</i>					
Maximum stress (N)	9.51 ± 0.62	8.34 ± 0.26 ^a	8.19 ± 0.32 ^a	9.16 ± 0.38 ^{bc}	9.32 ± 0.68 ^{bc}
Tensile strength (MPa)	0.62 ± 0.06	0.31 ± 0.04 ^a	0.28 ± 0.24 ^a	0.45 ± 0.02 ^{abc}	0.42 ± 0.09 ^{abc}
<i>Compressive mechanical property</i>					
Loading (N) of 50% deformation in compression	2.261 ± 0.165	1.669 ± 0.149 ^a	1.414 ± 0.284 ^{ab}	1.818 ± 0.148 ^{ab}	1.681 ± 0.107 ^{ab}
Elastic modulus (N/mm ²)	0.598 ± 0.129	0.404 ± 0.024 ^a	0.334 ± 0.058 ^a	0.51 ± 0.074 ^{bc}	0.496 ± 0.053 ^{ac}

ANOVA, compared with the native trachea (^a $p < 0.05$), TPM (^b $p < 0.05$), DEM (^c $p < 0.05$)

Fig. 1 A Shows the control groups. The characteristics of the decellularized tracheal matrix for TPM (B) and DEM (C) were observed by H&E. The matrix was cross-linked by genipin after

decellularization by TPM (D) and DEM (E). Compared with that in the uncross-linked trachea, the structure was tighter in the cross-linked trachea (scale bar: 20 μ m)

Fig. 2 Masson trichrome staining of the native trachea (A) and trachea harvested from TPM (B) and DEM (C). The matrix was cross-linked by genipin after decellularization by TPM (D) and DEM (E) (scale bar: 20 μ m)

Fig. 3 Compared with that of native tracheas (**A**), the results showed that the level of glycosaminoglycan, which is stained red in the picture, was slightly decreased for TPM (**B**), DEM (**C**). **D**, **E** Show samples cross-linked by genipin after decellularization using TPM

and DEM, respectively. No obvious change was observed in safranin O staining after cross-linking by genipin, compared with that of the uncross-linked trachea (scale bar: 20 μ m)

Fig. 4 DAPI staining of native trachea (**A**) and trachea treated by the TPM (**B**) and DEM (**C**). **D**, **E** Show samples cross-linked by genipin after decellularization using the TPM and DEM, respectively (scale bar: 200 μ m)

Fig. 5 A great quantity of cilia with a regular array was observed on the outer surface of the native trachea (A). The cilia were removed completely, and the basement membrane was relatively smooth; however, the trachea was slightly disrupted by the TPM (B). After the genipin treatment, the structure of the basement membrane was still

intact but more compact (D). The results also demonstrated that the cellular components of the trachea decellularized by DEM were removed completely without any obvious damage to the basement membrane (C); however, the structures were more compact after the genipin treatment (E) ($\times 3.0$ k)

Fig. 6 Scanning electron microscopy image of the cells in tracheal stents: native tracheas (A), tracheas treated by TPM (B), tracheas treated by DEM (C), tracheas treated by TPM with genipin treatment

(D), and tracheas treated by DEM with genipin treatment (E). The BMSCs grown on each group of tracheal stents for 24 h attached, and the morphologies of the cells were favourable ($\times 3.0$ k)

genipin, the basement membrane structure was still intact and more compact (Fig. 5D). However, the cellular components of the trachea that was decellularized by the DEM were mostly removed without causing any obvious damage to the basement membrane (Fig. 5C), which was better than those of the trachea decellularized by the TPM; additionally, the structure was more compact after the genipin treatment (Fig. 5E), suggesting that genipin improved the structure. The BMSCs grown on each group of tracheal stents for 24 h attached, and the morphology of the cells was favourable (Fig. 6A–E).

3.5 Biocompatibility evaluation of the different groups of tracheas

It was observed that the cells adhered to the surface of the scaffold and continued to proliferate within 1–5 days and that the number of cells continued to increase. However, due to the lack of subsequent passages, the inhibition of cell contact and the death of self-aging, the number of cells decreased in 7 days. The OD values of each group were statistically analysed and are listed in Table 2. The number of cells in the cross-linked trachea was greater than that in the uncross-linked trachea, while the number of cells in the control group was the lowest ($p < 0.05$). Although there were more cells in the trachea treated by the DEM than in the trachea treated by the TPM, there was no significant difference ($p > 0.05$, Table 2).

3.6 In vivo implantation and biocompatibility testing

3.6.1 General morphology

In addition to group B, two experimental animals died with urine and blood. The other experimental animals performed well within 30 days after the surgery, and the body masses increased. The wounds healed well. At 30 days, compared with the tracheas before planting, all embedded plants were decreased in length and increased in weight. Inflammatory connective tissue surrounded the embedded tracheas of

groups A and B. The structure of the tracheal lumen collapsed and was clogged. It adhered to the implanted bed and was not easily stripped. A large amount of pus accumulated in the cystic tracheal embedment and affected the normal tracheal anatomy (Fig. 7a, b). Among all groups, the connective tissue around the tracheal stent in group C was thinnest. There was no obvious sign of rejection, and neovascularization occurred on the tracheal stent surface (Fig. 7c). The luminal structures of groups C, D and E were still maintained (Fig. 7c, d, e). Compared that of group D, less inflammatory connective tissue surrounded the connective tissue in group E (Fig. 7d, e).

3.6.2 Histological staining

The embedded plants were histologically evaluated by H&E and the CD68 molecule. The experimental results showed that the structures of group A and group B were disordered. The cartilage tissue was almost completely destroyed, and the inflammatory cells infiltrated. Compared with that of group B, cartilage structure of group D was preserved, and the degree of infiltration of the inflammatory cells was slightly less; however, the morphology of the chondrocytes was abnormal (Figs. 8B, D, 9B, D). There was no infiltration of inflammatory cells, destruction of tracheal structures, or adverse reactions, such as calcification, in groups C and E, and there was no abnormality in the morphology of the chondrocytes (Figs. 8C, E, 9C, E).

4 Discussion

TE is considered a potential option for repairing damaged organs or tissues [19]. Tissues and organs derived from TE methods have been successfully used in preclinical animal experiments and clinical trials in humans [20, 21], providing extensive space for the replacement of tissue and organ [22]. There is currently a need to search for effective acellular methods, and the preparation of the acellular matrix is an extremely important step. The extracellular matrix (ECM), which is a complicated mixture of structural

Table 2 Results of the CCK-8 test under different methods

Method	1 day	3 days	5 days	7 days
Native	0.082 ± 0.002	0.137 ± 0.007	0.423 ± 0.018	0.413 ± 0.018
TPM	0.095 ± 0.003	0.209 ± 0.012 ^a	0.476 ± 0.02 ^a	0.416 ± 0.011
DEM	0.118 ± 0.019 ^a	0.217 ± 0.021 ^a	0.5 ± 0.015 ^a	0.452 ± 0.024 ^a
TPM with genipin treatment	0.123 ± 0.018 ^{ab}	0.365 ± 0.016 ^{abc}	0.626 ± 0.025 ^{abc}	0.539 ± 0.027 ^{abc}
DEM with genipin treatment	0.144 ± 0.02 ^{abc}	0.371 ± 0.028 ^{abc}	0.658 ± 0.019 ^{abc}	0.561 ± 0.029 ^{abc}

ANOVA, compared with native trachea (^a $p < 0.05$), TPM (^b $p < 0.05$), DEM (^c $p < 0.05$)

Fig. 7 Morphology images of the trachea before (A) and after (B) implantation. ('a' indicates native tracheas, 'b' indicates tracheas treated by TPM, 'c' indicates tracheas treated by DEM, 'd' indicates

tracheas treated by TPM with genipin, 'e' indicates tracheas treated by DEM with genipin)

Fig. 8 H&E staining of the grafts after implantation: native tracheas (A), tracheas treated by TPM (B), tracheas treated by DEM (C), tracheas treated by TPM with genipin (D), and tracheas treated by

DEM with genipin (E). Eosinophils migrated throughout the graft (arrow) (scale bar: 20 μ m)

and functional proteins [23], can be obtained from native tissues after decellularization. Many decellularization methods, including the physical, chemical and enzymatic methods, have been widely used in the field of TE [22, 24]. Nevertheless, an optimal method that can completely remove the cellular ingredients and preserve the structural and biomechanical properties of extracellular matrix has still not been found.

To prepare a suitable ECM, different decellularization methods are available. Triton-X 100 is a commonly used

non-ionic detergent, as it breaks the lipid–lipid and lipid–protein interactions instead of the protein–protein interactions; thus, many membrane proteins are solubilized in non-ionic detergents without affecting the structure of the protein [25]. Although the improved method increased the removal efficiency of epithelial cells, the matrix structure of the stent was slightly damaged. Furthermore, the safe concentration of Triton-X 100 was difficult to be defined, and a high concentration greatly damaged the ECM. In the allograft embedding experiment, two of the New Zealand

Fig. 9 IHC staining of CD68 in the grafts after implantation: native tracheas (A), tracheas treated by TPM (B), tracheas treated by DEM (C), tracheas treated by TPM with genipin (D), tracheas treated by

DEM with genipin (E). The inflammatory cells migrated throughout graft (arrow) (scale bar: 20 μ m)

rabbits in which the embedded tracheal tissue was treated with Triton-X 100 had haematuria and eventually died. The cause of death could be that the rabbits were unable to tolerate the 2% concentration of Triton-X 100. Therefore, the safety of Triton-X 100 still needs to be verified, which limits its application in the clinic. In addition, the removal efficiency of the nuclear materials depends on the type of tissue. For example, in the nerves [26] and pericardium [27], nuclear materials are completely removed by Triton-X 100, while the same effect is not observed in the artery [28].

DEM has been widely used in various fields, such as the throat [29] and blood vessel [13]. Acellular tracheal matrices of rat [30] and pig [31] have been achieved, further confirming that DEM is an ideal decellularization method. The only method applied to the clinic to prepare acellular tracheal matrices is DEM based on sodium deoxycholate and DNase-I [28]. Sodium deoxycholate is a commonly used ion detergent and dissolves the cells and nuclear membrane effectively. DNase-I is used to hydrolyse the remaining nuclear material. The results showed that the epithelial cellular components were removed and that the structure of the matrix and the mechanical properties are similar to those of the native samples. This indicated that DEM did not damage the tracheal matrix. However, DEM was superior because the epithelial cellular components were removed without destroying the structure of the matrix. Although the result of Masson trichrome staining

and DAPI staining showed no significant difference between the TPM and DEM-treated tracheal extracellular matrices, The result of HE staining showed that the DEM-treated tracheal extracellular matrix was tighter and the epithelial cell component clearance was more thorough compared to TPM-treated, which means Its rejection is smaller. Thus, DEM is considered the more ideal method for constructing tissue engineered tracheal matrices. This method will need to be considered in the long term, which may increase the possibility of contamination and may be unsuitable for patients who need a tracheal replacement immediately [32]. Next, we will try to shorten the cycle of decellularization by improving the concentration of DNase-I to reduce the possibility of contamination and destruction of the tracheal matrix.

Due to the damage of the matrix structure, the rapid degradation of the decellularized scaffold was observed after the transplantation; as a result, the clinical application was affected. To maintain the mechanical strength of the natural biological materials, the method of modifying the trachea using genipin was adopted [33]. Genipin cross-links the intramolecular and intermolecular cyclic structures within the collagen fibres. It has anti-inflammatory, anti-thrombotic and antioxidant activities and can inhibit the inflammatory activation of blood vessels and endothelial cells [34, 35]. Baiguera demonstrated genipin-treated decellularized tracheal matrices showed no toxicity to the cells [36]. Compared with that of traditional chemical

cross-linking agents, such as glutaraldehyde [37], the cytotoxicity of genipin is lower, and it can significantly improve the mechanical properties of tissues [38]. Glutaraldehyde can only cross-link collagen molecules that are next to each other while genipin can cross-link collagen molecules that are separated at a distance [39]. And the results of SEM and CCK-8 test showed that the scaffold with genipin treatment microscopically formed a network staggered structure, which is more conducive to attach and grow for cells.

To evaluate the *in vivo* biocompatibility of the TPM, DEM and genipin treatments, the tissues were implanted into New Zealand rabbits to achieve allograft transplantation. The implants were left for 30 days, and immunosuppression was not applied. The results of the general observation and histological staining showed that the decellularized trachea had a lower inflammatory response and that there was no obvious histological sign of local or transplant rejection; in addition, inflammatory reactions were significantly reduced. Compared with the uncross-linked tracheal tissue, the cross-linked tracheal tissue performed better in mechanical compression tests and showed no obvious inflammatory response. Genipin tightens the connection between the collagen molecules and thus improves the mechanical properties of the materials [17].

In conclusion, the trachea of rabbit that was decellularized by DEM had better biocompatibility and lower immunogenicity than that by TPM, and the structural and mechanical characteristics of the acellular matrix were effectively improved after cross-linking by genipin. The tracheal stents treated with DEM and genipin have better biocompatibility and biomechanics and are suitable for engineering replacement tracheal tissue.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement All animal experiments were approved in compliance with the rules and regulations of Laboratory Animals formulated by the National Ministry of Science (IACUC no. SYXK2016-0041).

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