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Development of the novel biotube inserting technique for acceleration of thick-walled autologous tissue-engineered vascular grafts fabrication

Ning Ma · Zhenyu Wang · Hao Chen · Yanjun Sun · Haifa Hong · Qi Sun · Meng Yin · Jinfen Liu

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Abstract To accelerate the fabrication of thick-walled autologous tissue-engineered vascular grafts (TEVGs), a novel biotube inserting technique was developed. After 2 weeks of subcutaneous embedding in rabbits, silicone rods (diameter, 3 mm; length, 35 mm) became encapsulated in connective tissues. Single-layered biotubes were obtained after removing the silicone rods. One silicone rod encapsulated in tissues was inserted into a single-layered biotube to form two layers of autologous tubular tissues. Three layers of autologous tubular tissues were also obtained by applying the same technique. Following a 2-week re-embedding procedure, two layers or three layers of autologous tubular tissues were integrated to form two-layered or three-layered TEVGs. Both wall thickness and burst pressure of three-layered TEVGs were significantly higher than those of two-layered and single-layered TEVGs ($P < 0.05$). The two-layered TEVGs could be applied as small-caliber vascular grafts, and three-layered TEVGs could be applied as medium- or large-caliber vascular grafts.

1 Introduction

Vascular bypass grafting is commonly used to relieve angina and other peripheral ischemic symptoms. An autologous native vessel such as an internal thoracic artery or a saphenous vein is the most preferable graft. However, the supply of native vascular grafts may be not adequate [1], especially in elderly patients with diabetes. Historically, tissue-engineered vascular grafts (TEVGs) have relied on synthetic materials such as Dacron and expanded polytetrafluoroethylene (ePTFE) for constructing scaffolds. Synthetic materials can provide adequate mechanical strength and suturing characteristics [2]. However, the small-caliber synthetic vascular grafts have shown poor patency rates, particularly in the vessels of the lower limbs where low blood flow and high resistance increase the risks of thrombosis which can result in early graft occlusion [3, 4]. Although some strategies to increase patency have focused on synthetic graft pacification, such as various protein coatings [5, 6], or seeding endothelial cells on the lumen of synthetic grafts [7], synthetic vascular grafts may induce graft infections [8] and chronic foreign body inflammation which can lead to late graft failure [9]. Furthermore, despite vast improvements in degradable biomaterials and nanotechnology, a completely biocompatible material for the fabrication of vascular grafts is not yet available [2]. Cryopreserved arterial or venous allografts may also be unacceptable for routine use because of serious limitations, including limited availability and durability due to calcification, aneurysmal dilation, and poor patency rates, which may be partly attributed to the immunologic rejection to the allografts by the recipient [1].

A living, biological, and autologous TEVG may be an appropriate alternative. On one hand, the traditional approach to fabricating autologous TEVGs is based on the in vitro cell sheet rolling technique. Recently, skin fibroblast sheets rolled in three revolutions were applied to fabricate tissue-engineered blood vessels as arterial-venous (A-V) shunts for dialysis patients [10]. The rolled fragile sheets required to be fused by in vitro incubation to form matured tubular tissues with thick walls. However, at least 10 weeks were required to fuse the fibroblast sheets.

N. Ma · Z. Wang · H. Chen · Y. Sun · H. Hong · Q. Sun · M. Yin · J. Liu (✉)
Department of Pediatric Cardiothoracic Surgery, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai 200127, China
e-mail: liujinfen2007@yahoo.cn

On the other hand, Nakayama developed autologous tubular tissues called "biotubes" by applying in vivo tissue engineering [11]. This approach is based on the phenomenon of encapsulation of foreign materials by connective tissue in living bodies [12]. The biotubes consist only of autologous fibroblasts and ECMs without any artificial materials [11]. However, the wall thickness of the biotube was less than a few hundred micrometers after several months of embedding, though the wall thickness could be somewhat regulated by changing the type and embedding period of the foreign material [13]. As a result, the burst pressure was not more than 1000 mmHg in 3-mm biotubes and less than 100 mmHg in 10-mm biotubes, which is not enough for large-caliber arterial system [14]. To improve the mechanical properties of the biotubes, a wing-attached rod mold and a nicotine-coating mold were innovatively designed [14, 15].

In this work, our purpose is to fabricate autologous TEVGs with thick and firm walls in a short period. To this end, a novel biotube inserting technique was developed as an alternative method. The two-layered TEVGs or three-layered TEVGs were obtained by layering the single-layered biotubes in only 4 weeks and then examined histologically and mechanically.

2 Materials and methods

2.1 Animal preparation

Eighteen New Zealand White rabbits were prepared for this study (2.65 ± 0.11 kg in weight). The study was approved by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine. All animals in this study were maintained in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

2.2 Fabrication of autologous TEVGs

Anesthesia was induced by intramuscular injection of ketamine hydrochloride (60 mg/kg), atropine sulfate (0.05 mg/kg) and xylazine (8 mg/kg) for all animals. One, two or three silicone rods (3 mm in diameter, 35 mm in length, made in China) were embedded into the dorsal subcutaneous pouches of the rabbits ($n = 6$) via small skin incisions. After a 2-week embedding period, the silicone rods which were covered with connective tissue capsules were harvested (Fig. 1A). The silicone rod was removed (Fig. 1B) to obtain a single-layered biotube (Fig. 1C). One silicone rod with covering connective tissues was inserted (Fig. 1D) into a single-layered biotube to form two layers of autologous tubular tissues which were ligated with 4-0 silk threads from the outside (Fig. 1E). This two layers of autologous tubular tissues could also be inserted into another single-layered biotube (Fig. 1H) to form three layers of autologous tubular tissues tied with 4-0 silk threads (Fig. 1I). After another 2-week re-embedding period, the silicone rods with integrated autologous tubular tissues were harvested to obtain single-layered, two-layered, or three-layered TEVGs (Fig. 1G, K) after removal of the silicone rods (Fig. 1F, J). The single-layered TEVGs were used as the control.

2.3 Histological examination

Some parts of the TEVG specimens were fixed in 10% formalin solution and embedded in paraffin. Then TEVG sections were cut into pieces 3–5 mm thick for hematoxylin and eosin staining for light microscopy. The wall thickness of the TEVGs was measured by microscopic examination of cross sections.

2.4 Measurements of mechanical properties

The mechanical properties were measured using a modified Power Lab data acquisition system (AD Instruments, Australia). Burst pressure of the TEVGs could be obtained by increasing hydrostatic pressure in the TEVGs at a rate of 10 mmHg/s until rupture. The pressure was recorded. Longitudinal slits of TEVGs provided an indication of obvious rupture. Compliance (C) of the TEVGs was analyzed in terms of the dilatation rate of the external diameter (D), which could be calculated by measuring the change in external diameter as the pressure (P) was increased from 80 to 120 mmHg, at a rate of 5 mmHg/s. The luminal pressure was recorded simultaneously by a pressure transducer. High-resolution images were used to measure the external diameter. The TEVG wall was assumed to be incompressible. Compliance was calculated using the equation:

$$C = (D_{120} - D_{80}) / (D_{80} \times \triangle P) \, [10]$$

2.5 Statistical analysis

All data are presented as mean with standard deviation (mean ± SD). For the wall thickness and mechanical testing, the significance was evaluated using one-way analysis of variation (ANOVA, SPSS, Chicago, IL, USA). P values that were less than 0.05 were considered statistically significant.

3 Results

3.1 Fabrication of autologous TEVGs

The novel biotube inserting technique was developed to fabricate completely autologous TEVGs instead of using

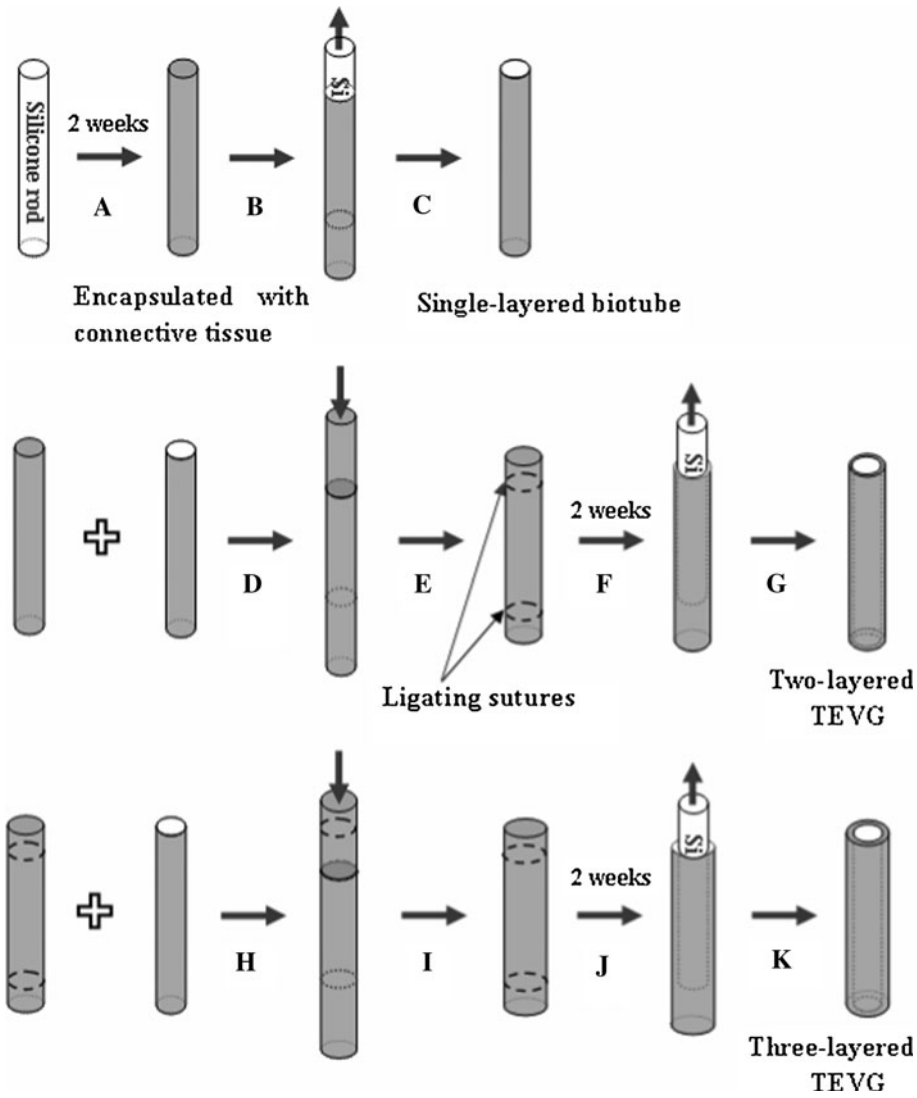

Fig. 1 Schematic diagram of the novel biotube inserting technique for fabricating autologous TEVGs. A silicone rod which was encapsulated with connective tissues was harvested after a 2-week embedding period (*A*) and then the silicone rod was removed (*B*) to obtain the single-layered biotube (*C*). Another silicone rod encapsulated with connective tissues was inserted into a single-layered biotube (*D*) to obtain a silicone rod with two layers of autologous tubular tissues ligated from the outside (*E*) and then the silicone rod was removed (*F*) to obtain the two-layered TEVG after another 2-week re-embedding period (*G*). The silicone rod with two layers of autologous tubular tissues could also be inserted into another single-layered biotube (*H*) to obtain a silicone rod with three layers of autologous tubular tissues ligated from the outside (*I*). After another 2-week re-embedding period, the silicone rod was removed (*J*) to obtain the three-layered TEVG (*K*)

the traditional tissue/sheet rolling technique (Fig. 1). Two or three silicone rods were embedded into the dorsal subcutaneous pouches of six rabbits via small skin incisions. After a 2-week embedding period, all silicone rods were completely encapsulated with connective tissues (Fig. 2a, b), which were very thin and inhomogeneous. One silicone rod encapsulated with connective tissues was inserted into a single-layered biotube (Fig. 2c) to obtain a silicone rod with two layers of autologous tubular tissues ligated from the outside (Fig. 2d). This structure could be inserted into another single-layered biotube (Fig. 2e) to obtain a silicone rod with three layers of autologous tubular tissues ligated from the outside (Fig. 2f). Then the silicone rod with two layers or three layers of autologous tubular tissues was re-embedded for another 2-week period. Subsequently, the silicone rods with two layers or three layers of autologous tubular tissues, which were covered by connective tissues, were harvested. Finally, two-layered or three-layered TEVGs were obtained after the silicone rods were removed.

As the control, simple silicone rods covered with connective tissue capsules (Fig. 2a) were harvested after a 2-week embedding period and then re-embedded for another 2 weeks. After the silicone rods were removed, single-layered TEVGs were obtained, which were relatively thin with a wall thickness of less than 200 μm (Fig. 4).

3.2 Histological properties of autologous TEVGs

All autologous TEVGs mainly consisted of collagen fibers and fibroblasts (Fig. 3a–c). The wall thickness of the two-layered TEVGs was 260 ± 59 μm, which was significantly thicker than that of the single-layered TEVGs (Fig. 4; $P < 0.05$). Furthermore, the wall thickness of the three-layered TEVGs was 415 ± 84 μm, which was significantly thicker than that of both the single-layered and two-layered TEVGs (Fig. 4; $P < 0.05$). In addition, the walls of both the two-layered and three-layered TEVGs were completely

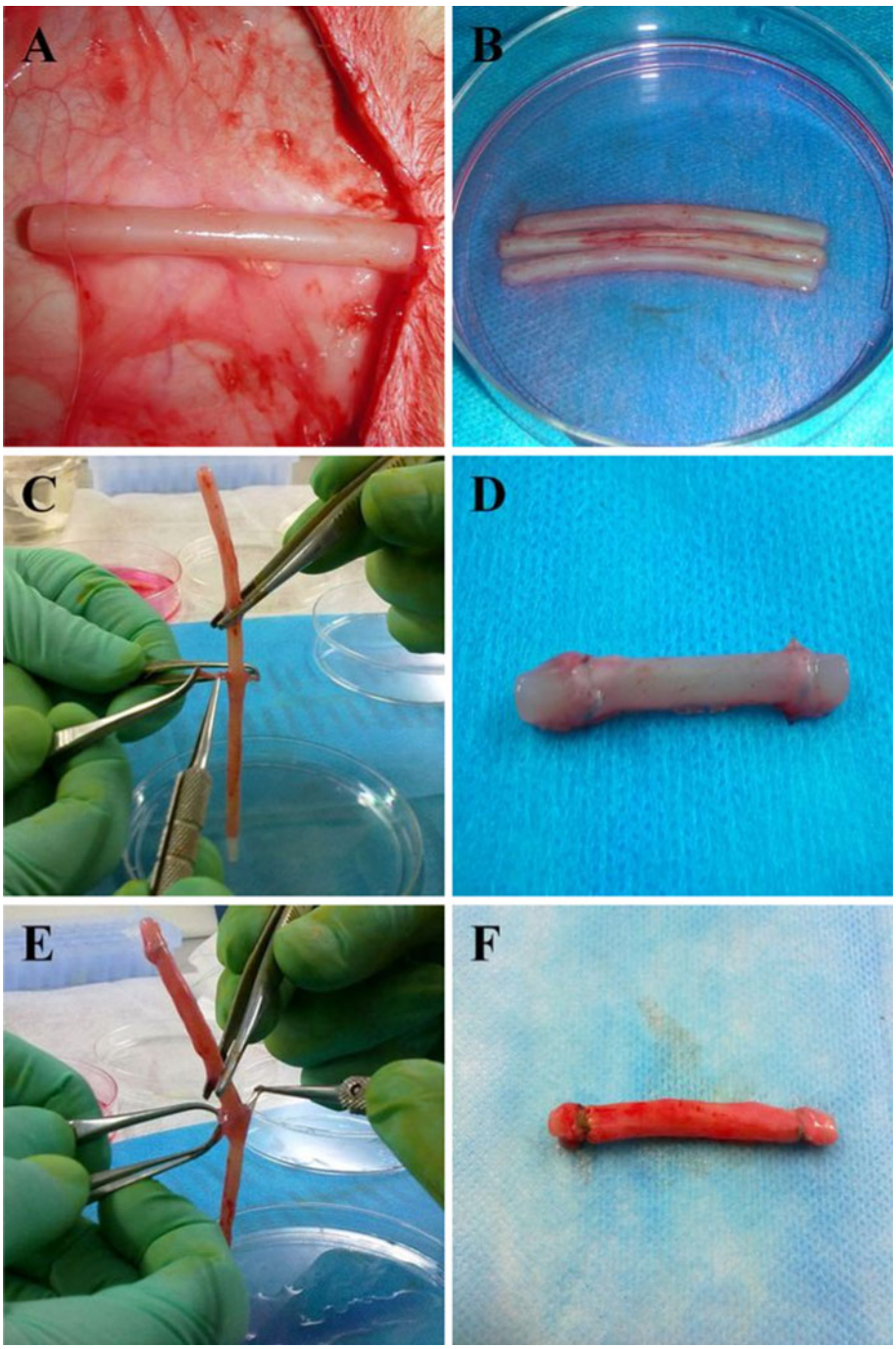

Fig. 2 Photographs illustrating the novel biotube inserting technique for fabricating autologous TEVGs. A silicone rod encapsulated with connective tissues was formed in the dorsal subcutaneous pouches after a 2-week embedding period (**a**) and then harvested into a Petri dish filled with D-Hanks solution (**b**). One silicone rod with encapsulating tissues was inserted into a single-layered biotube (**c**) to obtain a silicone rod with two layers of autologous tubular tissues ligated from the outside (**d**), which could also be inserted into another single-layered biotube (**e**) to obtain a silicone rod with three layers of autologous tubular tissues ligated from the outside (**f**)

integrated without gaps between each set of two layers (Fig. 3b, c) and rich angiogenesis occurred throughout the entire wall of the three groups (Fig. 3d).

3.3 Mechanical properties of autologous TEVGs

The burst pressure of the two-layered TEVGs was 1714 ± 367 mmHg. This was significantly higher than that of the single-layered TEVGs (Fig. 5; $P < 0.05$). Moreover, the burst pressure of the three-layered TEVGs was 2648 ± 428 mmHg, which was significantly higher than that of both the single-layered and two-layered TEVGs (Fig. 5; $P < 0.05$).

The compliance based on the intraluminal pressure and external diameter change of all the autologous TEVGs is indicated in Fig. 6. The dilatation rate of the outer diameter in the three-layered TEVGs was 3.03 ± 0.35%/100 mmHg when hydrostatic pressure increased from 80

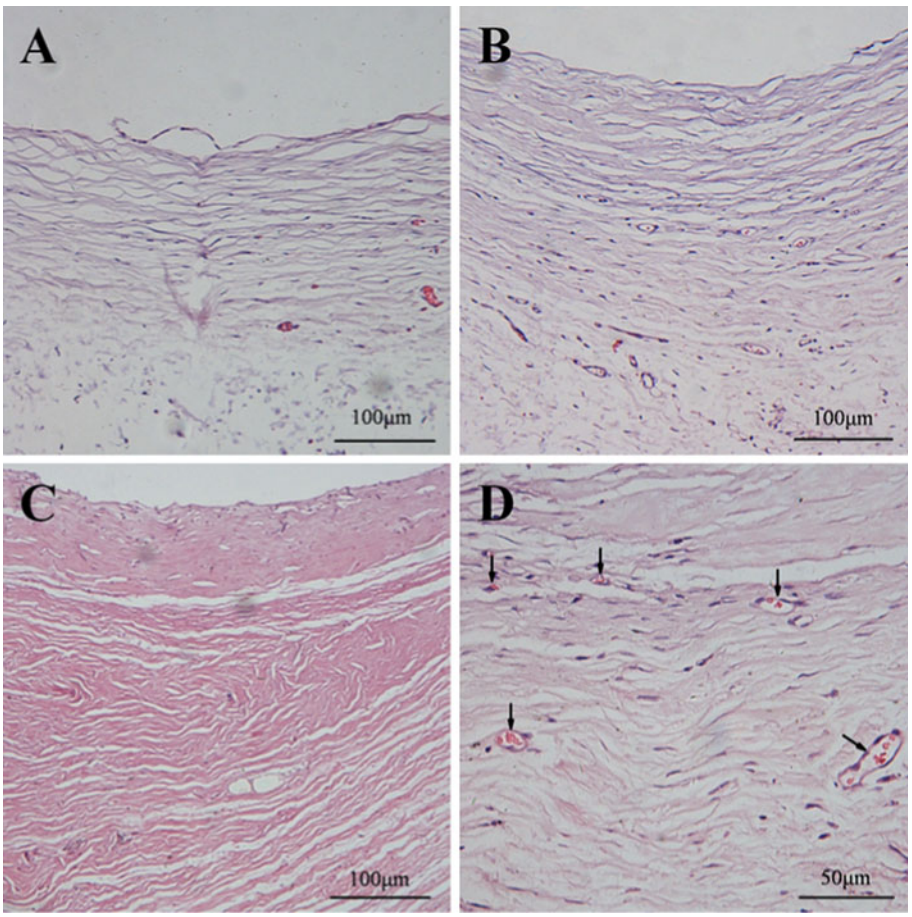

Fig. 3 Light microphotographs of the single-layered TEVG (**a**), two-layered TEVG (**b**) and three-layered TEVG (**c**) (hematoxylin and eosin staining). Rich angiogenesis (indicated by *arrows*) occurred throughout the entire wall (**d**)

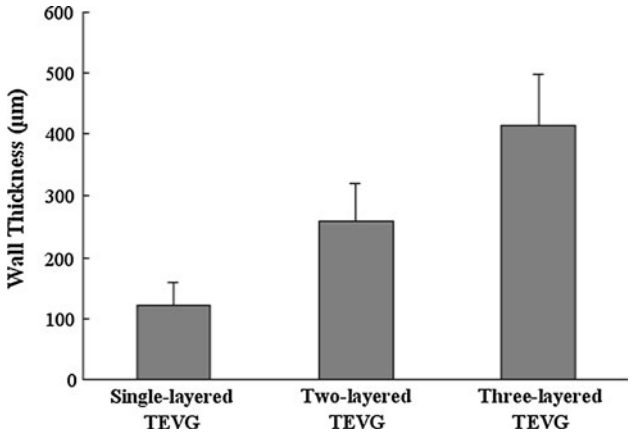

Fig. 4 The wall thickness of the single-layered TEVG, two-layered TEVG, and three-layered TEVG. The wall thickness was measured for hematoxylin- and eosin-stained cross sections under a light microscope

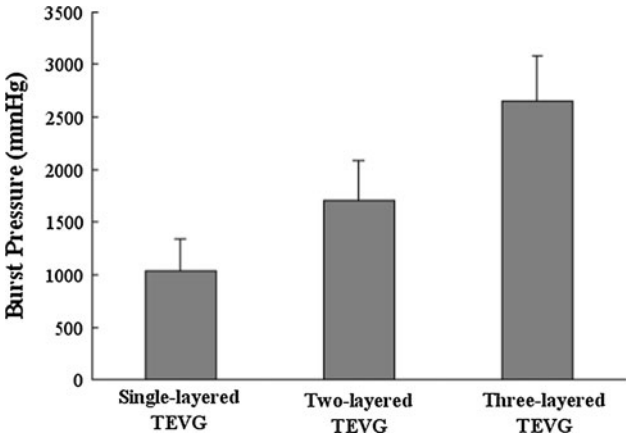

Fig. 5 The burst pressures of the single-layered TEVG, two-layered TEVG, and three-layered TEVG. Hydrostatic pressure was increased in the lumen of the autologous TEVGs until rupture at a rate of 10 mmHg/s

to 120 mmHg. This dilatation rate was significantly higher than that of both the single-layered and two-layered TEVGs (Fig. 6; $P < 0.05$). In addition, the dilatation rate of the two-layered TEVGs was 1.96 ± 0.31%/100 mmHg, which was significantly higher than that of the single-layered TEVGs (Fig. 6; $P < 0.05$).

4 Discussion

For the fabrication of autologous vascular grafts, an in vivo tissue engineering technique was developed based on the phenomenon of connective tissue encapsulation of foreign materials in vivo [12], instead of traditional in vitro tissue

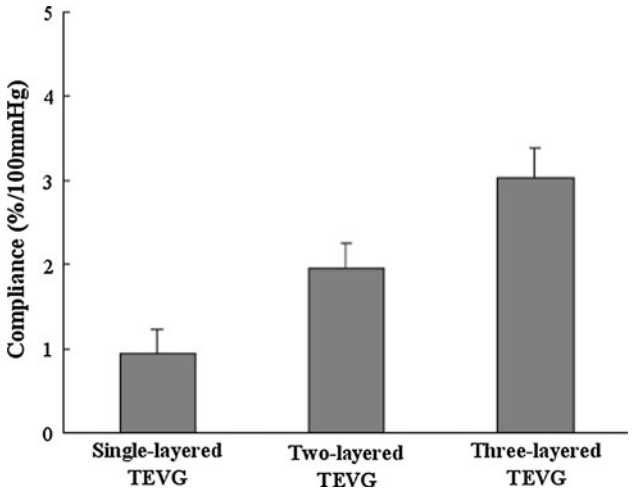

Fig. 6 Compliance of the single-layered TEVG, two-layered TEVG, and three-layered TEVG. Hydrostatic pressure was increased in the lumen of the autologous TEVGs from 80 to 120 mmHg at a rate of 5 mmHg/s

engineering technique [16–21]. An autologous tubular tissue known as a biotube was autonomically formed around the material mold in the recipient's body [11]. The biotubes consisted of autologous fibroblasts and collagen-rich ECMs without any artificial support materials [22]. Consequently, they exhibited nontoxic biocompatibility, induced no immunological rejection, and had growth potential. In addition, neither cell culture in vitro nor special laboratory facilities were necessary. Previously, a wing-attached rod mold based on the traditional tissue rolling technique was designed to accelerate the formation of a biotube [14]. During a two-step in-body tissue incubation process, a thick and robust biotube was obtained in 4 weeks. Other investigators reported that autologous TEVGs could be fabricated in vitro using the cell sheet rolling technique. However, a period of at least 10 weeks was required to fuse rolled cell sheets in vitro and this was unfavorable in terms of time and expense [10, 20, 23, 24]. Therefore, the recipient's body could be an ideal alternative bioreactor for fabrication of autologous vascular grafts [25–28].

In this study, biotube inserting technique was developed as an alternative method to the traditional tissue/sheet rolling technique for accelerating the formation of TEVGs. Biotube inserting technique has several advantages as follows: the fabrication of TEVGs was significantly time-saving due to the formation of thick and firm autologous tubular tissues over a short period of only 4 weeks. In addition, the TEVGs were found to have adequate mechanical properties and could be fabricated in a wide range of shapes and sizes for each recipient. This could be accomplished by changing the diameter, length and number of the silicone rods. Most importantly, rich angiogenesis occurred throughout the walls of the TEVGs and could supply tissue nutrition even in the thick wall. However, the current method is minimally invasive and has a potential risk of infection during the procedures of the embedding of silicone rods and re-embedding of the tubular tissues.

As expected, histological observations indicated that the two-layered and three-layered tubular connective tissues were fused to each other to fabricate thick and integrated walls. Furthermore, as Fig. 6 shows, the compliance increases with increased number of layers and increased wall thickness, which is similar to the previous study [14]. The reason of this phenomenon may be lack of smooth muscle cells in the walls of the two- and three-layered TEVGs in present study, which can lead to the difference in the structures between the TEVGs and native arteries.

The application of biotube inserting technique using biotubes with the same diameter was mainly dependent on the compliance of the biotubes. We found that the two-layered and three-layered TEVGs were easy to fabricate but four-layered or five-layered TEVGs were difficult to assemble as a result of the limited compliance of the biotubes. The four- or five-layered TEVGs require additional larger-caliber biotubes, which are obtained by increasing the number and diameter of the silicone rods.

Nonthrombogenicity is one of the most critical inherent characteristics of vascular grafts. This characteristic is obtained by endothelialization at the luminal surfaces of grafts [29]. However, during the in vivo tissue engineering process for preparation of TEVGs, endothelialization at the luminal surfaces was extremely difficult to obtain because of the direct contact with silicone rods [14]. Therefore, additional strategies such as seeding of endothelial cells [30] and coating with antithrombogenic agents [31] could be performed to promote nonthrombogenicity at the blood-contacting luminal surface of the TEVGs. Further evaluations of the TEVGs will be performed by graft implantation in vivo in the near future.

5 Conclusions

The biotube inserting technique is a novel approach for fabrication of autologous TEVGs. The two-layered TEVGs may be applied for small-caliber vascular bypass grafting and the three-layered TEVGs may be applied for medium- or large-caliber vascular bypass grafting.

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