Comparison of ethanol concentration as stock solution on mechanical properties of iBTA-induced collagenous tubular tissue “Biotube”

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Abstract Collagenous tubular tissues “Biotubes,” formed by in-body tissue engineering, is implanted already as clinical vascular grafts in hemodialysis surgery. In almost all previous studies, Biotubes were stored in 70% alcohol before implantation, but the influence of alcohol concentration on the mechanical properties of Biotubes has not been investigated in depth. In this study, the mechanical properties of Biotubes stored at room temperature in two different concentrations of ethanol (10% or 70%) were compared through fatigue and tensile tests. Biotubes with an internal diameter of 6 mm and wall thickness of ca. 2 mm was prepared by subcutaneous embedding of molds into goat for two months, and stored in 10% or 70% alcohol solution for 20 d. In the fatigue test, performed by repeatable loading of tension corresponding to arterial pressure (700,000 cycles at 10 Hz), Biotubes stored in 10% solution elongated by approximately 80%, but in 70% solution, the elongation was <20%. Storing Biotubes in high concentration ethanol solution improved practical compliance and prevented stretching. The preservation of ECM with 70% ethanol is a cost effective, safe and easy method.

Keywords alcohol, Biotube, collagenous tube, physical property, store solution

1. Introduction

As tissue-engineered blood vessels (TEBV), cultured blood vessels, such as cytograft [1] and decellularized blood vessels [2], have been developed according to in vitro cell management protocol. Recently, cultured vessels using three-dimensional bio-printers [3] have also been developed. Furthermore, Biotube collagenous tubular tissues have been produced in vivo [4, 5]. The mechanical strength of these tissue-engineered vessels is maintained by the extracellular matrixes (ECMs) mainly consisting of collagen. It is desirable to store these tissues easily, without the tissue being deteriorated for a long period until implantation. Methods to store these tissues include cryopreservation, wet preservation, and freeze-drying. Conventional cryopreservation methods damage the ECMs and reduce their mechanical strength [6, 7], and therefore, a special equipment has been developed; however, highly reliable sterilization method has not been established [8].

As ethanol can help maintain ECMs cost-effectively at room temperature, it is used to store amniotic membranes [9] or Biotubes [4, 5]. Ethanol prevents hydrolysis by water, growth of residual microorganisms, and prevents collagen degradation by residual endogenous enzymes [10]. On the contrary, as ethanol dehydrates tissues, the degree of contraction and deformation of tissue varies depending on the concentration of ethanol, leading to changes in their strength or compliance.

In this study, the mechanical properties of Biotubes stored in two different concentrations (10% or 70%) of ethanol at room temperature were compared by fatigue and tensile tests.

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2. Materials and Methods

2.1 Ethical approval

All animals received care according to the Principles of Laboratory Animal Care (National Institutes of Health, No. 56–23, received 1985) and the research protocols were approved by the Ethics Committee of National Cerebral and Cardiovascular Center (No. 17013).

2.2 Preparation of Biotube

According to our previous report [5], Biotubes were prepared by embedding molds (four per goat) in the abdominal subcutaneous pouches of a goat (approximately 40 kg). Briefly, anesthesia was induced with intramuscular (IM) xylazine 2 mg/kg after intra-tracheal intubation, and was maintained by isoflurane inhalation. Two months after the embedding procedure, the molds were harvested and Biotubes (internal diameter 6 mm, wall thickness ca. 2 mm) were extracted by removing the molds. The Biotubes were stored in 10% or 70% ethanol solution for 20 d at room temperature prior to the measurement of mechanical properties.

2.3 Measurement of wall thickness

Biotubes and goat carotid arteries (CA, internal diameter ca. 5 mm) were cut into 5-mm width pieces (n = 5 in 10%, n = 6 in 70%, n = 5 in CA), and then opened to obtain rectangular samples. The wall thickness mapping of all samples on the whole surface was constructed by optical coherence tomography system (OCT, IVS-2000; Santec, Aichi, Japan). Briefly, the spatial resolution of the OCT was 0.0391 mm in surface length and 0.008 mm in depth. The sample was placed with its smooth surface in contact with the stage and laser light was irradiated perpendicularly to the stage surface. An algorithm was developed to calculate the thickness distribution by determining the local maximum point of reflection intensity closest to the depth direction from the irradiation position as the sample surface position. Subsequently, the thickness data along the longitudinal axis close to the breaking position was obtained; the average value of sampling data was defined as the thickness at the breaking point.

2.4 Fatigue test

The samples were fixed with clamps (distance: 5 mm) and repeatedly loaded by a tensile system (MMT-250NV-10; Shimadzu, Japan). For each sample, a tension of 0.2–0.32 N, corresponding to the aortic pressure of 80–120 mmHg, was applied by upper chuck moved at a frequency of 10 Hz and was repeated 700,000 times (7 d in terms of heartbeat of 70 bpm). The sample was then bathed with saline circulated by a pump and maintained wet. The elongation of sample was measured by the distance between the chucks, and the load applied to the sample was also measured. After the cyclic load test, the thickness of tissue was measured by OCT.

2.5 Measurement of breaking strength

Tissue strength was measured using cyclic loaded samples and unloaded control sample with a uniaxial tensile tester (EZ-LX; Shimadzu, Kyoto, Japan). The sample was fixed at a distance of 5 mm with two chucks in order to extend it in the circumferential direction. The distance between the chucks was preloaded with a load of 0.02 N and thereafter the initial length of the sample obtained. The load was applied at a speed of 0.5 mm/s until it fractured. The breaking force was defined as the maximum load value (N) at the highest point of the obtained load-extension curve. The unit-breaking force was defined as a value obtained by dividing the breaking force by the sample width.

2.6 Calculation of mechanical values

True stress/true strain was calculated according to a previously published method [11], using the thickness of the breaking position measured with the OCT, and the load and displacement were measured with a tensile tester. The ultimate tensile strength (MPa) was determined from the maximum value of stress and Young’s modulus (MPa) was determined from the maximum slope of the stress-strain curve.

The physiological modulus was calculated according to a previously published method [11]. From the Laplace equation, the relationship between the stress in the circumferential direction as following formula:

\[ \sigma = \frac{P_i \times D_i}{2 \times t} \]

Where, \( \sigma \) is the circumferential stress of tube, \( P_i \) is the blood pressure, \( D_i \) is the initial internal diameter, \( D_i \) is the internal diameter, \( \varepsilon \) is the strain of each sample, and \( t \) is wall thickness. Physiological modulus is a gradient value calculated from slope from circumferential stress and strain of 80 mmHg and 120 mmHg.

The burst strength was calculated using the following formula:

\[ P = \frac{2F}{L_0D_i} \]

where, \( P \) is the estimated burst pressure, \( F \) is the breaking force, and \( L_0 \) is the initial sample length of the ring sample.

2.7 Statistical analyses

The results are expressed as mean ± standard deviation.
The differences in mean values between groups were examined by the *t* test and the differences with a *p* value of <0.05 were considered significant.

### 3. Results

Biotubes were obtained as a tubular tissue in spiral shape with an internal diameter and length of approximately 6 mm and 25 cm, respectively (Fig. 1a). A part of Biotube was randomly cut to a width of 5 mm, and then a rectangular sample (5 mm × 18 mm) was obtained by cutting in longitudinal direction (Fig. 1b). A typical example of the wall thickness mapping of the original samples after storing in each ethanol solution constructed by OCT system is shown in Fig. 1c, d. When stored in 10% solution, the averaged thickness of the sample was 1.84 ± 0.14 mm, but was 1.52 ± 0.29 mm after storing in 70% solution (Fig. 1e, f).

![Images of the Biotube obtained from the mold (a) and cross-sectional view of the Biotube and Biotube rectangular sample stored in 70% ethanol solution (b). Thickness mapping by OCT of the sample stored in 10% (c) or 70% ethanol (d) before and after the fatigue test. The average and standard deviation of the thickness of the tensile stress area of the samples stored in 10% (e) or 70% ethanol (f) before and after the fatigue test. The significance levels by statistical analysis are ** *p* < 0.01.](image)
After the fatigue test, the thickness of the 10%-sample decreased significantly to 1.14 ± 0.22 mm, but there was no significant change in the 70%-sample (Fig. 1e, f). The length of the samples continuously elongated during the fatigue test in both ethanol solutions (Fig. 2). The difference in the elongation between two samples was gradually spread, with a significant difference at \( p \) value < 0.05 after 350,000 cycles, \( p < 0.01 \) after 450,000 cycles. After 700,000 cycles, the elongation ratio of the 10%-sample was 76.8 ± 24.2%, and that of the 70%-sample was 17.8 ± 11.5%.

The typical stress-strain curves of samples before and after the fatigue test were shown in Fig. 3. Almost similar curves were obtained in 70%-sample, but in 10%-sample marked deviation in the stress was observed. The mechanical properties obtained from the stress-strain curves are summarized in Fig. 4. The ultimate tensile strength after the fatigue test was significantly higher in the 10%-sample, but the unit length-breaking load representing the actual strength was equivalent before and after the fatigue test. In the 70%-sample, no significant difference was observed between tensile strength and breaking force before and after the fatigue test. The ultimate tensile strength of goat carotid artery was at least 8 times higher than any conditions of the Biotubes. The difference in Young’s modulus was also found to be significant before and after the fatigue test in the 10%-sample. The physiological modulus of the 70%-sample was higher than that of the 10%-sample. The physiological modulus, which is rigid in the strain range at arterial pressure, was higher in the 70%-sample than in the 10%-sample. In addition, the physiological modulus of 10%-sample also increased significantly before and after the fatigue test. The goat carotid artery was lower in physiological modulus than the Biotube, but Young’s modulus was higher. The fracture strain and estimated burst pressure did not exhibit significant difference before and after the fatigue test. And also, the fracture strain was significantly smaller in the after fatigue test than in the 70%-sample. In the comparison after the fatigue test, the fracture strain of the 10%-sample was significantly smaller than the 70%-sample. The goat carotid artery was about 3 times the fracture strain of Biotubes.

4. Discussion

In the present study, we focused on the concentration of ethanol used for the store of Biotubes and studied the relationship between shape and mechanical properties. Even with the wall tension generated at the blood pressure level, elongation occurred when stored in low concentration (10%) of ethanol, and the wall thickness was thin. Although the tensile strength and Young’s modulus of the sample increased before and after stretching, the breaking force was equivalent. After the fatigue test, physiological modulus increased in the 10%-sample, the fracture strain of the 10%-sample was smaller than the 70%-sample and it was difficult to stretch and fracture strain of the 10%-sample was also decreased. Contrarily, store in 70% ethanol showed no significant changes in ultimate tensile strength, and breaking force was observed before and after the fatigue test.

The transplantation of amniotic membrane into the cornea has been reported. The interstitial tissue, which is the substantial tissue of the amniotic membrane, is a tissue composed mainly of collagen, and it has been conventionally cryopreserved [12]. However, cryopreservation not only requires large-scale facilities, but also there is a concern that the ECM will be destroyed [13]. A special dry method has also been developed with need of complicated
protocols [14, 15]. On the other hand, glutaraldehyde solution can improve the tissue strength and elastic modulus by chemical cross-linking [16]. However, it is one of major factors for calcification after implantation [17]. In clinical organ transplantation, human aorta was stored by refrigeration with Euro-Collins solution known as an organ preservation solution [18]. There was no significant change in its mechanical property even after store at 4°C for 31 days. However, because there is no sterilization function in the preservation solution, careful clean management is needed.

Differences based on ethanol concentrations appeared in the strain and physiological modulus of the Biotube. The elongation of Biotube preserved in 10% ethanol was higher than that of Biotubes stored in 70% ethanol. With the elongation of the wall of Biotube, the internal diameter of the tube increases. According to Laplace’s law, the internal diameter of the tube is proportional to the wall tension, and therefore, expansion of the internal diameter results in an increase in wall tension. Therefore, even if the breaking force is equal, the burst pressure decreases as the internal diameter increases. There is also the possibility of development of aneurysm when transplanting a Biotube preserved with low concentration ethanol into an artery. As a result, a thrombus might form within the artery with aneurysm, causing cerebral infarction or myocardial infarction [19, 20]. Although no significant difference was observed in this study, the degree of decrease in the burst average value was high when the Biotubes were stored in 10% ethanol. Theoretically, it is thought that the influence of elongation increases as the internal diameter of the tube increases. It seems that ethanol can increase practical compliance and prevent elongation. Therefore, untreated biotubes are deemed inappropriate for transplantation, and from the viewpoint of ease of expansion, it may be better to treat 70% ethanol.

The rate of elongation was significantly different between the samples at 350,000 cycles, which is equivalent to 3.5 d at a heart rate of 70 bpm. Approximately 15% strain occurred even when stored in 70%, it may be better for the concentration to be higher, so its optimization is a future task. In fact, it is thought that the Biotube made of collagen functions as a cell scaffold in vivo after transplantation and the tube is strengthened by matrix formation of the migrated cells. Further studies are needed to determine how dynamically the changes in physical property occur.

5. Conclusion

There was no significant decrease in the strength of Biotubes stored in 10% and 70% ethanol solutions at room temperature even with repeated tension generated by aortic
pressure. However, after repeated loading, the elongation of Biotubes stored in 10% ethanol was higher than that of Biotubes stored in 70% ethanol; this might induce aortic aneurysm when transplanted into the aorta. Storing Biotubes in high concentration ethanol solution improved practical compliance and prevented stretching. The preservation of ECM with 70% ethanol is a cost effective, safe and easy method.

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References


