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Comparison of five preservation methods for fascia allograft

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ABSTRACT

Importance: Research on tissue preservation, including cortical bone, skin, nerves, and vessels in glycerol and cortical bone in honey, has shown positive results. On the other hand, relatively few studies have been performed on fascia preservation, and comparisons between different fascia preservation methods remain scarce.
Objective: This *in vitro* study compared the biomechanical properties of five different methods of preserving fascia lata.
Methods: The control group underwent biomechanical testing immediately after decellularization, while the other five groups were stored in glycerol, honey, deep freezer, lyophilizer, and liquid nitrogen for 30 days. The ultimate load, elongation at failure, and stiffness for each group were determined from a load–elongation curve.
Results: A comparison of the ultimate load showed that the control group had the highest value, followed by the glycerol group. The glycerol group was the only group that did not show a significant difference from the control group, while all the other groups showed a significantly lower ultimate load. A comparison of elongation at failure revealed the glycerol group to have the highest value at failure among all groups and was significantly higher than the deep freeze, honey, and cryopreservation groups.
Conclusions and Relevance: Glycerol can be used as an effective method for preserving fascia allografts because the resulting allografts show a similar ultimate load to the control group and the highest mean elongation at failure.

Keywords: Allograft; biomechanics; decellularization; fascia; rabbits

INTRODUCTION

Reconstructive surgery using fascia grafts is used widely to treat congenital defects, traumatic injuries, and wide resections of tumors, especially in the abdominal wall, perineum, diaphragm, patellar ligament, cruciate ligament, tendon, urethra, and oral mucosa [1-7]. Although autografts have the most significant advantage of not causing any host responses, the shape and sizes are limited and have donor morbidity, which may delay recovery [3,8].

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Conflict of Interest
The authors declare no conflicts of interest.

Xenografts have high availability, but there may be a risk of cross-contamination because viruses like bovine spongiform encephalopathy or porcine endogenous retrovirus cannot be screened. In addition, porcine DNA has been detected in swine intestinal submucosa, which is markedly acellular, indicating the induction of immunogenic responses [9].

Although allografts may also induce immunological responses, rejection is less vigorous compared to xenografts [10]. The main advantages of allografts are a lack of donor morbidity, which can shorten the operation time and availability in various shapes and sizes that can be used in multiple reconstructions [11,12]. Allografts also have high biocompatibility, making them an effective biological scaffold to be used in reconstructive surgery.

Before transplantation, the preservation of allogenic tissues is essential because they are not used immediately. Only studies on the preservation of canine fascia by lyophilization have been reported [13]. Other methods of preserving fascia, such as deep freezing, cryopreservation, and lyophilization, have been reported in other species, such as humans and porcine [14,15].

Studies on the preservation of tissues, such as cortical bone, skin, nerve, vessels in glycerol, and cortical bone in honey, were reported to have positive outcomes [16-21]. In addition, honey and glycerol were found to be cost-effective and convenient because they can be stored at ambient temperature without any special equipment [17,21]. Hence, conservation in honey and glycerol, which were used in other tissues, such as bone, skin, and vessel, appears applicable in fascia because of their satisfying results.

To the best of the author's knowledge, only a few preservation methods of fascia have been presented in previous studies, and comparisons of the preservation methods of fascia are very limited [13-15]. A direct comparison of various methods under the same conditions is needed to reduce the confounding factors and find the best option for the preservation of fascia lata. Canine cadavers cannot be compared under the same conditions because of the difference in size, breed, and time of death, and there is difficulty in obtaining sufficient samples for statistical analysis. Therefore, New Zealand White rabbit cadavers were used as an alternative to canine cadavers to harvest the most similar-sized fascia lata sample. This study compared five methods of preserving fascia to find the optimal conservation method for fascia allografts using New Zealand White rabbits.

METHODS

Animals

Thirty female New Zealand White rabbit cadavers weighing approximately 3 kg were used. The fascia lata from both hindlimbs was collected from 30 New Zealand White rabbits. The Institutional Animal Care and Use of Committee of Konkuk University approved this study (approval number: KU 23049).

Group design

Sixty samples were divided randomly into six groups: 1) glycerol, 2) honey, 3) deep freeze, 4) lyophilization, 5) cryopreservation, and 6) control groups. Ten samples in each group were divided randomly into two groups: five samples from the right leg and five samples from the left leg.

Graft harvest

Both hindlimbs were clipped and aseptically prepared. A craniolateral skin incision was made from the level of the coxofemoral joint to the tarsal joint. The subcutaneous tissue was bluntly dissected to expose the fascia lata. The fascia lata was harvested within the following margins: the sartorius muscle cranially, the biceps femoris muscle caudally, the tensor fascia lata muscle proximally, and the distal third of the femur distally. The fascia lata was manipulated carefully to minimize tissue damage.

Decellularization of fascia lata

Decellularization processes were conducted at room temperature besides those indicated, with agitation at 100 rpm using a digital orbital shaker (SK-O180-S, DLAB Scientific, China). First, the samples were stored in an antibiotic–antimycotic solution (100 UI/mL penicillin, 100 μg/mL streptomycin, 200 μg/mL neomycin) for 30 min. Subsequently, the samples were frozen at −80°C for 15 min and thawed at room temperature for 20 min. The freeze-thaw cycle was repeated three times, and fascia samples were washed in distilled water (DW) for two days. A treatment cycle consisted of 0.5M NaCl for four h, 1M NaCl for four h, and washing in DW for one day that was repeated once, and the samples were treated with 0.25% Trypsin/EDTA (37°C) for two h. After washing DW for two h, the samples were moved to 1% Triton X-100 solution for five days, which was changed once per day and washed in DW overnight. The samples were processed with 0.5 mg/mL DNAase (37°C) for three h and washed in DW for two days. The decellularized samples were then stored in an antibiotic–antimycotic solution (100 UI/mL penicillin, 100 μg/mL streptomycin, and 200 μg/mL neomycin) for 30 min.

Preservation of fascia lata

The samples were cut into a symmetrical arciform shape to ensure that failure occurred within the narrowest portion rather than the edges during the biomechanical test. The fascia lata was flattened out carefully using vascular debakey tissue forceps by only manipulating the edges of the sample. The sample was secured using two vascular debakey tissue forceps, each pressing down on the proximal and distal parts of the fascia lata. Three symmetrical points were marked on the cranial and caudal parts of the fascia sample using the tip of a #15 blade. The distance between the three symmetrical points was 1.5 cm, 0.5 cm. and 1.5 cm, with 0.5 cm being the narrowest portion and 1.5 cm being the widest portion of the fascia. A rectangular-shaped disposable razor blade was bent to form a 120° angle, and the fascia was cut off by placing razor blades at the three points (**Fig. 1**).

The control group was tested immediately after trimming. Samples, besides the control group, were stored in a glycerol solution (glycerol: ethanol = 4:1), manuka honey (UMF 20+), deep freezer (CLN-51U, Nihon Freezer, Japan) at −80°C, lyophilizer (LP03, Ilsin Bio Base, Korea), and liquid nitrogen at −196°C.

All samples in the preservation groups were flattened out, and two fascia samples were placed in each petri dish. For the glycerol and honey groups, the samples were fully submerged in a glycerol solution and honey, respectively. The petri dishes were sealed with parafilm and kept in the dark at room temperature. Regarding the lyophilization group, the samples were placed flat on a petri dish without adding any solution, and primary drying was conducted for 12 h at −40°C under vacuum (4 mTorr). For secondary drying, the shelf temperature increased by 0.2°C/min until 22°C and kept under vacuum for 12 h. After lyophilization, the petri dishes were sealed with parafilm to minimize exposure to air and stored in the dark at room temperature. For the samples in the deep freeze group and

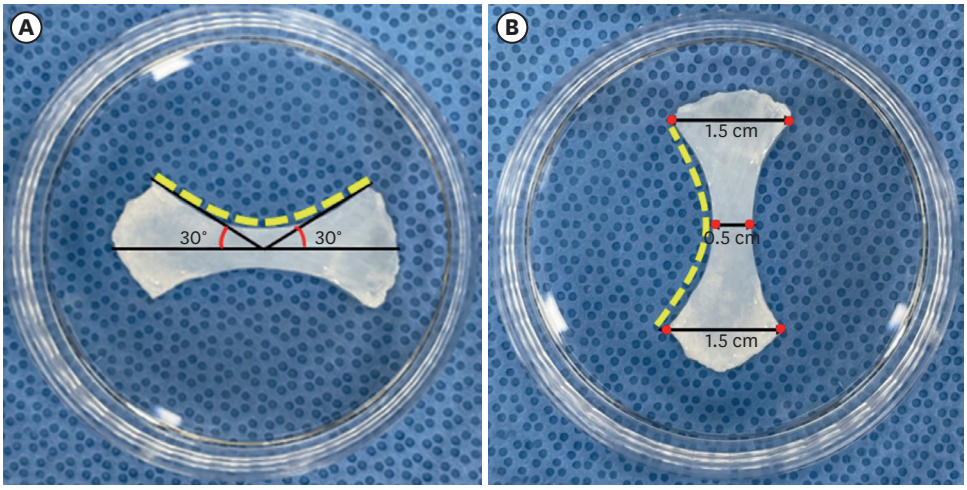

Fig. 1. Photograph of the trimmed fascia lata. (A) A razor blade was bent to an arciform shape with an angle of 120° (yellow dash line). (B) The razor blade is placed on the three points (red dots) of the cranial and caudal edge of the fascia sample for symmetry. The narrowest and widest part of the fascia is set up to a width of 0.5 cm and 1.5 cm to make failure occur at the narrowest part.

cryopreservation group, the petri dishes were sealed with parafilm without adding a solution and stored in a deep freezer and liquid nitrogen. All samples were stored for 30 days except for the control group.

Biomechanical testing

All samples were washed with DW on an agitator for at least one h at room temperature before testing. The biomechanical properties of the fascia lata samples were determined by uniaxial tensile testing using a universal testing machine (Instron 3369, Instron Corp, USA). The ends of the samples were secured in clamps with 1.5 cm of the centrally tapered portion exposed between the clamps (**Fig. 2**). The loading was applied uniaxially, and the crosshead was moved at a speed of 10 mm/min until failure. A load–elongation curve was collected for each sample. The ultimate load, maximum elongation at failure, and stiffness were measured. The ultimate load (N) is the highest point of the load-elongation curve reached before failure, and the stiffness (N/mm) is the gradient of the linear part of the load–elongation curve. The elongation at failure (mm) is the point of the extended length at the failure point.

Statistical analysis

Statistical analysis was performed using R studio 2021.09.1. The results for tensile testing are presented as mean ± standard deviation. The six groups were compared using Kruskal–Wallis ANOVA on ranks followed by pairwise comparison analysis (Dunn's method). The p values < 0.05 were considered significant.

RESULTS

Table 1 lists the results of the biomechanical properties of the fascia samples.

Ultimate load

Fig. 3 compares the ultimate load values of the biomechanical test. The mean ultimate load of the glycerol, honey, deep freeze, lyophilization, cryopreservation, and control group were

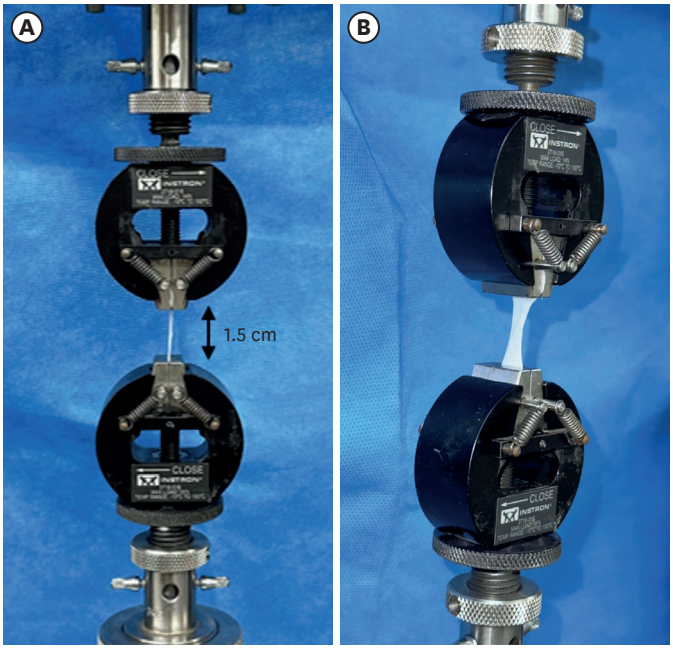

Fig. 2. Photograph of the fascia lata sample placed on the biomechanical testing machine. (A) The distance between the clamps was adjusted at 1.5 cm. (B) The narrowest portion of the sample is exposed between the clamps.

Table 1. Mean ± SD of the ultimate load, elongation at failure, and stiffness for six groups under biomechanical testing using fascia lata of New Zealand White rabbits

Test	Control	Glycerol	Honey	Lyophilization	Deep freeze	Cryopreservation
Ultimate load (N)	21.5 ± 4.6	17.0 ± 2.1	3.4 ± 0.9	10.5 ± 3.1	9.8 ± 3.3	10.5 ± 1.6
Elongation at failure (mm)	8.7 ± 3.2	13.9 ± 2.5	6.9 ± 1.4	9.3 ± 3.1	5.4 ± 1.4	7.6 ± 2.0
Stiffness (N/mm)	4.9 ± 1.5	2.6 ± 0.9	0.8 ± 0.2	1.7 ± 0.9	2.7 ± 1.2	2.6 ± 0.7

The results are presented as mean ± SD.
SD, standard deviation.

17.0 ± 2.1 N, 3.4 ± 0.9 N, 10.5 ± 3.1 N, 9.8 ± 3.3 N, 10.5 ± 1.6 N, and 21.5 ± 4.6 N, respectively. The control group showed the highest mean ultimate load that was significantly higher than the other groups ($p < 0.05$) except for the glycerol group, which did not show a significant difference. Among the groups other than the control group, the glycerol group showed the highest mean ultimate load. In addition, the glycerol group showed a significantly higher ultimate load than the honey group ($p < 0.001$) and deep freeze group ($p = 0.007$).

Elongation at failure

Fig. 4 compares the elongation at failure. The mean elongation at failure of the glycerol, honey, deep freeze, lyophilization, cryopreservation, and control groups was 13.9 ± 2.5 mm, 6.9 ± 1.4 mm, 9.3 ± 3.1 mm, 5.4 ± 1.4 mm, 7.6 ± 2.0 mm, and 8.7 ± 3.2 mm, respectively. The glycerol group showed the highest mean elongation at failure among the groups. The elongation at failure of the glycerol group was significantly higher than the deep freeze group ($p < 0.001$), honey group ($p = 0.001$), and cryopreservation group ($p = 0.011$). The lyophilization group showed a significantly higher elongation at failure than the deep freeze group ($p = 0.011$).

Stiffness

Fig. 5 compares the stiffness values of the biomechanical test. The mean stiffness of the glycerol, honey, deep freeze, lyophilization, cryopreservation, and control groups were 2.6 ± 0.9 N/mm,

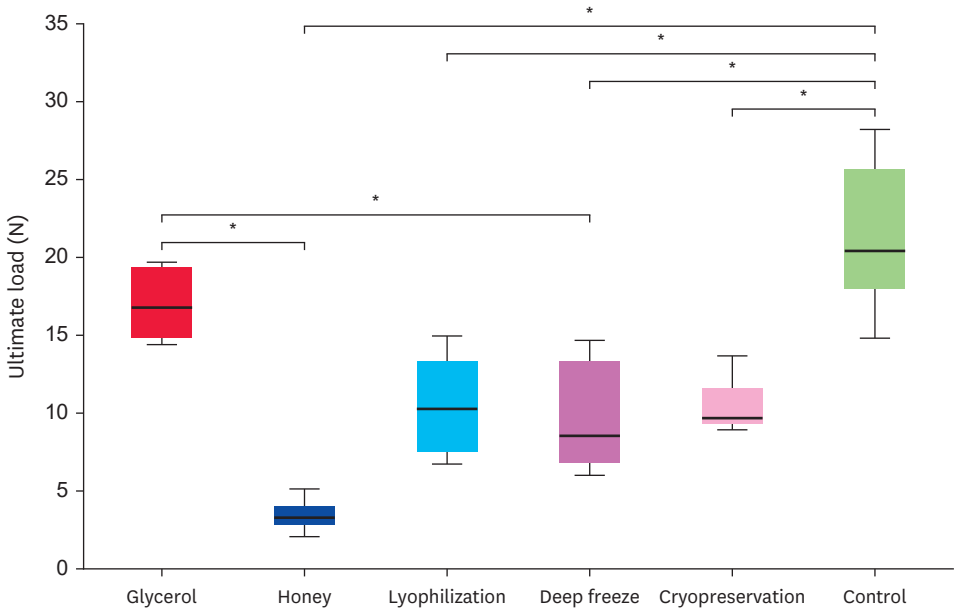

Fig. 3. Box plot of the ultimate load of five preservation groups and control group. Statistical significance was set at $p < 0.05$. The statistically significant groups are marked with an asterisk (*). The ultimate load of the control group is significantly higher than all groups ($p < 0.05$) except for the glycerol group. The glycerol group shows a significantly higher ultimate load than the honey group ($p < 0.001$) and the deep freeze group ($p = 0.007$).

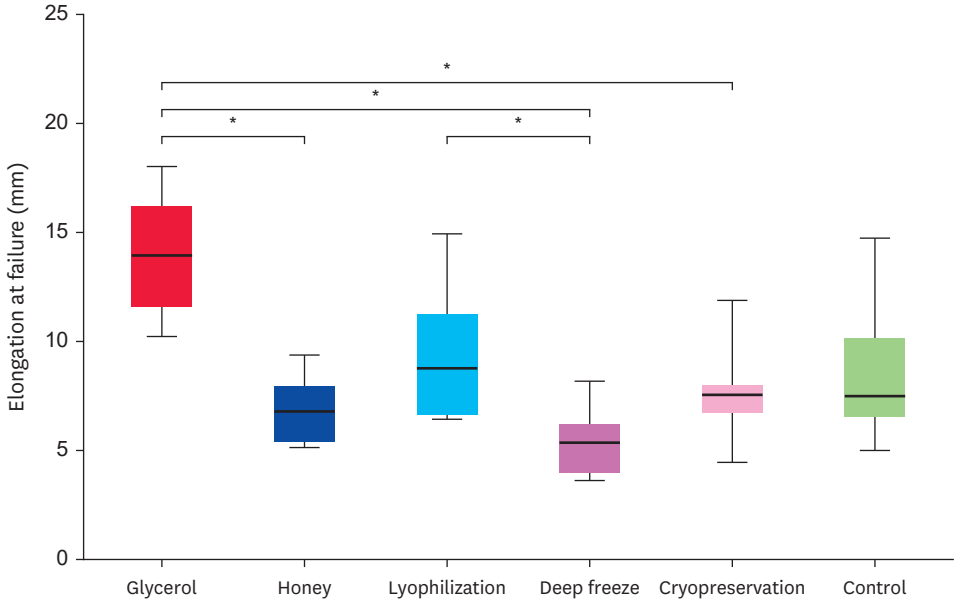

Fig. 4. Box plot of the elongation at failure of five preservation groups and control group. Statistical significance was set at $p < 0.05$. The statistically significant groups are marked with an asterisk (*). The elongation at failure of the glycerol group is significantly higher than the deep freeze group ($p < 0.001$), honey group ($p = 0.001$), and cryopreservation group ($p = 0.011$). The lyophilization group showed a significantly higher elongation at failure than the deep freeze group ($p = 0.011$).

0.8 ± 0.2 N/mm, 1.7 ± 0.9 N/mm, 2.7 ± 1.2 N/mm, 2.6 ± 0.7 N/mm, and 4.9 ± 1.5 N/mm, respectively. The honey group showed the lowest mean stiffness among the groups. The stiffness of the honey group was significantly lower than all the other groups ($p < 0.05$) except for the lyophilization group ($p = 0.095$). In addition, the stiffness of the lyophilization group was significantly lower than the control group ($p < 0.001$).

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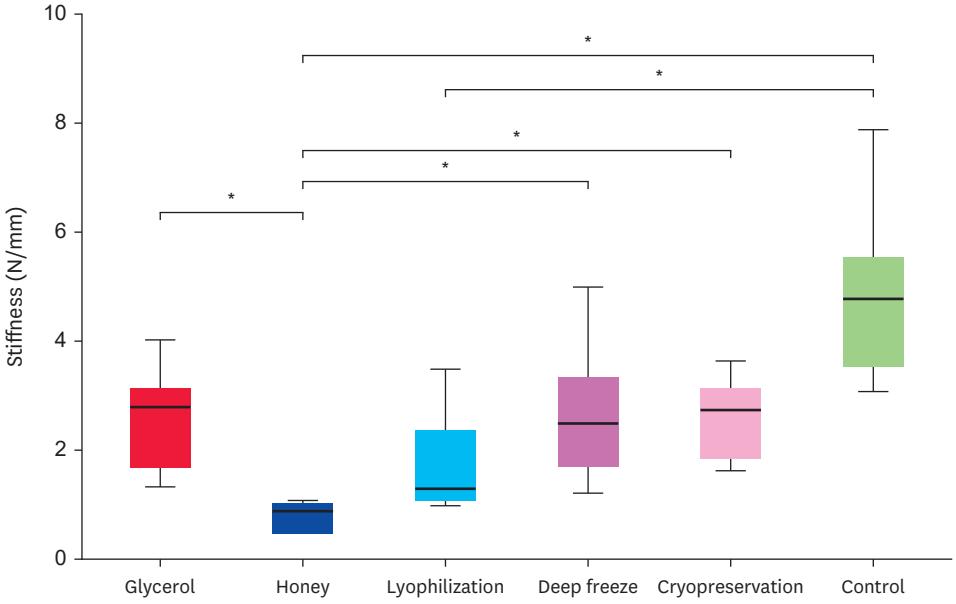

Fig. 5. Box plot of the stiffness of five preservation groups and control group. Statistical significance was set at $p < 0.05$. The statistically significant groups are marked with an asterisk (*). The stiffness of the honey group was significantly lower than all groups ($p < 0.05$) except for the lyophilization group. The lyophilization group showed significantly lower stiffness than the control group ($p < 0.001$).

DISCUSSION

A fascia autograft is generally used in reconstructive surgery because of the absence of host response [2-4], but it has limitations in shapes and sizes, as well as the disadvantages of donor morbidity and longer operation time [11]. Therefore, allografts can be considered a good alternative. On the other hand, the different storage methods for fascia were compared because the preservation of allograft tissues is inevitable.

New Zealand White rabbits were selected as the experimental animals in this study because the point of death, size, and breed differ in canine cadavers. Tissue deformation could occur during the freeze and thaw process, and the duration of the freezing time could affect the properties of the tissues [12,17,22]. Therefore, comparing the preservation methods under the same conditions using samples from canine cadavers is not possible. In contrast, the point of death, size, and breed could be unified using New Zealand White rabbits, which makes them suitable for comparison under equal conditions. Furthermore, this study will serve as the foundation for future research on allografts in small animals because the size of the New Zealand White rabbits used in this study is comparable to that of small animals.

In this study, unifying the shape of the fascia lata sample was critical because it could significantly influence the result. The sample was manipulated more when using a #15 blade to cut off the fascia by tracing a template placed over the fascia lata, which could damage the sample before biomechanical testing. On the other hand, pressing down the razor blade on the sample could cut off the fascia neatly at once, which minimized the manipulation of the sample. The shape of the samples could be seen as identical by unifying the angle of the razor blade and the width of the widest and narrowest portion of the fascia lata.

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Lyophilization and deep freezing are commonly used to preserve the fascia, but special equipment is needed, and adverse effects in the collagen microstructure can occur because of ice crystal formation when freezing the tissue [22]. Cryopreservation can be used to preserve fascia, blood vessels, bone, nervous tissue, and cornea, but it is inconvenient because it requires special equipment [12,23,24]. Theoretically, all chemical and biological processes stop when preserved in liquid nitrogen (–196°C), and indefinite preservation can be achieved [23]. On the other hand, tissue damage can occur because of changes in temperature during the cryopreservation process [24]. After the fascia was washed in DW before biomechanical testing, the fascia in the lyophilization, deep freeze, and cryopreservation groups were limper than the other groups. The ultimate load and stiffness were similar in the cryopreservation, lyophilization, and deep freeze groups, and all three groups showed a significantly lower ultimate load than the control group.

Honey has hypertonic, sterile, and bactericidal properties and has been used to preserve skin, cornea, and bone [17,25]. It is inexpensive, easy to preserve, and can lower the possibility of infection [25]. The conservation of fascia in honey was first attempted in this study because previous research on other tissues preserved in honey showed positive results. On the other hand, the honey group had the lowest mean ultimate load and stiffness and the second shortest mean elongation at failure among the groups. Stiffness is the ability to resist the force needed to cause deformation. Low stiffness means irreversible change occurs with low energy [26]. The honey group showed a significantly lower stiffness than the other groups, except for the lyophilization group. Moreover, the honey group showed the lowest mean ultimate load and elongation at failure. Hence, the tissue deforms easily with low force, cannot endure high strength, nor can it stretch compared to other groups.

Research on honey-infused scaffolds used in wound healing has reported that the strength and elasticity decrease when honey is incorporated into the scaffold [27]. This could explain why the honey group showed insufficient results in ultimate load, stiffness, and elongation at failure. In addition, there may be an inconvenience in the clinical application of honey-preserved fascia because repetitive washing is needed owing to the viscous properties of honey. Nevertheless, this study is the first to attempt to preserve fascia in honey. Therefore, further research will be needed on the exact interaction between honey and fascia.

For the control group, the biomechanical test was conducted immediately after the decellularization process. The control group showed the highest mean ultimate load and was significantly higher than all the groups except for the glycerol group. The fascia preserved in glycerol appears expansible and tolerates high force in that only the glycerol group did not show a significant difference in the ultimate load from the control group and had the longest mean elongation at failure.

Although glycerol is a common solution for preserving skin and bone, it has not been used to preserve fascia. Preservation in glycerol is beneficial because it is inexpensive, has virucidal and antibacterial characteristics, and is convenient in that it can be kept at room temperature [20]. In addition, compared to the deep freeze, cryopreservation, and lyophilization methods, shorter preparation times will be needed because thawing and rehydrating procedures are unnecessary when preserved in glycerol [21]. Therefore, glycerol can be regarded as an effective preservation method for fascia when aggregated with satisfactory biomechanical test results.

According to an existing study of glycerol-preserved cornea, glycerol effectively protects the glycosaminoglycan in the cornea and maintains the structure of the extracellular matrix [28]. Moreover, the microscopic morphology was well maintained even after long-term preservation of skin in glycerol [20,29]. Glycerol, a commonly used plasticizer, has been reported to increase the intermolecular spacing of collagen and reduce the relative content of triple-helical structures, facilitating structural elongation [30]. These studies may support the relatively superior biomechanical results of the glycerol group in this study. Nevertheless, further research on the mechanism of how glycerol affects the fascia will be needed because this study is the first to evaluate the biomechanical properties of glycerol-preserved fascia.

Glycerol-preserved fascia also required thorough washing because of its high viscosity. A previous study reported that glycerol-preserved skin required a minimum of one h to remove most of the glycerol [28]. Although glycerol is considered safe, adverse effects can occur because of the residual concentrations [22]. Therefore, additional research will be needed on the adequate washing time required to remove glycerol from the fascia.

Although decellularization was conducted in this study, further studies on *in vivo* healing and the possible host responses will be needed to overcome the limitation of an *in vitro* study. In addition, further research will be needed on the optimal time for the preservation of fascia allografts because the duration of preservation could affect the biomechanical properties. Future research is warranted to conduct histological analysis and further determine the biomechanical. This will enable an evaluation of decellularization effectiveness and any degenerative changes induced by the preservation processes. Various sterilization techniques have been used to prevent infection in allografts, including chemical processing, gamma irradiation, thermal treatment, antibiotic soaks, and beta-propiolactone [31]. Nevertheless, most of these methods compromise the biomechanical properties of the grafts [31]. In this study, only an antibiotic-antimycotic solution was used after decellularization. Further research will be needed to evaluate the detrimental effects of different sterilization methods on the biomechanical properties of fascia allografts.

Nevertheless, these results should be interpreted with caution because the biomechanical properties are not the sole factors determining the applicability of preservation methods. The use of multilayered allografts may compensate for the reduced biomechanical strength. An allograft does not need to exceed the predicted strain; rather, it must possess sufficient strength to function effectively for its intended purpose. In addition, allografts must exhibit biocompatibility with the host. Therefore, preservation methods should be selected based on a comprehensive evaluation of these factors.

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