Abstract

Purpose: To evaluate the effectiveness of combining a customized mold with frozen conventional clamps against other freezing and non-freezing methods.

Methods: Forty-five porcine and 45 chicken tendons were evenly divided into five groups (n = 9 + 9/group): control group, non-freezing with gauze placed between tendon and clamp (gauze), non-freezing with suture fixation at tendon ends (suture), freezing with dry ice pocket placed at the clamps (pocket), and freezing using a templated liquid nitrogen clamp with a customized mold (mold). Tension tests were used to measure failure modes and loads.

Result: Slippage and avulsion were observed in non-freezing groups with significantly lower failure loads compared to freezing methods. With freezing, rupture occurred near the central point only in the mold group. The failure loads for porcine tendons in the mold group were higher (2121.651 ± 73.101 N) than the pocket group (1746.337 ± 68.849 N). The failure loads of chicken tendons in the mold (243.552 ± 15.881 N) and pocket groups (260.647 ± 22.161 N) were not statistically different.

Conclusion: Freezing clamps represent the better choice for soft tissue clamping. The customized mold method could improve gripping effectiveness.

Keywords: Biomechanical phenomena, Mechanical tests, Tensile strength, clamping, Tendon

Introduction

The fixation of tendons is key point during the tension test for the mechanical properties of tendons [1–3]. If slippage or damage of the tendon occurred in the clamping position during the test, it would lead to errors and affect the accuracy of conclusions about its mechanical properties. The style of tendon fixation on the clamps can be divided into freezing and non-freezing treatments. Non-freezing treatments include modifying the clamp interface geometry [4, 5], or adding additional materials [5, 6]. The modified surface of the clamps can increase the contact area and friction between tendon and clamp, but the equipment fabrication and application techniques are highly demanding. Adding additional materials such as sandpaper, adhesive and winding as the interposition only applies force to the superficial layers which may cause the inner fibers to displace and increase transverse shear forces. The slippage caused by low friction between the clamp and the soft, wet collagen tissue can easily damage the orientation of tendons due to extrusion from the clamps and reduce their effectiveness [4, 7, 8]. Freezing treatment has long been considered the gold standard for high load mechanical testing of soft tissues, [5, 9]. It has been shown that frozen tendons are stiffer and more rigid, which can prevent damage to the tendon and slippage between the tendon and clamps.
Freezing methods include cryogenic and thermoelectric approaches [11]. The former method applies cold resources such as liquid carbon dioxide (CO2), dry ice, or liquid nitrogen [3, 9] to freeze the clamped ends of tendons. A thermoelectric approach uses a cooler to remove heat from the contact surface to achieve freezing. However, it requires long preparation time, which could be a problem for a large number of tests [5]. Recently, Hangody [11] reported a simple, convenient and enlightening method using pockets of dry ice on both sides of the clamps as a freezing method. This method provided a satisfactory outcome, but still required a modified clamp, a non-frozen asymmetrical nylon teeth jaw and an alloyed titanium clamp body designed by Shi et al. for clamping tendons [4]. Due to the high cost of cryogenic and thermoelectric approaches and the time-consuming process to customize the specialized clamps, we hope to explore a relatively simple and affordable alternative to make the conventional mechanical clamps that can achieve the clamping quality similar to cryogenic and thermoelectric approaches. Accordingly, we designed a freezing method using conventional (non-customized) clamps with a simple mold. The purpose of our study was to (1) explore if the new method could provide sufficient efficacy for mechanical tendon testing, and (2) compare its effectiveness against other freezing and non-freezing methods.

Materials and methods

Animals and preparation

Flexor digitorum profundus tendons from 45 porcine hind-legs and chicken feet were sourced from a local butcher (Fig. 1). The chicken tendons were acquired from the third digit, which was the longest and therefore considered more suitable for study [12, 13]. The cross section area (CSA) was calculated by approximating the cross section to an ellipse [14, 15] ($S = \frac{\pi \text{a}\text{b}}{4}$, where a and b are the average values of major and minor axes of the ellipse measured three times by a vernier-caliper with a precision of 0.02 mm, which was placed vertically to the long axis of the tendon). The CSA of tendons of porcine $(23.544 \pm 4.450 \text{mm}^2)$ and chicken $(2.076 \pm 0.511 \text{mm}^2)$ is significantly different, representing two different sizes of tendons to simulate the different sizes encountered in the clinic [16]. After the tendons were taken, some uniform marks, including the central point and the clamp line, were made on the tendons in the same location to ensure the areas of freeze and test were the same (Fig. 2A). The central point indicated the middle of the tendon, and the clamp line specified the location of the clamps during the test. These marks were determined with calipers (accuracy 1 mm).

Five different treatments were used to test the tendons using the general manual metal clamp, which was made of metal with four-sided pyramid structures in the cross-sectional view. Only one pair of clamps were used in all tests. Nine porcine tendons and nine chicken tendons were included in each group. The intact tendons without any additional treatment were used as the control group. The ends of tendons were wrapped with a layer of gauze in gauze group (Fig. 3A). The gauze represents a method of adding movable high friction
coefficient objects to the clamp and tendon. The tendons from the suture group were sutured with 4–0 medical thread (TianHe, China, M47193T(T-451)) at the part outside the clamp line according to the method shown in Fig. 4. There were five sutures perpendicular to the longitudinal axis of the tendon on two sides of each end of the tendon, to increase the friction. The pocket group was based on Hangody’s method [11]. Two non-woven fabric pockets with dry ice were placed on both sides of each clamp. In order to ensure the effectiveness of cold insulation, we encircled the surface of the pocket with an insulation layer composed of aluminum foil, thickened polyethylene (PE) and pearl cotton. Then, the clamp with the tendon was frozen for about 3 min according to the Hangody’s method as the study had shown that freezing for 3–5 min can achieve a firm freeze [9, 11] (Fig. 3B). In the mold group, we clamped the tendon at the clamp lines and aligned the center of the clamps with the central point of the tendon. Then, we placed the clamps and the tendon in a specified position using the specially made mold (Fig. 5). Liquid nitrogen flowed to the surface of the clamp along the drainage rod and the edge of the clamp at the clamping line, while warm water (30 °C) flowed continuously at the center of the tendon to prevent freezing. This process was run for five minutes before stopping the liquid nitrogen. Warm water continued to flow for another minute. Then, the clamps were taken out with cotton gloves for failure testing. (Additional file 1)

Biomechanical testing
A microcomputer-controlled electromechanical universal testing machine (Wance, China, ETM203A) was used for failure testing. The tendons gripped on clamp lines at both ends by non-special designed clamps (Fig. 6) were tested at a speed of 20 mm/min until they reached failure point, which was defined as either significantly slippage (or avulsion) or rupture of the tendon. When a tendon failed, the morphological change of the tendon represented the failure mode and the maximum force before failure was the failure load, which could be recorded by specialized software (Wance, China, TestPilot_E10C). If the tendon failure mode was slippage, the failure load represented the fixation capacity of the clamps. When a rupture occurred, it meant that the force loaded by the machine exceeded the stress of a tendon to maintain its integrity, indicating that the fixation capacity of the fixture was higher than the failure load. During the test, the tendon was kept parallel to the moving direction of the clamps to avoid forces in other directions affecting the failure mode (Fig. 2B). Keeping the
surface of the tendons moist was important during the test because an excessively dry surface could lead to desiccation, which may affect the mechanical properties of the tendons [1, 17].

Statistical evaluation
Statistics were performed using SPSS Version 22. Differences in tendon failure loads between groups were compared using t tests.

Results
Failure mode
The observed failure modes include slippage, avulsion, and rupture. Slippage means the tendon part is out of the clamp partly, or completely. It can be seen that the clamp is obviously out of the clamp line. Avulsion is tear of tendon. It should be noted that slip allows a small amount of tissue damage, caused by clamping teeth on the tendon surface of the clamping part. If the injury causes the tear of tendon, it is avulsion. Rupture means the tendon is
divided into two parts completely at the test area. (Fig. 7).
In order to test the true mechanical failure of the tissue, a firm and effective grip is indicated when the only failure mode of the tendon is rupture. In the control group, slippage occurred in the porcine tendon, while avulsion occurred at the clamp line occurred in chicken tendon. (Fig. 7A). In the gauze group, the gauze was always kept between the clamps. The tendon tissue detached from the gauze, and the tearing of the tissue surface was not obvious. This indicates that while the uneven surface may indeed play a role in increased friction, this failure was caused by insufficient friction between the gauze and the tissue (Fig. 7B). In the suture group, avulsion at the clamping area occurred, and tearing of the suture was also observed in the clamping area. The suture and a small amount of tissue were detached from the tendon and adhered to the clamp face (Fig. 7C). In the pocket group, the porcine tendons showed slippage from the clamp line or rupture inside the clamp area which may also be caused by slippage, while the chicken tendons showed rupture only near the central point (Fig. 7D). There was no significant visual slippage that happened before rupture, and the rupture position was near the central point of the tested area in both porcine and chicken tendon in the mold groups (Fig. 7E).

**Failure load**
Failure load is the amount of force required for a tendon to reach its failure point. The results of untreated tendons show that the test ended due to slippage with an average of only 135.654 ± 14.140 N in porcine tendons and 113.780 ± 10.135 N in chicken tendons (Table 1). Overall, the gauze group for both sizes of tendons showed the lowest failure load, which was significantly lower than the control group (p < 0.05), with 74.310 ± 12.707 N in...
porcine and 63.903 ± 14.616 N chicken tendons (Table 1, Fig. 8). Sutures may change the smoothness of tissue surface and increase the friction of tissue surface. The failure load reached 144.991 ± 21.064 N and 101.774 ± 16.623 N in porcine and chicken tendons, respectively, and there was no significant difference compared with the control group (Table 1). Finally, the average failure load-force for the porcine tendons was 1746.337 ± 68.849 N in the pocket group and 2121.651 ± 73.101 N in the mold group. The difference was statistically significant (p < 0.05) (Fig. 8). The force in the chicken tendons was 260.647 ± 22.161 N for the mold group and 243.552 ± 15.881 N for the pocket group. Failure loads for all freezing methods were significantly higher than that of the non-freezing treatments groups (Table 1, Fig. 8). In addition, the failure load of porcine tendon in the mold group was significantly higher than that in the pocket group (Fig. 8).

**Discussion**

In the presented study, the effects of different fixation methods for clamping tendons of different diameters were compared. The results showed that the avulsion or slippage around the clamping area was found in control group (general metal clamp) and unfrozen groups (gauze and suture groups) for both chicken and porcine tendon, while rupture near the central point occurred in frozen groups with the exception of porcine tendons in the pocket group. These data suggest that the ordinary conventional clamp may achieve a higher gripping force with freezing methods; however, the pocket method was only effective for smaller diameter tendons. In order to meet the demand of a high clamping force for large diameter tendon, an application of the mold method may be beneficial.

Non-freezing clamps are often considered an inferior method to freezing clamps for tendon fixation, mainly due to the viscoelastic characteristics of the wet soft tendons making it hard for the clamps to grip the tendon tightly [4]. The extrusion during the loading process increases the moisture content of the holding part, and

| Table 1 | Failure mode and failure load (mean ± standard deviation) of porcine and chicken’s tendons with different treatments |
|---------|------------------------------------------------------------------------------------------------------------------|
| Group   | Porcine (n = 45) Failure load (N)  | Failure mode | Chicken (n = 45) Failure load (N)  | Failure mode |
|---------|----------------------------------|--------------|----------------------------------|--------------|
| Control group | 135.654 ± 14.140 | Avulsion     | 113.780 ± 10.135 | Avulsion     |
| Unfrozen treatment |                        |              |                                  |              |
| Gauze group | 74.310 ± 12.707 | Slippage     | 63.903 ± 14.616 | Slippage     |
| Suture group | 144.991 ± 21.064 | Avulsion     | 101.774 ± 16.623 | Avulsion     |
| Frozen treatment |                        |              |                                  |              |
| Pocket group | 1746.337 ± 68.849 | Slippage or fracture inside the clamp | 260.647 ± 22.161 | Fracture near the central point |
| Mold group | 2121.651 ± 73.101 | Fracture near the central point | 243.552 ± 15.881 | Fracture near the central point |

Fig. 8 failure load of (A) porcine and (B) chicken’s tendons with different treatments. * p < 0.05 indicates significant differences between two groups. In all treatments, the failure load of porcine tendons was higher than chicken tendons. The failure load of freezing treatment was significantly higher than non-freezing treatment and the failure load of gauze group was significantly lower than the control group for both porcine and chicken tendons. The failure load of the mold group was significantly higher than the pocket group for porcine tendons.
spraying of saline to maintain the moisture of the tendon surface to avoid change in the mechanical properties of the tendon [17] may further reduce the friction between the clamp and tendon. Besides, due to the deformation of the tendon at the clamp line, uneven stress distributions can lead to more damage in the clamping area [18]. The relative displacement between the outer layer of the tendon (which is well fixed with the clamp) and the inner layer of the tendon can also create avulsion. The gauze treatment was even worse than untreated control tendons, because of decreased friction between the gauze and the tendon. In the suture group, the failure load was similar to the control group. This suggests that the conventional clamp with non-freezing methods could not provide enough grip strength for mechanical tendon testing.

Freezing clamps can fixate the tendon firmly and decrease injuries to the clamping area of the tendon because the frozen tendon is tightly attached to the clamp and is difficult to deform. Freezing can increase the friction coefficient between the clamp and the tendon, and the hardness of the clamping area. As a result, the effectiveness of clamping is increased, and the damage caused by the clamp’s teeth to the tendon and the displacement between the outer and inner layers of the tendon was reduced [5]. The freezing clamp was first proposed by Riemersa and Schamhardt in 1982 and was called the ‘cryo-jaw’ [19]. They expected to prevent the deformation of tissues in high loads by freezing the tendons with clamps with expansion of liquid CO2. In 2004, Wieloch et al. [20] soldered liquid nitrogen containers consisting of copper plates to the clamps. Ramachandran et al. [21] also designed a special freezing assembly that included a container to circulate coolant, the nitrogen and ice container to fixate the specimen. However, this costly and time-consuming method is not widely available and is also difficult to design and manufacture, so more affordable, efficient, and easy to operate methods are worth exploring. Recently, Hangody used dry ice pockets to freeze a modified clamp called Shi’s clamp to obtain a relatively satisfactory outcome. We applied Hangody’s method but used conventional metal clamps with dry ice (Fig. 6). Although slippage occurred in the larger diameter porcine tendons, the failure load significantly increased compared with non-freezing treatments. The reason may be that the fixation ability of our clamp was lower than the specially designed Shi’s clamp and unable to provide enough grip strength to reach the rupture point of porcine tendons. However, the slippage or avulsion around the clamp line was not observed in the pocket group of smaller chicken tendons which requires lower load to reach structure failure. This proves that Hangody’s pocket method works, but only to a limited extent. In the mold group, which is an improvement of Hangody’s pocket method, rupture was only observed where failure mode occurred near the central point in both porcine and chicken tendons. Moreover, porcine tendons in the mold group reached the failure threshold with significantly higher loads than tendons in the pocket group. However, the difference was not observed in chicken tendons. These findings suggest that our mold method works for both large- and small-sized tendons, while conventional clamps pocketed with dry ice are only effective for small-size tendons. Our method was especially effective in the test of large-size tendons.

The customized mold strictly controls the position and direction of the clamps during freezing, which avoids the difficulty of clamp installation caused by tendon torsion and bending. Studies have shown that there is fiber stretching and sliding during tendon loading [2], but the fiber sliding of frozen tendons may be limited and the tendon may present a similar brittle failure behavior [22] in the failure testing, which will lead to abnormal data. Therefore, it is necessary to avoid the effects of freezing in the test area. Since running water does not freeze easily, in this study, a continuous flow of water was placed above the center of the tendon to avoid the test area of tendon freezing. Since the water was spread along the surface of the tendon, the spread range is related to the height, width and diameter of the water flow, which was adjusted according to the actual situation. After the liquid nitrogen infusion, it is necessary to continue water perfusion for 1 min to offset the secondary freezing of tendon by low-temperature clamps and avoid the possibility of insufficient non-frozen area for mechanical testing of the tendon. On the other hand, the water perfusion could not be too long to decrease the freezing effect on clamping area of tendons. Our preliminary findings did show inconsistent melting times under different surrounding temperature conditions. Accordingly, the temperature of the experimental environment was maintained at 18–20°C for standardization.

In this study, we found several advantages of our conventional clamp frozen with our customized mold. First, it could provide enough holding strength for tendon mechanical test as high as 2000 N, which represents nearly 16 times enhancement over the control group. Moreover, this technique prevents the tendon test area from freezing and the possibility of further tissue properties changes caused by the frozen state. The slippage and avulsion in the clamping area did not occur in our mold method, but they occurred in all other groups. Secondly, the mold method demonstrated high efficiency. In the Hangody’s study [11], the frozen method of dry ice pocket took about 8 min which is similar to what we found in our pocket group. Scholze
[7] reported their preparation time for 3D printing technology clamp would be 10–15 min. The cryo-jaw would spend further more time for preparation [7, 9]. On average, preparation for test of tendon was 5–6 min for our mold method, which would be time-saving and convenient for measuring large sample. Thirdly, the cost of our mold method was relatively low. The special customized clamp for roughen clamp surface that was used in other researches costs between 35 and 100 US dollars [8, 9]. The cryo-jaw would be more complicated and also cost more [4]. In our study, the non-special designed clamp, which came with the testing machine in the original package, and the poly foam box for making molds cost next to nothing. The only cost for our mold method is liquid nitrogen (approximate 0.4–0.5 US dollar per sample).

This study has some limitations. First, the experimental conditions of this study are affected by the type of clamps and the experimental environment. The clamping force of different types of clamps is different, and the perfusion time may be slightly different under various experimental environments. Second, the determination of slippage was done by the naked eye, and there may still be very small slippage that was not observed. This may cause a certain degree of error. Third, there was a large amount of liquid nitrogen and water dripping in the experimental operation of the mold group. Therefore, a better method to collect nitrogen and water separately and maybe recycle them should be considered for future study. This may reduce the cost even further.

Conclusions
The freezing clamp could be a better choice for soft tissue clamping scheme. The templated freezing of clamp through a customized mold and liquid nitrogen could improve the effectiveness of gripping force for tendon’s biomechanical tests. This method is simple, high efficiency and affordable. Applying this method to non-special designed clamps can achieve a relatively satisfactory effect.

Supplementary Information
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Additional file 1. Warm water continued to flow after the liquid nitrogen stopped.

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TW was a major contributor in writing the manuscript. HY has made a substantial contribution to the concept or design of the article and revised it critically for important intellectual content. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Domnick C, Wieskotter B, Raschke MJ, Schulze M, Kronenberg D, Wefelmeier M, et al. Evaluation of biomechanical properties: are porcine flexor tendons and bovine extensor tendons eligible surrogates for human tendons in in vitro studies? Arch Orthop Trauma Surg. 2016;136(10):1465–71.
2. Lee AH, Elliott DM. Freezing does not alter multiscale tendon mechanics and damage mechanisms in tension. Ann N Y Acad Sci. 2017;1409(1):85–94.
3. Pearsall AW, Hollis JM, Russell GV, Jr Scheer Z. A biomechanical comparison of three lower extremity tendons for ligamentous reconstruction about the knee. Arthroscopy. 2003;19(10):1091–6.
4. Shi D, Wang D, Wang C, Liu A. A novel, inexpensive and easy to use tendon clamp for in vitro biomechanical testing. Med Eng Phys. 2012;34(4):516–20.
5. Jiang M, Lawson ZT, Erel V, Pervere S, Nan T, Robbins AB, et al. Clamping soft biologic tissues for uniaxial tensile testing: A brief survey of current methods and development of a novel clamping mechanism. J Mech Behav Biomed Mater. 2020;103:103503.
6. Soden PD, Kershaw I. Tensile testing of connective tissues. Med Biol Eng. 1974;12(4):510–8.
7. Scholze M, Singh A, Lozano PF, Ondruschka B, Ramezani M, Werner M, et al. Utilization of 3D printing technology to facilitate and standardize soft tissue testing. Sci Rep. 2018;8(1):11340.
8. Cheung JT, Zhang M. A serrated jaw clamp for tendon gripping. Med Eng Phys. 2006;28(4):379–82.
9. Kiss MO, Hagemeister N, Levasseur A, Fernandes J, Lussier B, Petit Y. A low-cost thermoelectrically cooled tissue clamp for in vitro cyclic loading and load-to-failure testing of muscles and tendons. Med Eng Phys. 2009;31(9):1182–6.
10. Omar M, Dratzidis A, Klintschar M, Kwisda S, Krettek C, Ettinger M. Are porcine flexor digitorum profundus tendons suitable graft substitutes

Omar M, Dratzidis A, Klintschar M, Kwisda S, Krettek C, Ettinger M. Are porcine flexor digitorum profundus tendons suitable graft substitutes
for human hamstring tendons in biomechanical in vitro-studies? Arch Orthop Trauma Surg. 2016;136(5):681–6.

11. Hangody G, Panics G, Szebenyi G, Kiss R, Hangody L, Pap K. Pitfalls during biomechanical testing - evaluation of different fixation methods for measuring tendons endurance properties. Physiol Int. 2016;103(1):86–93.

12. Farkas LG, Thomson HG, Martin R. Some practical notes on the anatomy of the chicken toe for surgeon investigators. Plast Reconstr Surg. 1974;54(4):452–8.

13. Kadar A, Thoreson AR, Reisdorf RL, Amadio PC, Moran SL, Zhao C. Turkey model for flexor tendon research: in vitro comparison of human, canine, turkey, and chicken tendons. J Surg Res. 2017;216:46–55.

14. Ge XJ, Zhang L, Xiang G, Hu YC, Lun DX. Cross-sectional area measurement techniques of soft tissue: a literature review. Orthop Surg. 2020;12(6):1547–66.

15. Yang CC, Yu X, Guo ZH, Fu YW. The biomechanical study of rupture of achilles tendon and repair by different suture techniques. Pak J Med Sci. 2018;34(3):638–42.

16. Weber JF, Agur AM, Fattah AY, Gordon KD, Oliver ML. Tensile mechanical properties of human forearm tendons. J Hand Surg Eur. 2015;40(7):711–9.

17. Lozanno PF, Scholze M, Babian C, Scheidt H, Vielmuth F, Waschke J, et al. Water-content related alterations in macro and micro scale tendon biomechanics. Sci Rep. 2019;9(1):7887.

18. Mommersteeg TJ, Blankevoort L, Huskens R, Koolooi MJ, Kauer JM. Characterization of the mechanical behavior of human knee ligaments: a numerical-experimental approach. J Biomech. 1996;29(2):151–60.

19. Riemersa DJ, Schamhardt HC. The cryo-jaw, a clamp designed for in vitro rheology studies of horse digital flexor tendons. J Biomech. 1982;15(8):619–20.

20. Wieloch P, Buchmann G, Roth W, Rickert M. A cryo-jaw designed for in vitro tensile testing of the healing Achilles tendons in rats. J Biomech. 2004;37(11):1719–22.

21. Ramachandran N, Koike Y, Poitras P, Backman D, Uhthoff HK, Trudel G. Dual cryogenic fixation for mechanical testing of soft musculoskeletal tissues. IEEE Trans Biomed Eng. 2005;52(10):1792–5.

22. Fu Q, Jia W, Lau GY, Tomsia AP. Strength, toughness, and reliability of a porous glass/biopolymer composite scaffold. J Biomed Mater Res B Appl Biomater. 2018;106(5):1209–17.

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