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ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

The Role of Hyaluronidase for the Skin Necrosis Caused by Hyaluronic Acid Injection-Induced Embolism: A Rabbit Auricular Model Study

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Abstract

Background Skin necrosis is considered the most serious complication of hyaluronic acid dermal filler injection procedures. To effectively treat skin necrosis, hyaluronidase injection is one of the essential preventative treatments, and yet optimal complication management remains an unmet need. Therefore, this paper investigates the effects of hyaluronidase injection timing on the treatment of skin necrosis.

Methods In an in vitro experiment, the carbazole method was used to determine the degradation time of hyaluronic acid gels in a large volume of hyaluronidase. In vivo experimental rabbit ear models were developed to simulate the skin necrosis caused by hyaluronic acid and the test animals distributed into five groups. Except one control group, the other four groups were injected with a large volume of hyaluronidase as treatment at 2 h, 4 h, 8 h and 16 h, respectively, after models were built. The necrosis degree of models was analyzed with necrotic area and histologic examination on the postoperative 7th day. Besides, temperatures of rabbit ears were observed to demonstrate the healing process of flap models.

Results The average necrotic area of flaps in the 2-h and 4-h injection groups showed a significant difference compared with that of the control group ($p < 0.05$; $p < 0.05$). The histologic examination showed that there were HA embolisms, vascular thrombolytic recanalization and arteriovenous thromboses in the survival area. In addition, the mean temperatures of the rabbit ear flaps fluctuated over time and showed clear differences between distal and proximal parts.

Conclusions The area of flap necrosis positively correlates with injection timing of the large volume of hyaluronidase. More importantly, when injection timing is within 4 h, treatment effectiveness will be significantly improved.

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Keywords Hyaluronic acid · Complication · Skin necrosis · Hyaluronidase · Embolism

Introduction

Soft tissue augmentation is widely performed to replenish volume loss or improve facial appearance over the past several years. This procedure is gaining in popularity for its remarkable anti-aging outcome without much surgical risks [1, 2]. Among various kinds of fillers used for this procedure, hyaluronic acid (HA) fillers have become the most popular due to several benefits: sustained volumizing effect, biocompatibility, immunologically inert, biodegradability, viscoelasticity and easy correction after treatment [3, 4].

In general, native HA in the body is distributed throughout the extracellular matrix and can be degraded into D-glucuronic acid and *N*-acetyl-D-glucosamine with

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endogenous hyaluronidase within 2 h in lymph nodes and the liver [5–7]. However, cross-linked HA fillers with 1,4-butanediol diglycidyl ether (BDDE) could reduce the susceptibility to endogenous hyaluronidase, thus sustaining esthetic results for up to 18 months in vivo.

While HA injection is considered safe, the incidence rate of complications has risen rapidly in recent years with increasing usage of HA fillers, especially in illegal operations [8]. Most of the complications are mild and reversible, such as overcorrection, filler misplacement, hypersensitivity and chronic inflammation. However, vascular occlusion as a severe complication can result in serious consequences, such as skin necrosis, blindness and cerebral infarction [9–14]. These are caused by improper operations of HA filler injections. A certain amount of HA filler is accidentally injected into the blood vessel, and then HA emboli are formed and carried by blood flow into smaller-sized vessels where they cannot pass through any further distally and thus result in partial or total blockage of blood flow. Moreover, high injection pressure may cause retrograde flow of HA emboli to larger vessels as well as other branches. Vascular obstruction and tissue ischemia at both proximal and distal sites might occur, generating a vicious cycle between inflammation and hypoxia [15]. Among the above-mentioned serious consequences, skin necrosis has also aroused a great concern from doctors and patients because of its high incidence rate. The main symptoms are pain and skin color changes [16–20], which may occur immediately or several days post-operation. The areas susceptible to skin necrosis include the glabella, nasolabial folds, lips and nose due to the lack of rami anastomoticus [21].

To deal with vascular complications effectively [22–26], exogenous hyaluronidase, a soluble protein which enables enzymatic degradation of hyaluronic acid, is recommended for injection. Many works of the literature explored the optimal use of hyaluronidase. For now, the current protocol for vascular occlusion caused by HA injection starts with infiltrating 450–1500 units of hyaluronidase over the entire area including the course of the vessel by serial puncture. Then massage and heat are applied. Reassessment is done after 1 h to ensure capillary refill is less than 4 s; if not, the protocol above should be repeated at hourly intervals up to 4 cycles [22, 27, 28].

The three most important factors in the current protocol are the dose, injection type and timing based on earlier studies. Firstly, about the dose, the hyaluronidase dose depends on the volume of ischemic tissue. A high-dose pulsed protocol (450–1500 units) is proposed, and injection should be hourly repeated as early as the diagnosis is made or even merely suspected [29]. The dose required will be influenced by the physical properties of the different HA fillers, regarding the amount of cross-linking, particle size and concentration [26, 30–33]. Secondly, about the injection type, Claudio DeLorenzi reported that perivascular hyaluronidase could permeate vascular walls [20]. A large volume of hyaluronidase should be injected throughout the entire blanching area including the course of the vessel [34]. Thirdly, about the injection timing, treatment should be initiated no later than 3 days after the operation; otherwise, the healing process may result in scar formation [35]. Kim et al. [36] reported that hyaluronidase lost its effect in vivo within 3–6 h and successful engraftment of reinjected HA filler could be accomplished 6 h after the first injection. Deok-Woo Kim et al. found that rabbit full-ear models treated with 750 IU hyaluronidase in 4 h after 0.25 ml HA filler injection had significantly smaller necrotic areas than the untreated group, while no differences was observed between the 24-h intervention groups [20, 37]. Despite providing a fundamental principle for our in vivo research, these works' inaccuracy especially in the calculation of necrotic areas of the rabbit full-ear model and a limited number of experiment samples urge more accurate models and more complete, specified experiment samples to meet the research requirements.

The purpose of this study was to accurately evaluate the time–response relationship between skin necrotic areas and large volumes of hyaluronidase injection as well as to examine the tissue destruction consequence correlated with time wasted before intervention, laying an experimental basis for the treatment of skin necrosis caused by HA filler.

Materials and Methods

In Vitro Study

To validate the time for complete degradation of 80 µl cross-linked HA gel (BioHyalux, Bloomage BioTechnology Corporation Limited, Jinan, China) in 1500 U hyaluronidase/ml of PBS (1500 U/vials; Shanghai No. 1 Biochemical and Pharmaceutical Co., Ltd, Shanghai, China), a modified carbazole assay was used to detect the concentration of HA residue for different degradation times in vitro, which was based on the reaction between carbazole and glucuronic acid [38–40].

In the degradation tests, 80 µl cross-linked HA gel was incubated with 1 ml of 1500 U/ml hyaluronidase in a hybridization oven and rotated with the speed of 30 rpm at 37 °C. These steps were repeated four times with different durations of incubation for 10 min, 15 min, 30 min and 45 min, respectively. After adding 3.5 mL anhydrous ethanol, the solution was centrifuged with a high-speed centrifuge at 10,000 r/min for 15 min, and then, 1 ml supernatant was collected. The reaction was stopped by adding 0.025 mol/L sodium borate sulfuric acid (5 ml) into

the collected 1 ml supernatant in an ice water bath. After that, the solution was placed in boiling water bath, heated for 10 min before reacting with 0.1% carbazole ethanol solution (0.2 ml) and then heated for another 15 min. The absorbance value of each sample was measured by an ultraviolet spectrophotometer at a wavelength of 530 nm with water as the blank control. In addition, hyaluronidase interference was eliminated.

In Vivo Study

This study was approved by the Animal Ethics Committee of the Sichuan University. All animal procedures were carried out according to the *Guide for the Care and Use of Laboratory Animals*.

Fifteen New Zealand rabbits, weighing 2.5–3.0 kg, were obtained from the Animal Center of Sichuan University. After one-week adaptation, the rabbits were anesthetized with pentobarbital sodium (1 ml/kg) via the auricular vein. The posterior surface of both side ears was shaved and prepared with povidone–iodine and alcohol. An axial pattern flap sized 6 × 2.5 cm^2 was designed with the central auricular artery as the long axis, and the proximal border of the flap was marked 1 cm proximal to the level of the central vein giving off the medial branch. The skin on the border of the flap was infiltrated with 1% lidocaine. Two-thirds of the flap were prepared from distal to proximal in a plane directly above the perichondrium of the ear cartilage under sterile conditions. And for the proximal one-third, the flap was prepared above the muscle to make sure that the flap consisted of the skin layer. The stumps of the auricular artery and vein on the border were ligated after flaps were established and closed [37, 40, 41]. A skin incision was made to expose the central auricular artery before the artery was punctured with a 30-gauge needle, and 80 μl cross-linked HA was injected at a speed of 10 μl per 15 s (Fig. 1). Erythromycin ointment was applied to the skin wound after surgery. Thirty flaps were prepared and averagely divided into five groups. Then, 1500 U hyaluronidase was injected evenly into the flaps at 2 h, 4 h, 8 h and 16 h for each group except for one blank control group. The above processes and photographs on the post-operative seventh day (POD7) are illustrated in a process flowchart (Fig. 2).

Necrotic Area and Temperature

Each flap was observed and photographed consecutively for 7 days after surgery. Photoshop software was used to calculate the percentage of necrotic area by analyzing the photographs on POD7. The procedure of necrosis image assessment was blinded. Two members in our group were responsible for data calculation, without knowing the relation between the groups and the photographs. The correlation between authors' measurements was proved by ANOVA. Some of the photographs are shown in Fig. 2. The temperatures of the proximal, middle and distal one-third parts of flaps were recorded, respectively, each day by a non-contact infrared thermometer. The data recorded were the mean values of 3 continuous measurements (accurate to 0.1 °C). The measured 3 points are marked in the photograph in Fig. 1.

Histologic Study

For this part, two researchers were involved in the paraffin histology assessments, and any differences between the two researchers were discussed, settled by consensus after consulting the third researcher. The proximal, middle and distal one-third parts of flaps sized 1 × 1 cm^2 were harvested for HE staining histology examination on POD7 to observe the changes in and around the vessel. The specimens were fixed with 10% neutral formalin and made into paraffin sections. The sections were stained with hematoxylin–eosin, followed by pathological observation under a light microscopy (Eclipse 80i, Nikon, Japan) with a digital camera.

Data Collection and Statistical Analysis

Data were analyzed by Statistical Package for Social Sciences software (SPSS, Chicago, IL, USA) 24.0. The data were presented by mean differences ± standard deviations. The Kruskal–Wallis H test was used to determine the statistical significance of temperature data between different parts of rabbit ear flaps. Necrotic area data between groups with different hyaluronidase injection time were analyzed by the Pearson correlation test, one-way analysis of variance and least significant difference procedure. A p value (< 0.05) was considered statistically significant.

Results

In Vitro

The Carbazole Assay

As shown in Fig. 3, the absorption value measured by the ultraviolet spectrophotometer reached the maximum at about 15 min and remained unchanged afterward, meaning that glucuronic acid produced by the degradation of 80 μl hyaluronic acid reached the maximum at about 15 min and all 80 μl of HA were degraded.

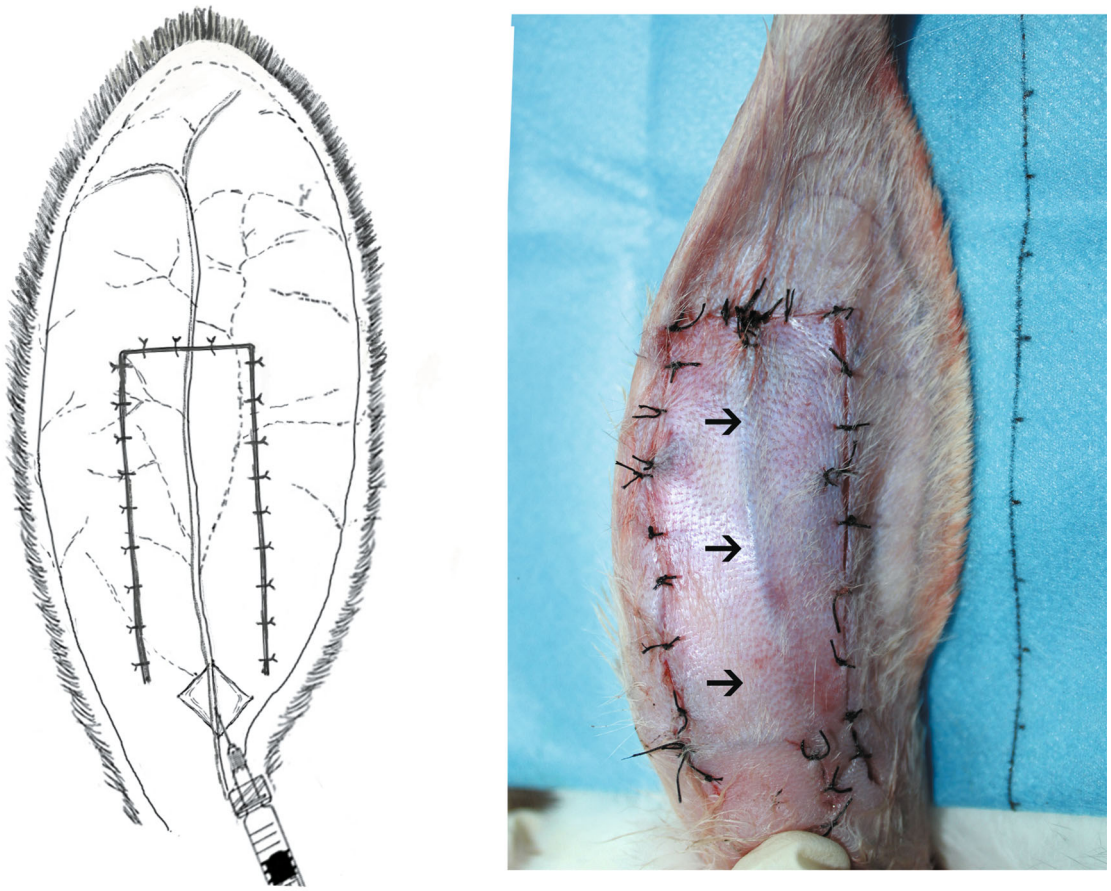

Fig. 1 The ear flap model. The diagram on the left shows a 6 cm × 2.5 cm rabbit ear flap with a reliable pedicle of the central auricular artery. The photograph on the right shows the rabbit ear flap on dorsal surface. Three arrows (↑) mark the measurement positions of temperature

In Vivo

All the rabbits were restored to normal activity and dieted 2 h after surgery, and they all survived during the 7-day postoperative period. After the cross-linked HA gel was injected into the artery, the ears appeared to be pale along the arterial course and part of the area remained dark blue during the first 2 days. Then, most of these flaps showed congestion and edema which turned into necrosis in the end (Fig. 2).

Necrotic Area

As shown in Fig. 4, the calculated mean necrotic area was 3.39 ± 1.98 cm^2 (26.65%) in the 2-h group, 4.05 ± 1.63 cm^2 (29.32%) in the 4-h group, 5.36 ± 1.23 cm^2 (35.76%) in the 8-h group and 6.95 ± 2.93 cm^2 (46.35%) in the 16-h group, while 8.17 ± 2.17 cm^2 (54.49%) in the control group. Positive correlation between injection timing of hyaluronidase and necrotic area of flaps can be concluded. Moreover, the necrotic area was significantly smaller in the 2-h and 4-h groups, and the difference in necrotic area between the blank control group and the 2-h or 4-h group was statistically significant ($p_{b-2h} = 0.03$; $p_{b-4h} = 0.03$). However, there was no significant difference between the blank control group and the 8-h or 16-h group ($p_{b-8h} = 0.085$; $p_{b-16h} = 0.46$).

Temperature

The mean temperature of the distal one-third part of rabbit ear skin flaps was 4 °C lower than that of the proximal one-third part during the seven post-operation days, which was statistically significant ($p = 0.001$, Fig. 5). However, the temperatures of rabbit ear flaps fluctuated with time (Fig. 6). On POD1 and POD2, most of the rabbit ear flaps' temperatures decreased and then increased a bit on POD3 and POD4. Then, the temperatures dropped again from POD4 to POD5, which was even lower than that on POD1. Most flaps' temperatures rose at POD6, some of which began to recover at POD7.

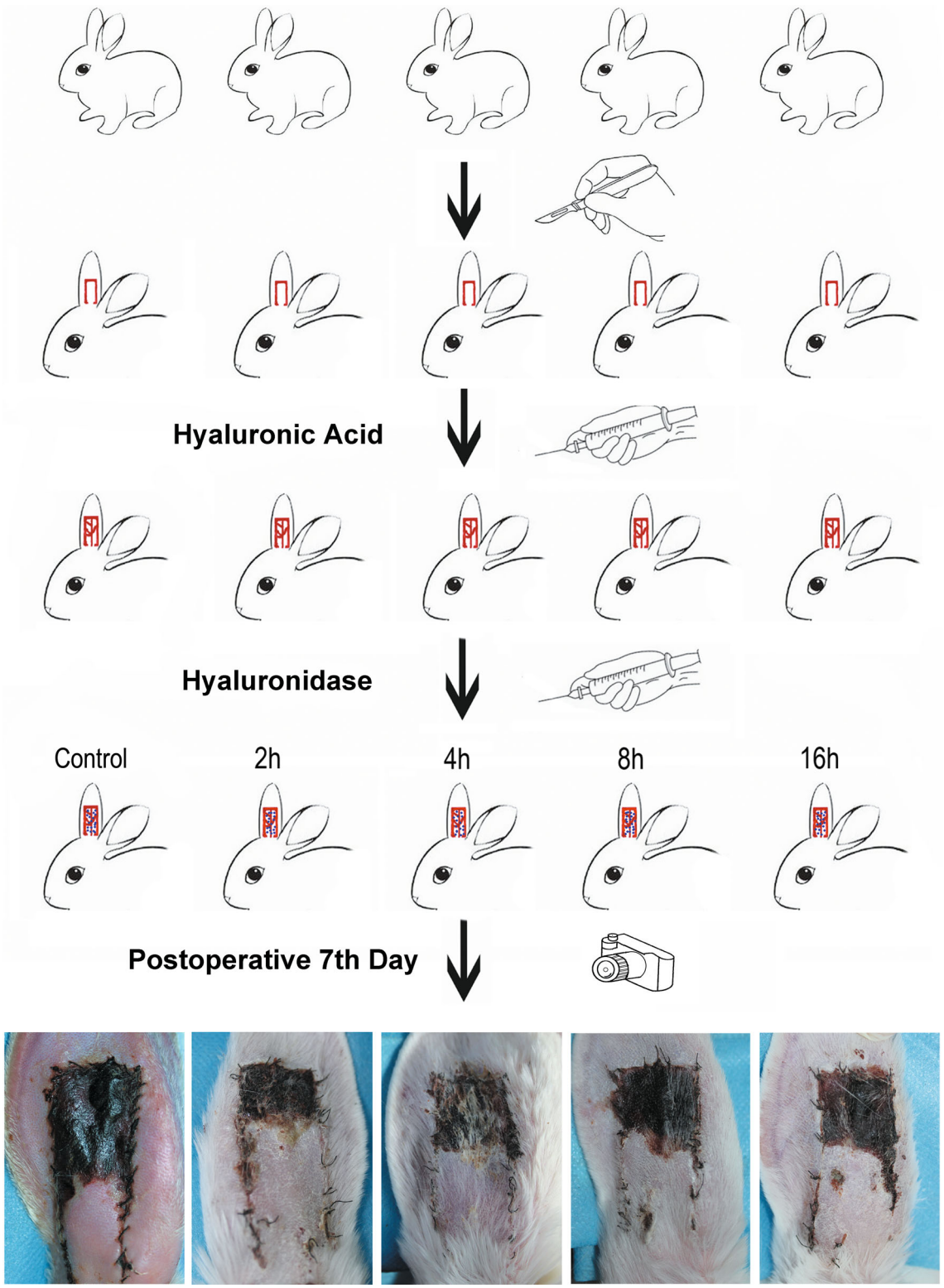

Fig. 2 The experimental flowchart of in vivo study. The experimental flowchart shows the simplified process of the in vivo study, which includes classifying all rabbits into five groups (the first is control group), elevating of ear flap model, injecting 80 μl cross-linked HA for each group simultaneously and then injecting hyaluronidase at 2 h, 4 h, 8 h and 16 h for each group, respectively. The photographs for ear flaps on the seventh day after surgery are shown in the last row

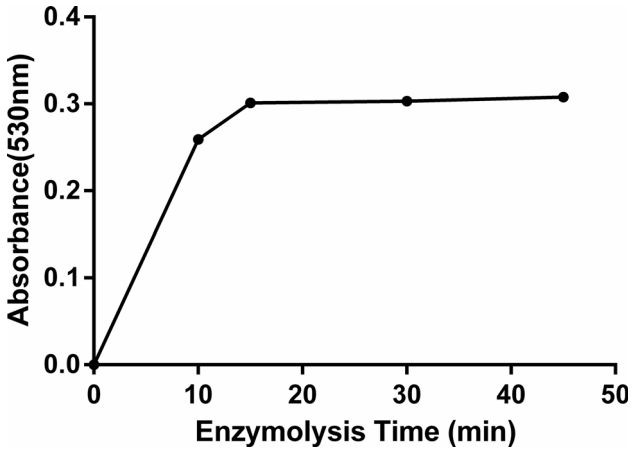

Fig. 3 The enzymolysis time of ha degraded by high dosage hyaluronidase in vitro. The absorption value of carbazole assay processed HA residue measured by ultraviolet spectrophotometer reached a maximum at about 15 min, indicating that 80 μl cross-linked HA gel can be degraded by 1500 U hyaluronidase under 15 min in vitro

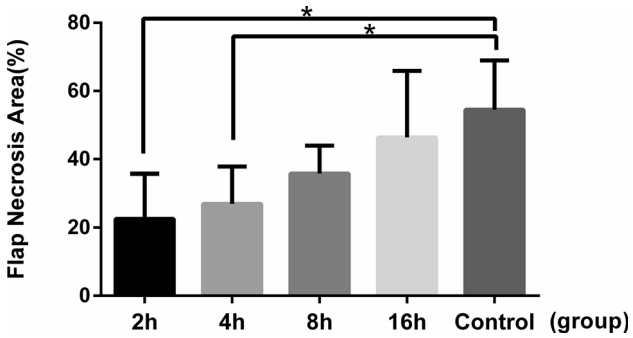

Fig. 4 The necrotic area of different groups. The bar graph displays the average necrotic area of flap models in each group on the postoperative seventh day. *The differences of necrotic areas between the blank control group and 2-h or 4-h group are statistically significant ($p_{b-2h} = 0.03$; $p_{b-4h} = 0.03$)

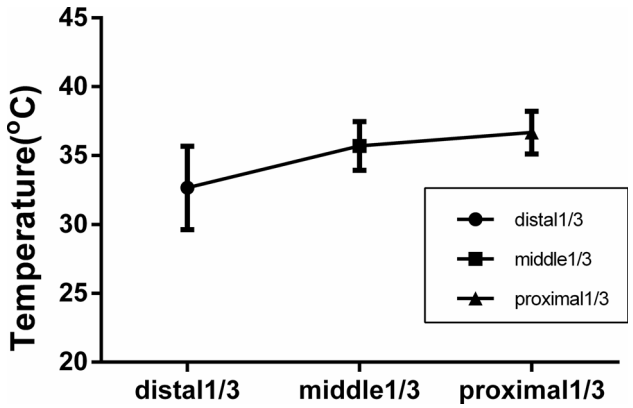

Fig. 5 The mean temperatures of different parts in the rabbit ear flaps. The line chart shows the mean temperature of the distal one-third of rabbit ear skin flaps was 4 °C lower than the proximal one-third during the seven post-operation days

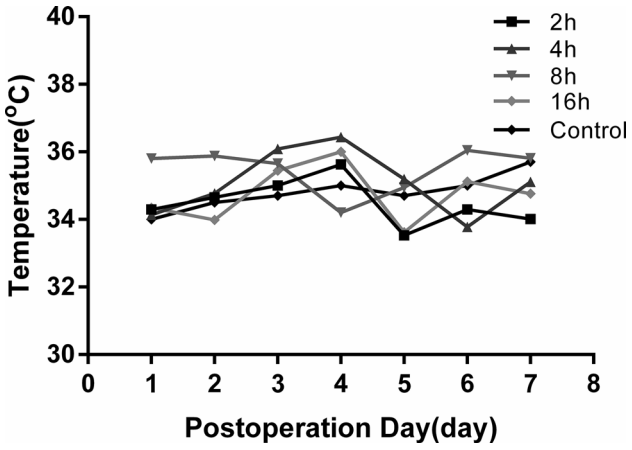

Fig. 6 The temperature change of each group during the observation. The time line graph depicts the temperatures of whole rabbit ear flaps fluctuated during 7 days after surgery which may be related to ischemia, surgical trauma and the development and relief of inflammation

Histologic Study

As shown in Fig. 7, cross-linked HA granules stained into homogeneous blue color were found in the vessel of whole flaps, and large amounts of arteriovenous thrombosis were observed in the pathological examination on POD7. However, recanalization of thrombosis was presented in hyaluronidase-treated groups, which was absent in the control group. Furthermore, marked dilatation and multiplication of blood vessels were seen to compensate for the lack of vascularization due to embolisms.

In this study, there were histologic differences among groups. More eosinophilic granulocytes and mononuclear cells were observed with therapy time delayed. More HA embolisms blocked in arteries in the 16-h group and blank control group than that in the 2-h group as well. In the treatment groups, vascular thrombolytic recanalization can be found in the 8-h and 16-h groups but not observed in the control group.

The necrosis formed along vessels from distal to proximal. In the distal one-third of flaps, tissue destruction was severe with large areas of necrosis and disintegration. Significant infiltration of eosinophilic granulocytes and mononuclear cells around in both dorsal and ventral parts of flaps was detected. In the proximal one-third sections, necrosis was less noticed and the severity of eosinophilic granulocyte infiltration was milder compared to the distal one-third, where HA embolisms and thrombi were present in veins, arterioles and venules. In the proximal one-third section, HA embolisms and thrombi were still seen in veins and venules, but were rare in arteries.

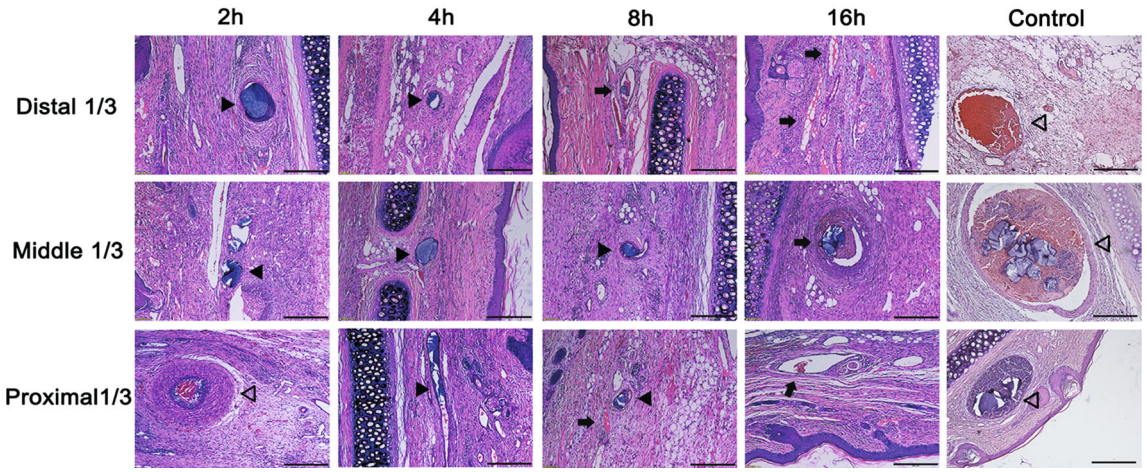

Fig. 7 Histologic examination of the rabbit ear flaps. HE staining for all three parts of the flaps in each group on the postoperative seventh day, showing HA emboli (filled triangle), arteriovenous thrombosis (open triangle) and vascular thrombolytic recanalization (up arrow). Original magnification ×10

Discussion

To develop extensive skin necrosis by injecting HA, we found that 80 µl HA of fillers was adequate to form an appropriate necrosis area for evaluating the effectiveness of hyaluronidase from our previous work.

In our in vitro study, to measure the degradation time, HA gel was degraded by 1500 U/mL hyaluronidase, much higher than the previously recommended clinical concentration (150 U/ml), since a large volume of hyaluronidase (450–1500 units) was recommended by a new high-dose pulsed protocol recently [29]. Degradation time was examined by adding carbazole assay at different time points [41, 42].This method is mostly used for testing the tolerance capability of HA to hyaluronidase; however, it has not been applied to measuring degradation time in previous studies. The results showed that glucuronic acid reached the maximum at about 15 min, indicating that 80 µl hyaluronic acid was completely degraded in about 15 min. In the previous study, nearly all the HA was degraded by 150 U/mL hyaluronidase in an hour. This is most likely related to a higher concentration of hyaluronidase [41]. Although the degradation of the in vitro experiment is not necessarily analogous to the in vivo experiment, ischemic tissue can benefit from the rapid degradation of intravascular HA emboli caused by a high-dose injection of hyaluronidase.

In the in vivo study, the necrosis flap model was designed considering the skin necrosis caused by HA filler injections has commonly been reported in the glabellar and nasal area where the collateral blood flow is limited. The use of rabbit ear flaps also helped to increase the accuracy of necrosis area calculation, and the grouping method based on different hyaluronidase injection times was more detailed than in other works.

The injection methods and region of treatment were also considered in this study. Some reports showed cutaneous circulation of ischemic tissues was recovered only by directly injecting hyaluronidase into the affected artery [22]. However, it has been reported that increased resistance caused by the HA filler in artery makes the intra-arterial injection of hyaluronidase difficult to reach the obstructed vessels [20, 43]. Some researchers described that the subcutaneously injected hyaluronidase would penetrate into obstructed vessels and degrade HA filler in vivo, as the in vitro experiments had proved that hyaluronidase would diffuse into human arteries and degrade HA filler within 4 h [20]. In addition, subcutaneous injection is a far easier clinical approach than direct intravascular injection because of the vasoconstriction and insufficient perfusion of related vessels. Although the exact degree of vascular occlusion needs to be assessed with sophisticated examination, the sufficient concentration of hyaluronidase injected can be judged by color change of flaps and capillary refilling. Moreover, the treatment area should include the course of the vessel because histologic examination showed HA embolisms and arteriovenous thromboses even in the survival area. The concentration of hyaluronidase injected along the course of the vessel should be less, since HA embolus was more dispersive and allowed collateral vessels to provide sufficient blood supply.

As the temperature results indicated, there was a large difference between the temperature of distal and proximal parts of flaps. After intravascular injection, cross-linked HA gel dispersed with blood flow and mainly obstructed at

the distal one-third part of flaps, resulting in severe hypoperfusion and ischemia. The temperature of rabbit ear flaps fluctuating with time might be related to the inflammation and surgical trauma.

In this experiment, the effect of hyaluronidase injection timing was accurately measured by using a rabbit ear flap model. The calculated necrosis area of flaps was closely related to the injection timing of hyaluronidase. Injection with a large volume of hyaluronidase within 4 h was remarkably effective, but the efficacy after 4 h was limited.

For future works, studies of repeated hyaluronidase treatments should be conducted, especially for the first 2 h to see how much of the necrosis could be prevented by earlier hyaluronidase injection and to find the adequate timing when changes may be reversible. Another ongoing challenge is to reach a better understanding of cellular and subcellular mechanisms triggered by ischemic injury. In addition, clinical guidelines of pharmacologic choices and different endovascular manipulation approaches have not been well established. With an improved definition of successful treatment, that is, complete recovery of ischemia with no secondary changes, more experimental data are needed to facilitate early endovascular recanalization strategy. Although the rabbit model has greatly contributed to understanding of pathological cutaneous necrosis, knowing the interspecies particularities of injury is fundamental to bridge the gap between preclinical and clinical studies [44].

Though most HA filler injection procedures are safe and severe complications are rare, surgeons should notice the signs of impending necrosis and take appropriate first-aid measures at an early stage.

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Compliance with Ethical Standards

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

Human and Animal Rights All applicable institutional and/or national guidelines for the care and use of animals were followed.

Informed Consent For this type of study, informed consent is not required.

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