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Original Article

Biocompatibility of an injectable gel composed of modified hyaluronic acid and adipose-derived mesenchymal cells

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Abstract: Background: Some biomaterials are currently available for soft-tissue augmentation. Hyaluronic acid (HA) is recently introduced as a kind of dermal fillers, but its biocompatibility is poorly understood. This study aimed to investigate the biocompatibility of an injectable gel composed of modified hyaluronic acid and adipose-derived mesenchymal cells. Methods: The physical and chemical characteristics of this injectable HA gel was evaluated, and the biocompatibility of HA gel with adipose-derived mesenchymal cells was explored in nude mice. Results: All the physical and chemical characteristics of the injectable HA gel satisfied the requirements of Pharmacopoeia of People's Republic of China and the occupation standard of hyaluronic acid (YY 0308-2004), and the cytotoxicity and intradermal reaction met the requirements of national standards (GB/T 16886). Histological evaluation showed a favorable biocompatibility of HA gel in nude mice. Conclusion: Our results reveal that the modified HA gel has a favorable biocompatibility and suitable degradation *in vivo*, and can be used for soft-tissue augmentation.

Keywords: Hyaluronic acid, biocompatibility, mesenchymal cells

Introduction

Injectable dermal fillers have recently been paid attention to for the aesthetic and cosmetic improvement of skin as well as tissue augmentation owing to their simple and short surgical procedures and rapid facial rejuvenation after surgery [1]. To develop filler materials suitable for plastic and cosmetic surgery has been an ongoing effort for decades in numerous studies [2]. There are mainly 3 types of soft tissue fillers: autologous, allogenic and synthetic. Encouraging results have been reported with autologous fat transplants, although the resorption rate is high, and better efficacy can be achieved by repeated operations. However, studies on bovine collagen report the allergic reactions and lack of persistence [3, 4]. The ideal filler should be safe and effective; it should be biocompatible, non-immunogenic, easily obtainable, non-absorbable, low in cost, and easily stored [5, 6]. It should also be easy to remove if necessary [7].

Hyaluronic acid (HA) is a polymer of disaccharides and composed of D-glucuronic acid and D-N-acetylglucosamine. Pure HA has a favorable biologic compatibility [8], and acts as an important extracellular matrix. However, the natural HA, even in its longest polymeric form, may be degraded quickly in tissues. HA derivatives have been developed by modifying its physical and rheological characteristics, which makes them suitable for tissue augmentation. A kind of cross-linked HA gels with sodium hyaluronate and 1,4-Butanediol diglycidyl ether (BDDE) were developed to extend the degradation of HA *in vivo*. In general, all injectable substances exerting a positive effect may be expected to also cause negative side effects. All current dermal fillers are associated with adverse effects [9, 10]. Biological substances such as collagen or HAs may cause lumps, allergies, long-lasting redness, sterile abscesses, and eventually early foreign body granulomas. Thus, the biocompatibility of these gels was also evaluated *in vivo*. Our results showed

these gels had favorable physical properties and biocompatibility and may be used as good biomaterials for soft-tissue augmentation.

Materials and methods

Sample preparation

Our injectable gel was a kind of modified HA. It is a non-animal-derived product obtained by fermentation of nonpathogenic *Streptococcus* bacterial strains and stabilized by a chemical cross-linking process using BDDE. It presents as a homogeneous gel that is stable at room temperature.

Detection of physical and chemical characteristics of injectable gels

Physical and chemical characteristics (pH value, osmotic pressure, swelling degree, protein content, sterility and bacterial endotoxin content) of injectable gels were detected *in vitro*.

Detection of swelling degree

In brief, 0.2 g of samples were placed on a dish and heated in an oven at 80°C. The samples were weighed until the weight remained stable. The weight served as dry weight (m^1). Then, water was added until the samples completely swelled and excess water was removed. The samples were weighed again and the weight served as wet weight (m^2). The swelling degree was calculated as follow: $Q=(m^2-m^1)/m^1$. Detection was performed five times.

Detection of sterility

Media were prepared for aerobic bacteria, anaerobic bacteria (thioglycollate fluid medium) and mold (modified Martin medium). These media were sterilized at 115°C for 30 min. Then, control bacterial solution was prepared. After incubation at 30-35°C for 16-18 h, the bacteria were diluted at 1:106 with sterilized normal saline. Then, samples were added to 10 ml of medium for aerobic bacteria (n=4), anaerobic bacteria (n=4) or mold (n=3; 1 ml per 10 ml of medium). In addition, 10-100 CFU of staphylococcus aureus was added to medium for aerobic bacteria (n=1) and anaerobic bacteria (n=1) as positive controls. In addition, media for aerobic bacteria, anaerobic bacteria and mold without inoculation of bacteria and samples independently served as negative controls. After inoculation, media for aerobic bacteria and anaerobic bacteria were incubated at 30-35°C for 14 d, and the medium for mold at 23-28°C for 14 d. The bacterial growth was observed once daily.

Detection of bacterial endotoxin

The bacterial endotoxin was detected on the basis of agglutination between limulus agent and endotoxin. This detection was conducted according to the instructions specified in the Pharmacopoeia of People's Republic of China. The detection was based on the agglutination between tachypleus amebocyte lysate (TAL) and bacterial endotoxin. TAL at specified concentration is mixed with sample solution. The formation of firming gel as in positive control group is indicative of presence of endotoxin, and absence of firming gel as in negative control group suggests no endotoxin.

Detection of biological characteristics of injectable gels

The biological characteristics of injectable gels were also evaluated in fibroblasts using DMEM as a negative control and 64 g/L phenol as a positive control. The cytotoxicity that has been widely used in different medical devices and biomaterials was evaluated according to the national standards (GB/T 16886). In brief, cells were seeded into 96-well plates at a density of 1×10^4 cells/well. Cells in logarithmic growth phase were used in the detection of cytotoxicity by MTT assay. At 2, 4, and 7 days after incubation with sample solution, cells were treated with 5 mg/ml MTT solution (20 μl/well) at 37°C for 4 h. Then, DMS0 was added to each well, and the absorbance was measured with a microplate reader at 570 nm. RGR was calculated as follow: $RGR=(absorbance_{sample}/absorbance_{negative})\times100$. Cytotoxicity was graded as follow: RGR≥100, grade 0; RGR=75-99, grade 1. Grade 0 and grade 1 suggest a low cytotoxicity and cells can survive. RGR≤50 suggests a high cytotoxicity, and cells can not survive. In our study, RGR was ≤20 in positive control group.

Hematoxylin-eosin (HE) staining for detection of injectable gel degradation

The injectable gels were injected into the back of SD rats and their biological degradation was

Table 1. Physical and chemical characteristics of injectable gels

Sample	pH	Osmotic pressure (mOsmol/L)	Swelling degree	Protein content (%)
1	7.02	327	15.79	0.07
2	6.92	339	18.21	0.08
3	6.97	341	19.34	0.06
4	7.05	336	17.45	0.06
5	7.08	329	16.96	0.07
Means ± SEM	7.01±0.06	334.4±5.5	17.55±1.19	0.07±0.01

Table 2. Physical, chemical and biological characteristics of injectable gels

Sample	Sterile test	Content of bacterial endotoxin	Intradermal reaction test
1	S	Q	Q
2	S	Q	Q
3	S	Q	Q
4	S	Q	Q
5	S	Q	Q

Notes: S: sterile; Q: qualified; n=5.

Table 3. Toxicity evaluation

Cytotoxicity of 0.5 mg/ml HA			Cytotoxicity of 1.0 mg/ml HA		
Time point	RGR (%)	Toxicity grade	Time point	RGR (%)	Toxicity grade
Day 2	99.46%	0	Day 2	95.54	1
Day 4	105.84%	0	Day 4	118.11	0
Day 7	95.56%	1	Day 7	103.54	0

Notes: RGR≥75, qualified (grade 0 or 1); RGR≤50%, not qualified (3-5 grades); RGR at 2 d, 4 d, 7 d was all less-than 20% in positive control group.

evaluated by HE staining at weeks 4, weeks 8 and 12. The samples were dehydrated through a series of graded ethanol solutions to displace the water, and then embedded in paraffin, and cut into 5 μM sections. After being heated at 65°C for 20 min, the sections were treated in xylene twice (10 min for each), re-hydrated in absolute alcohol twice (5 min for each), in 95% alcohol for 2 min and in 70% alcohol for 2 min. After washing with distilled water, sectioned were stained with hematoxylin solution for 10 min, followed by washing in flowing water for 5 min, treatment in 1% acid alcohol for 30 s and washing in flowing water for 1 min. Counter-staining was performed in eosin-phloxine solution for 1 min, followed by dehydration in 95% alcohol. After treatment with xylene twice (5 min for each), sections were mounted and observed under microscope.

Evaluation of cytotoxicity

Samples were diluted with DMEM at 0.5 mg/ml and 1.0 mg/ml, 64 g/L phenol served as a positive control, and DMEM as a negative control. Fibroblasts seeded into 96-well plates at a density of 1×10^4 cells/well. Incubation was performed for 24 h. Then, sample solution, positive solution and negative solution were added independently. Relative cells proliferation (RGR%) was determined at 2, 4 and 7 days as follows: $RGR=[absorbance_{experiment\ group}/absorbance_{negative\ control\ group}]\times100\%$. Samples were graded 0-1 when RGR was ≥75, suggesting samples have a very low cytotoxicity and cells can survive; samples were graded 3-5 when RGR ≤50%, suggesting that samples have a very high cytotoxicity and cells cannot survive. In positive controls, the RGR was ≤20% at all the time points.

Evaluation of intradermal reaction

The intradermal reaction of HA gels was evaluated in healthy rabbits at 1 week after injection (0.2 ml per point) on the basis of primary irritation index (PII). PII refers to the scores of erythema/scar and edema which are scored as follows: Erythema/scar: no erythema, 0; mild erythema, 1; clear erythema, 2; moderate erythema, 3; severe erythema or even scar, 4; Edema: no edema, 0; mild edema, 1; clear edema (not exceeding the borderline), 2; moderate edema (~1 cm in height), 3; severe edema (>1 cm in height and exceeding the contacting area), 4. The sample and PBS were independently and subcutaneously injected at 3 sites of bilateral abdomen in the same animal. The average of sample score and PBS score was calculated independently, and the difference between sample score and PBS score was the PPI of the specific animal. The average PPI was calculated from 3 animals in each group and the maximum PPI is 8. The reaction was determined according to the PII (final score): very mild reaction, 0.0-0.4; mild reaction, 0.5-1.9; moderate reaction, 1.0-4.9; severe reaction, 5.0-8.0.

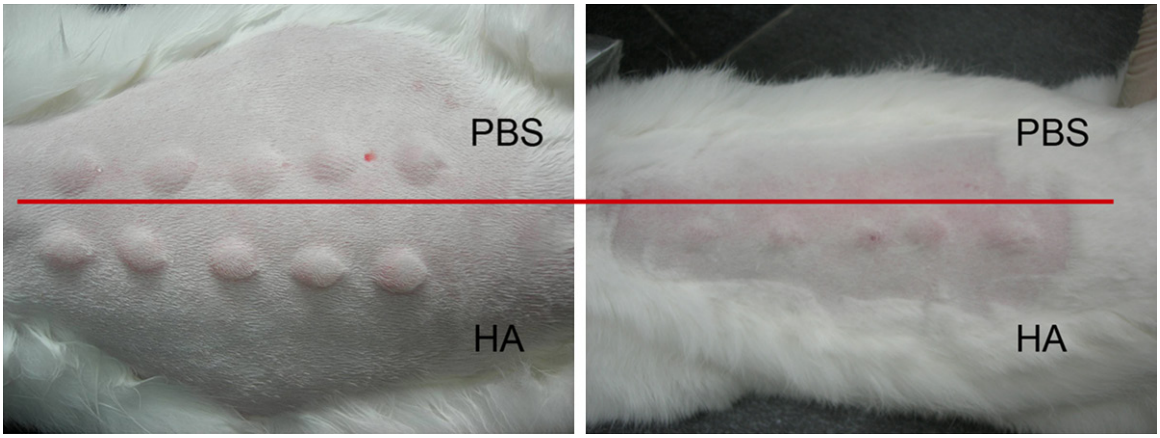

Figure 1. Intradermal reaction of HA gels in healthy rabbits. At 1 week after injection (0.2 ml per point), primary irritation index (PII) scores showed a very slight reaction.

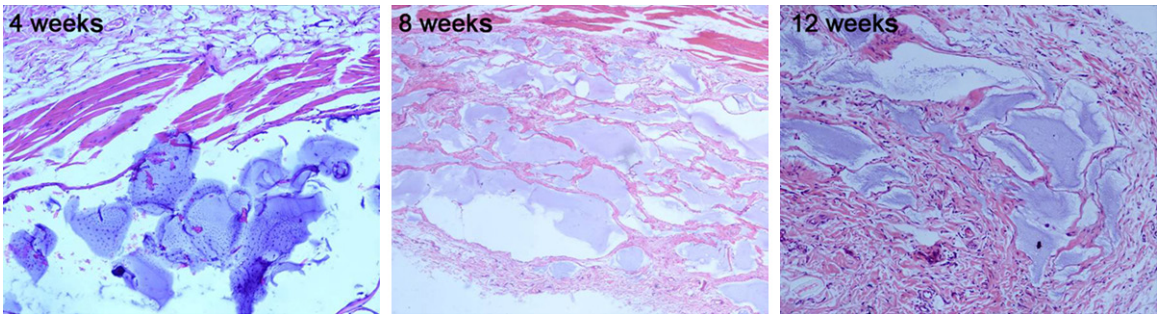

Figure 2. Test of hyaluronic acid gel degradation *by HE staining.* Hyaluronic acid gels were injected into SD rats subcutaneously, and observed at different time points. At 4 weeks, the majority of gels were not degraded and aggregated at the injection site (star); at 8 weeks, gels began to degrade and penetrated into surrounding tissues; at 12 weeks, the majority of gels were degraded and there were no evident residual gels at the injection site. After gel degradation, there was not by-products observed at the injection site, and infiltration of lymphocytes was not found in the surrounding tissues, indicating the absence of inflammation. These findings suggest that the hyaluronic acid gels have a good biocompatibility.

HE staining for evaluation of biocompatibility of injectable gels with adipose-derived mesenchymal cells in nude mice

HA gels with adipose-derived mesenchymal cells were injected into nude mice which were observed for 12 weeks and 24 weeks. The implants were collected, fixed in 4% paraformaldehyde and sectioned for HE staining as abovementioned. Inflammatory reaction was assessed by qualitative histological evaluation.

Statistical analysis

Data are presented as the means ± standard error (S.E.). Each experiment was conducted five times and means were calculated. Comparisons were conducted with one way analysis of variance (ANOVA) or repeated measures analysis of variance if appropriate, followed by SNP test. A value of $P<0.05$ was considered statistically significant. Statistical analysis was performed with SPSS version 20.0 for Windows.

Results

Physical, chemical and biological characteristics of injectable gels

Results showed all the physical and chemical characteristics of injectable gels met the requirements of the Pharmacopoeia of People's Republic of China and the occupation standard of hyaluronic acid (YY 0308-2004) (**Tables 1** and **2**).

The specific standards are as follows: pH value is 6.5-7.5; osmotic pressure is 270 mOsmol/L-350 mOsmol/L; swelling degree is

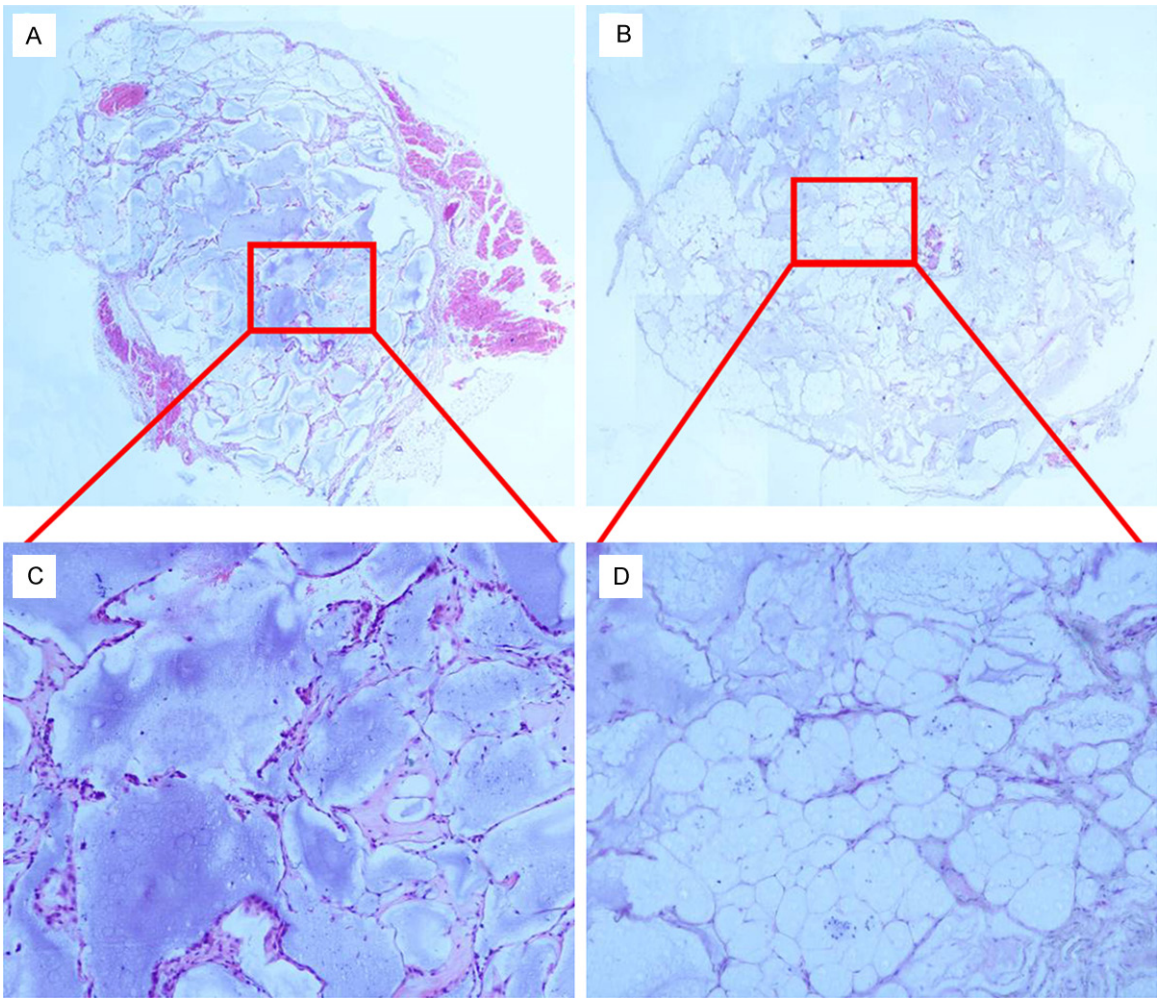

Figure 3. Test of *in vivo* biocompatibility of hyaluronic acid gels combined with adipose derived mesenchymal cells by HE staining. Hyaluronic acid gels were mixed with adipose derived mesenchymal cells. The mixture was inoculated into the back of nude mice subcutaneously, and tissues were collected at different time points and processed for HE staining. A, C: There was no evident inflammation in the surrounding tissues, suggesting a good biocompatibility (A: 40 × at 12 weeks; C: 200 × at 12 weeks); B, D: There was no evident inflammation in the surrounding tissues, but adipose like tissues were found at the injection site, suggesting a good biocompatibility (B: 40 × at 12 weeks; D: 200 × at 12 weeks).

less than 20; protein content is no more than 0.15%. In the sterile test, bacterial endotoxin detection and intradermal reaction should show being sterile and qualified. In the evaluation of cytotoxicity, relative growth rate (RGR) (**Table 3**) was calculated as follow: RGR= [absorbance$_{\text{experiment group}}$/absorbance$_{\text{negative control group}}$]×100%, and DMEM served as a negative control and 64 g/L phenol as a positive control. Results showed no intradermal reaction was observed in these samples (**Figure 1**).

Biological degradation of injectable gels

The injectable gels were injected subcutaneously into SD rats, and samples were collected at different time points. As shown in **Figure 2**, most samples were not degraded at 4 weeks and still aggregated. At 8 weeks, the injectable gels became to be degraded and permeated into tissues. At 12 weeks, most samples were degraded, and completely permeated into tissues, and gel pieces were not observed in samples.

Biocompatibility of injectable gels with adipose-derived mesenchymal cells in nude mice

The HA gels with adipose-derived mesenchymal cells were injected into nude mice and their biocompatibility was evaluated at 12 weeks and 24 weeks. Implants were collected and

processed for HE staining. As shown in **Figure 3**, there were residual materials at 12 weeks after injection, but newly generated tissues were found at the injection site. At 24 weeks after injection, the majority of materials were degraded, and adipose like tissues were found at the injection site. Inflammatory cells were not observed at both time points, and there was no inflammatory reaction at the injection site. These suggest a favorable biocompatibility.

Discussion

The ideal filler must be biocompatible, nonallergenic, nonmigratory, and must provide lasting and reversible effects. HA is the filler that closely meets these characteristics, and consequently, is being increasingly used in everyday practice by dermatologists and plastic surgeons [11].

HA is a glycosaminoglycan component of the extracellular tissue of various human organs, present with the same composition in all living beings. It is a polysaccharide comprising repeated units of disaccharides, D-glucuronic acid, and N-acetyl-D-glucosamine, with molecular weight proportional to the number of repetitions of these disaccharides [1]. The natural unmodified form of this acid is a linear molecule, biocompatible, with low potential for allergic and immunogenic reactions.

HA must undergo a purification process to eliminate the largest possible amount of protein derived from bacteria in order to reduce the antigenic potential and prevent hypersensitivity reactions [11]. Commercial preparations based on HA provide the product as sodium salt-sodium hyaluronate [12].

In its natural state, HA exhibits poor biomechanical properties as a dermal filler. HA has excellent biocompatibility and affinity for water molecules, but it is a soluble polymer that is cleared rapidly when injected into normal skin [13]. To increase its time of permanence in the tissue, a chemical process called “crosslink” is performed, whereby an artificial modification through the addition of chemicals alters its physical and mechanical properties. These modifying chemicals are called crosslinkers [14]. The most currently used crosslinker is BDDE, which forms irreversible carbon bridges between the hyaluronic acid molecules, causing an increase of the in vivo duration of the product [15]. Cross-linking strategies attempt to improve biomechanical properties while maintaining biocompatibility and biological activity. In this study, the injectable HA gels with sodium hyaluronate and BDDE were developed to extend the degradation of HA, and their biodegradation was investigated *in vivo* and *in vitro*. Our results showed the injectable HA gels had a favorable degradation.

The physical and chemical characteristics of these hydrogels were also evaluated. Results showed they had appropriate biochemical properties for biological application, with the pH value and osmotic pressure close to normal physiological levels. In addition, these hydrogels had an advantageous swelling ratio and a low protein content. The bacterial endotoxin content was also very low in these hydrogels.

Biocompatibility is a key characteristic for products that will be used as dermal filler materials [16]. In the present study, intradermal allergy test in New Zealand rabbits showed that these hydrogels failed to induce skin irritation and were non-cytotoxic, suggesting that the hydrogels may be used as biological materials. Moreover, the biodegradability of these hydrogels was also assessed after injection into the back of SD rats. Three months later, a large portion of hydrogels was observed, indicating that this hydrogel was biodegradable, but the degradation time was longer than 3 months. Finally, the biocompatibility of these hydrogels was evaluated *in vivo*. A mixture of adipose-derived mesenchymal mesenchymal cells and hydrogels was injected into nude mice, and the biocompatibility was assessed at 12 and 24 weeks by HE staining. The hydrogels were partially degraded after 12 weeks, indicating a favorable biocompatibility with surrounding cells and tissues. At 24 weeks, most of materials were degraded, and a small amount of adipose tissues formed.

On the basis of above findings, we speculate that the modified BDDE-HA hydrogel has appropriate biochemical properties and biocompatibility, and may be useful as a cosmetic dermal filler or a scaffold for tissue engineering.

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Disclosure of conflict of interest

None.

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