Studies on the Effects of Chemical Treatments of In-Body-Formed Collagenous Tissue Tubes on Vascular Implantation Performance

(体内形成コラーゲン組織管の化学処理が血管移植性能に及ぼす効果に関する研究)

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Chapter 1

General Introduction

1.1 Artificial vascular prostheses

Artificial vascular prostheses are used to maintain or restore normal hemodynamics in the body by replacing diseased blood vessels or connecting blood vessels to create bypasses for stenotic areas. Furthermore, artificial vascular prostheses are used during hemodialysis to create arterial-venous shunts that act as intermediaries between bodies and machines to transfer large amounts of blood from arteries into dialysis machines, and back into bodies through veins. Currently, polyester (Dacron) and expanded polytetrafluoroethylene (ePTFE) artificial vascular prostheses are widely used in clinical treatments. Additionally, research is being conducted to develop tissue-engineered vascular grafts by combining cells, tissues, plasma components, and artificial materials such as biodegradable polymers using tissue engineering techniques. Artificial vascular prostheses are generally classified based on their caliber. Those larger than 10 mm are classified as prostheses with large calibers, and those between 6–8 mm are classified as prostheses with medium calibers, and those below 6 mm are classified as prostheses with small calibers. Artificial vascular prostheses should possess the following characteristics:

1) good biocompatibility (low or no antigenicity or toxicity), 2) durability, 3) resistance to infection, 4) non-thrombogenicity, 5) good operability, 6) excellent hemostasis, and 7) moderate cost.

Artificial vascular prostheses are often used to treat aortic diseases as they are durable, and the incidence of prosthesis failure owing to stenosis or occlusion is low in large-caliber artificial vascular prostheses [1, 2]. However, artificial vascular prostheses possess a higher risk of infection owing to the use of artificial materials.

However, the blood flow in small-calilber artificial vascular prosthesis is often affected by intimal hyperplasia and thrombus formation. Currently, there are no small-caliber artificial vascular prostheses that possess excellent functinality [3–5]. Therefore, autologous vascular blood vessels such as femoral veins are the first choice of treatment for vascular diseases involving small-caliber blood vessels.

Most patients who require small-caliber vascular prostheses are middle-aged and elderly, especially those with underlying diseases such as diabetes or end-stage renal diseases. These patients often have weak autologous blood vessels, and reoperation may be required.

1.2 Tissue-engineered vascular grafts

In recent years, the development of tissue-engineered vascular grafts (TEVGs) has received increased attention. Several fabrication technologies have been reported for TEVGs, including the use of biodegradable polymers with cultured autologous cells such as endothelial cells or bone marrow-derived cells, or the use of a 3D bioprinter for constructing of tubular structures [6–8]. TEVGs fabricated mainly from autologous tissues or cells are thought to have high biocompatibility and cause lower immune

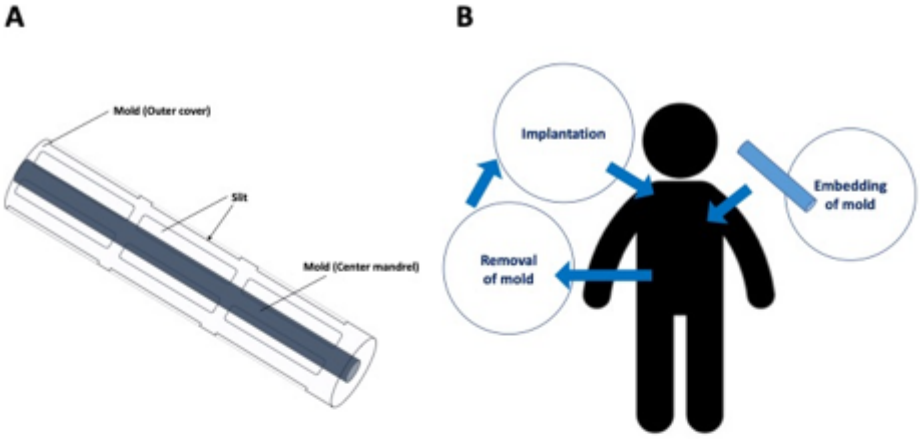
reactions. In addition, TEVGs can be reconstructed into a vascular-like structure. For engraftment in native tissues, TEVGs are thought to be durable, thereby reducing the risk of infection and deterioration, and self-organization occurs to control thrombus formation and intimal hyperplasia.

L'Heuwreux et al. [6] developed "Cytograft" based on human skin fibroblasts; this material has been used in clinical trials as a vascular access for hemodialysis. Another famous TEVG, "Humacyte", was developed by Niklason et al. [7]. Although these TEVGs show excellent properties, the cell culture step is time-consuming and the fabrication process requires special equipment and high costs for *in vitro* processes, as well as specialized technicians who are familiar with each technology, thereby limiting its mass production.

1.3 In-body tissue architecture technology

In-body tissue architecture (iBTA) is a tissue-engineering technology that differs greatly from the general TEVGs described above in that it does not require cell culture. The fundamental principle of iBTA is based on the application of encapsulation reactions to foreign materials *in vivo*. When a 3D mold made of silicone or stainless steel is implanted into subcutaneous pouches of a living body, fibroblasts under the skin enter the inner section of the mold and produce collagen fibers, filling the space of the mold and thus forming collagen-dominated tissue inside the mold (Figure 1). Collagen fibers are important in the formation of biological tissue and iBTA-induced tissues. Depending on the shape of the mold, the shape, size, and even thickness of the tissue can be controlled. Tissue with a tubular shape known as a "Biotube" can be obtained from a mold that is cylindrical or spiral [9, 10]. Regardless of the size and shape of the mold, Biotubes can

be fabricated in two months by embedding molds into subcutaneous pouches of living bodies.



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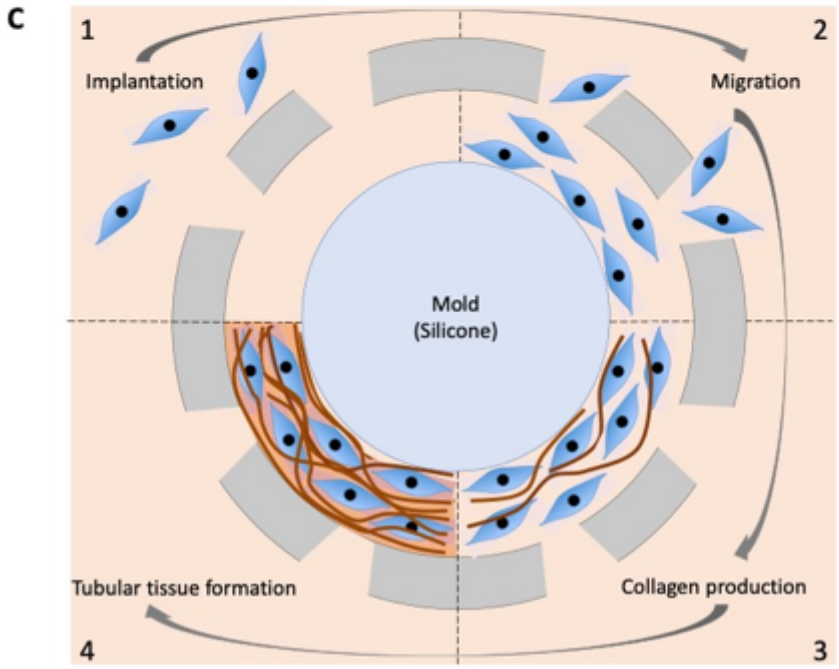

Figure 1. Schematic diagram of the mold for preparation of Biotube **(A)**. It assembled with center mandrel and outer cover with side slit. Image of the iBTA technology procedure **(B)**. During the embedding period of the mold, fibroblasts gather around the mold **(C1)**, then migrate into the space of mold **(C2)**. Inside of the mold, fibroblasts product collagen fibers **(C3)** and fill the space **(C4).**

Biotubes with calibers ranging from 0.6 to over 10 mm, and long Biotubes with length of up to 50 cm, have been developed [10, 11]. When the Biotube is removed from the mold immediately after fabrication, it can maintain its luminal structure independently without support from the mold and its pressure resistance is approximately 2000 mmHg, which is sufficient to withstand arterial pressure [9, 12]. The lumen of the Biotube is very smooth and flat, and is free of any irregularities. The tissue structure is composed almost entirely of collagen fibers, as described above, and is particularly dense on the luminal side. Few fibroblasts are present in the Biotube wall, with no cellular components on the lumen surface. Collagen fibers function as a scaffold for cells *in vivo*. In fact, when the Biotube is implanted between arteries *in vivo*, the luminal surface is covered by endothelial cells, smooth muscle cells invade the lumen side, and an elastin fiber layer is formed. Previous studies showed that within a few months of implantation, the Biotube tissue is remodeled and a vascular-like structure is established [10, 13, 14]. The Biotube self-assembles by engrafting to its own blood vessels in the living body unlike artificial materials, it is thought to have the same durability as the tissues in the living organ and exhibits growth potential in the body.

Previous studies showed that Biotubes with a small-caliber and several centimeters in length can maintain long-term patency in the medium when implanted between the arteries of small laboratory animals such as rats and rabbits [13, 14]. In this study, I used beagle dogs and goats as larger model animals and implanted these animals with Biotubes with a small-caliber and a length of more than 5 cm. I evaluated the *in vivo* patency and function of the Biotubes implanted not only between arteries, but also between arteries and veins with different vascular resistances.

1.4 Chemical treatment before implantation

Maintaining a strong structure of a vascular prosthesis will lead to improved operationality during implantation and contribute to long-term maintenance of patency after implantation.

Tissue fixation is often performed using formalin or ethanol [16, 17]. Formalin fixes issues by forming cross-links, whereas ethanol dehydrates tissues. The most commonly used method of fixing biological tissue for therapeutic purposes is glutaraldehyde-fixed autologous pericardium, as an aortic valve repair method [18, 19].

In previous studies, Biotubes intended for implantation were immersed in 70% ethanol solution to preserve the Biotubes from the fabrication stage to the implantation stage, and to ensure the shape memory of the Biotube. As described above, long-term maintenance of patency of the Biotube is possible when it is implanted between arteries *in vivo* [13, 14].

In addition to the ethanol immersion method, tissue fixation with glutaraldehyde was performed for Biotubes to determine if their shape changes after implantation.

1.5 Thesis outline

This thesis consists of four chapters. The background related to this research and the aims of the study are introduced in Chapter 1.

In Chapter 2, implantation of small-diameter and long length Biotubes more than 10 cm with a specific shape between the femoral arteries and veins of beagle dogs, and that of Biotubes 25 cm long between entire common carotid arteries of goats are described, along with the evaluation of patency and shape memory.

Chapter 3 describes the evaluation of the Biotube *in vivo* in an arterial-venous

environment with different vascular resistances. First, the Biotube for use in only ethanol immersion implantation models between the carotid arteries and jugular veins of beagle dogs was prepared, and short-term evaluation of three anastomosis methods was performed. Next, I evaluated the shape and patency of the Biotube *in vivo* during long-term observation by preparing the Biotube with two different chemical treatments: ethanol-immersion only and addition of glutaraldehyde fixation.

Finally, in Chapter 4, the research summary and conclusion are discussed.

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Chapter 2

Effect of ethanol treatment of Biotubes before implantation

2.1 Introduction

Flexibility is required for artificial vascular prostheses to follow the shape of the target implantation region. Dacron is fabricated from polyester cloth and thus is soft but has a weak shape-maintaining ability [1–3]. Artificial vascular prostheses composed of Dacron have a bellow structure that maintains their tubular structure, even in a bent state. However, uneven steps in the lumen are created by this bellow structure, which can easily lead to thrombus formation in the lumen, making it unsuitable for small-caliber artificial vascular prostheses. In contrast, ePTFE is relatively stiff but bends, easily; therefore, a plastic ring must be inserted around the ePTFE prostheses to hold and maintain the tubular structure. However, it is difficult to apply these procedures in TEVGs [4, 5]

Biotubes are fabricated from straight or spiral molds. Although they are soft and flexible immediately after fabrication, they become susceptible to kinking upon strong angulation after removal from the mold. In previous studies, short Biotubes of 3 cm or less were used [6–11], as it is thought that a short length will not kink in the living body. However, when vascular prostheses are used for treatment, they must often be at least 10 cm in length, and may need to be implanted across a joint or in a curved shape.

In this study, I performed vascular implantation into beagle dogs and goats using Biotubes with lengths of 10–25 cm. Biotubes were treated by conventional immersion in 70% ethanol solution. To prevent collagen aggregation, a mandrel with the desired shape was inserted into the lumen of the Biotube before immersion in ethanol solution.

In vascular surgery, straight vascular prostheses are mainly used for arterial bypass, whereas U-shaped vascular prostheses are often used to establish arterial-venous shunts for hemodialysis accesses. Therefore, in this study, the straight Biotubes were implanted into the carotid arteries of goats, and U-shaped and spiral shape Biotubes were

implanted between the femoral arteries and femoral veins of beagle dogs.

2.2 Methods

2.2.1 Ethical approval

In all experiments, all animals were treated humanely according to the Guide for the Care and Use of Laboratory Animals, published by the USA National Institutes of Health (NIH Publication No. 85-23, received 1996). All animal experiments were approved by the National Cerebral and Cardiovascular Center Research Institute Committee (No. 17013) and carried out at the National Cerebral and Cardiovascular Center Research Institute.

2.2.2 Biotube preparation and shape memory

The straight Biotube mold was constructed by inserting a 4-mm diameter silicone mandrel into a stainless-steel pipe (caliber 6 mm). These molds were embedded into the subcutaneous pouches of female beagle dogs (body weight ca. 10 kg). Atropine (25 μg/kg, intramuscular injection (IM)), ketamine (5 mg/kg, IM), and buprenorphine (0.02 mg/kg, intravenous injection (IV)) were used as pre-anesthetic medications. Anesthesia was induced using pentobarbital (15 mg/kg, IV) at a quarter or half of the initial doses. The molds were embedded for two months, and then harvested with the encapsulated connective tissues. The straight Biotubes with an internal caliber of 4 mm and wall thickness of approximately 1 mm were obtained by completely removing the internal mandrels.

The spiral-shaped mold was constructed with two pieces of outer shells (caliber 6 mm) and an inner spiral mandrel (external diameter 4 mm). These molds were

embedded into the subcutaneous pouches of beagle dogs or goats (body weight ca. 30 kg). In goats, xylazine (2 mg/kg IM) was used to induce anesthesia for performing intratracheal intubation, and the anesthesia effect was maintained by administering isoflurane as an inhalant. After two months of embedding, the molds were harvested with the encapsulated connective tissues. The surrounding connective tissues were completely removed, and then the two pieces of shells were separated. The mandrels covered with Biotubes were extracted. The mandrel was removed to obtain the spiral Biotubes (caliber; 4 mm, wall thickness ca. 1 mm).

The shape memory of the Biotubes was maintained by fixing them in the desired shape such as a straight shape, U-shape, or spiral shape by inserting a mandrel, and the Biotubes and mandrels were immersed in 70% ethanol solution.

2.2.3 Implantation procedure

To prepare an arteriovenous shunt, U-shaped Biotubes (length ca. 10 cm, n = 11) were allogenically implanted between the femoral arteries and veins of beagle dogs. Under the anesthetic management described above, small incisions were made in the femoral region. After five minutes of intravenous injection of heparin sodium (200 units/kg), the femoral arteries and veins were cross-clamped. Anastomosis of one edge of the Biotube and femoral vein was performed in a side (vein)-to-end (Biotube) manner by using continuous 7-0 polypropylene sutures. Anastomosis of the other edge of the Biotube and the femoral artery was performed using the same method.

Next, using spiral Biotubes (length ca. 25 cm, n = 4), an arteriovenous shunt was prepared. These spiral Biotubes were used for allogenic implantation between the carotid artery and jugular vein of beagle dogs. Under the aforementioned anesthesia management,

median incisions were made in the neck of the beagle dogs. After five minutes of intravenous injection of heparin sodium (200 units/kg), the carotid artery and jugular vein were cross-clamped. Anastomosis was performed between one edge of the spiral Biotube and the side of the jugular vein by using continuous 7-0 polypropylene sutures. The other edge of the spiral Biotube was anastomosed to the femoral artery in the same manner. After five minutes of heparin sodium (200 units/kg) intravenous injection, the carotid arteries were cross-clamped at both ends.

The straight Biotubes (length ca. 25 cm, n = 2) were used for allogenic implantation between the carotid arteries of goats. Under the aforementioned anesthesia management, small incisions were added on the proximal and distal sides of the neck. After three minutes of intravenous injection of heparin sodium (200 units/kg), both ends of the carotid arteries were cross-clamped. One end of the Biotube was anastomosed to the proximal side of the carotid artery in a side-to-end manner using 7-0 polypropylene sutures. The other side of the Biotube end was anastomosed to the distal side in the same manner.

After implantation, all animals were administered low-molecular-weight heparin (dalteparin, 1000 units/head for goat, 100 units/head for beagle dog, SC, SID) for one week and clopidogrel (50 mg/head for goat, 25 mg/head for beagle dog, PO, SID) for one month post-implantation. One month after implantation, pulsed wave Doppler observations and angiography were also carried out.

2.3 Results

2.3.1 Shape memory of Biotubes

The basic mold for Biotube fabrication had a straight cylindrical shape and was

constructed by inserting a silicone mandrel into a stainless-steel pipe with several slits (Figure 1A). The mold was designed so that there was a 1-mm gap between the mandrel and pipe. When the mold was embedded into a subcutaneous pouch of an animal for two months, the gap space was filled with collagen, resulting in the formation of tubular tissue (*i. e.,* Biotube) with a caliber of 4 mm and wall thickness of approximately 1 mm (Figure 1B). The obtained Biotubes were soft and flexible. However, when the Biotube was bent sharply, the Biotube luminal structure collapsed because of kinking (Figure 1C).

When the Biotube was immersed in 70% ethanol solution overnight with the straight mandrel in its lumen, its color changed from slightly reddish to whitish. The straight shape of the Biotube was maintained and the lumen of the Biotube remained firm even after removing the mandrel, with no signs of deformation. The surface of the lumen side was smooth and flat without wrinkles, whereas the outer surface had liner projections corresponding to the slits of the mold.

The Biotube in which a U-shape mandrel was inserted was similarly immersed in 70% ethanol solution overnight. Even after rinsing with saline solution, the Biotube maintained its U-shape (Figure 1E). Furthermore, when the mandrel shape was changed to an S-shape, the Biotube shape was maintained even after ethanol immersion (Figure 1G).

The spiral mold was constructed from an outer shell with many slits and a spiral mandrel. Similar to the straight shape mold, there was a 1-mm gap in the spiral mold between the mandrel and outer shell. When the spiral mold was embedded into a subcutaneous pouch of an animal for two months, the spiral-shaped Biotube with a caliber of 4 mm and a wall thickness of approximately 1 mm was formed around the mandrel (Figure 1F and G). When the Biotube with the spiral mandrel was immersed in

70% ethanol solution, the shape of the Biotube was fixed into a spiral shape. In contrast, when the Biotube was immersed in 70% ethanol solution after the mandrels was straightened, the Biotube stretched into a long straight shape (Figure 1H).

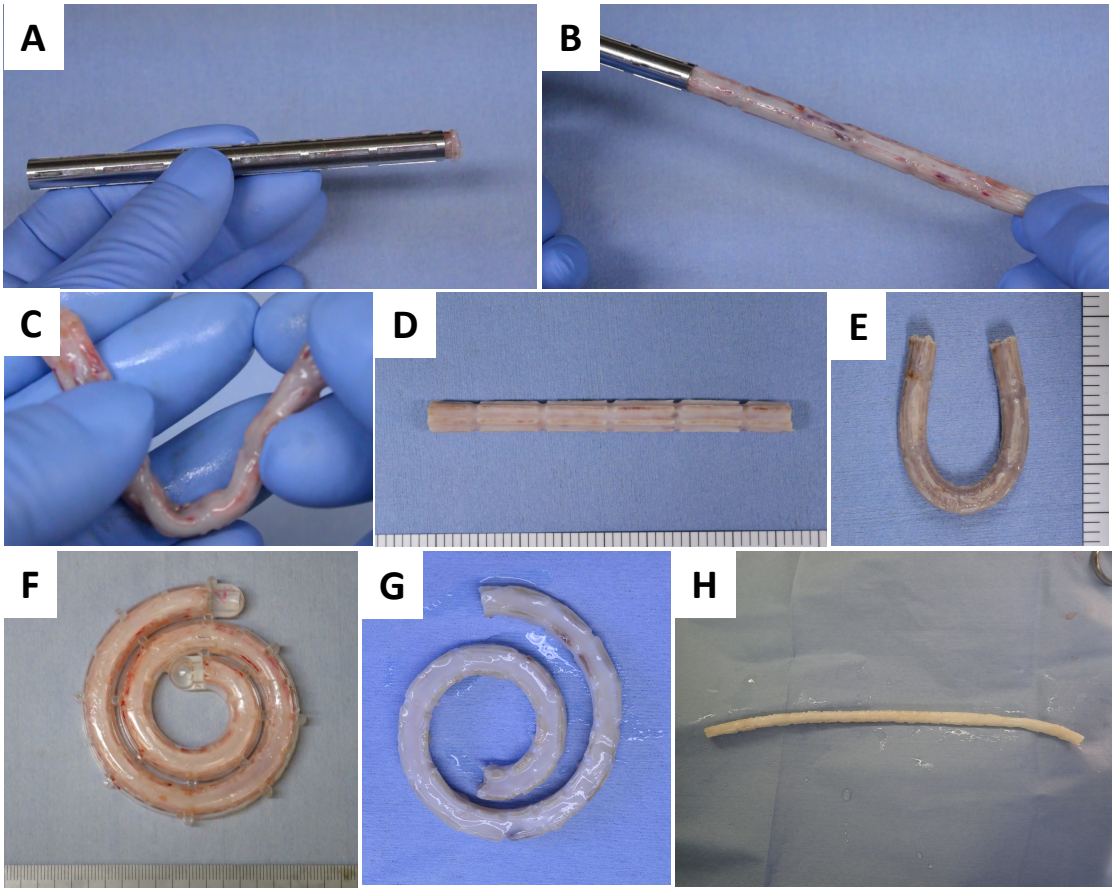

Figure 1. Photos of a straight mold for the preparation of the straight Biotube **(A)**, the Biotube formed in the mold **(B)**, and after curving and with a kink **(C)**. The straight Biotube was obtained from a straight mold after overnight immersion in 70% ethanol solution **(D)**. The U-shaped **(E)** Biotube was obtained from a straight mold after overnight immersion in 70% ethanol solution to fix the respective U-shape. Spiral mold and spiral Biotube in the mold obtained from the spiral mold **(F)**. The spiral-shaped Biotube was obtained by removing the outer shell and immersed in 70% ethanol solution with the mandrel in place **(G)**. A straight Biotube was obtained from a spiral mold after overnight immersion in 70% ethanol solution to fix its straight shape **(H).**

2.3.2 *In-vivo* evaluation of shape maintenance

Straight-shaped Biotubes were prepared from beagle dogs using a straight mold and fixed into a U-shape. Using 11 U-shaped Biotubes, arteriovenous shunts were prepared by allogenic implantation into the femoral region of beagle dogs (Figure 2A). The Biotubes were robust but showed limited elasticity, similar to a normal vascular anastomosis, and the Biotube could be easily sutured to the native vessels. When blood flow resumed, strong turbulent blood flow was palpated in the Biotube. One month after implantation, angiography was carried out (Figure 2B); in all 11 cases, no thrombus formation was confirmed on the luminal surface of the Biotubes, and complete patency was confirmed. In addition, all Biotubes maintained their curved shape without stenosis or kinks, and blood flow inside was smooth. Four spiral-shaped Biotubes from beagle dogs were fixed intact and implanted in the neck of beagle dogs as arteriovenous shunts (Figure 2C). Turbulent blood flow was observed in the spiral-shaped Biotubes for one month. Angiography one month after implantation showed that all Biotubes maintained their spiral shape with no deformation such as stenosis or aneurysm formation (Figure 2D).

The straight Biotube, approximately 25 cm in length, was prepared using a spiral mold from a goat (Figure 3A). Two Biotubes were implanted into the carotid arteries of two goats with minimal bleeding from the anastomosis sites, and no intraoperative hemostasis was required in either animal model. One month after implantation, pulsed wave Doppler observation revealed no abnormal vascular deformations such as stenosis or aneurysm in either Biotube, and smooth pulsatile blood flow was observed (Figure 3B).

On echocardiography, one case showed a blood flow velocity of 76.60 cm/s and blood volume of 151 mL/min, and the other had a blood flow velocity of 45.93 cm/s and

blood volume of 167 mL/min. Angiographic examination showed that the two Biotubes maintained their straight shape with smooth blood flow without vascular deformation or thrombus formation over the entire length.

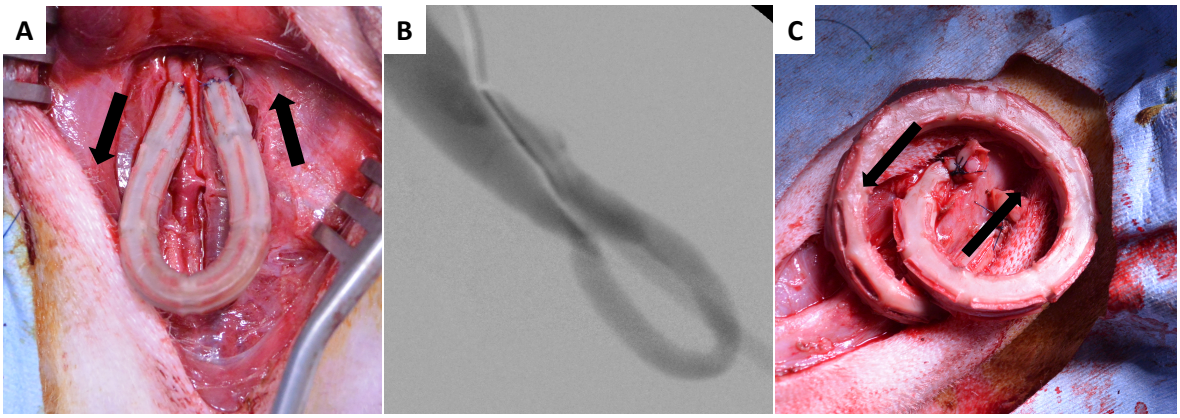

Figure 2. Photograph of the U-shaped Biotube implanted into the beagle femoral region to form an arteriovenous shunt **(A)**. An angiograph 1-month post-implantation **(B)**. Spiral Biotube implanted into the carotid region of a beagle to form an arteriovenous shunt **(C)**. Arrows indicate the direction of blood flow.

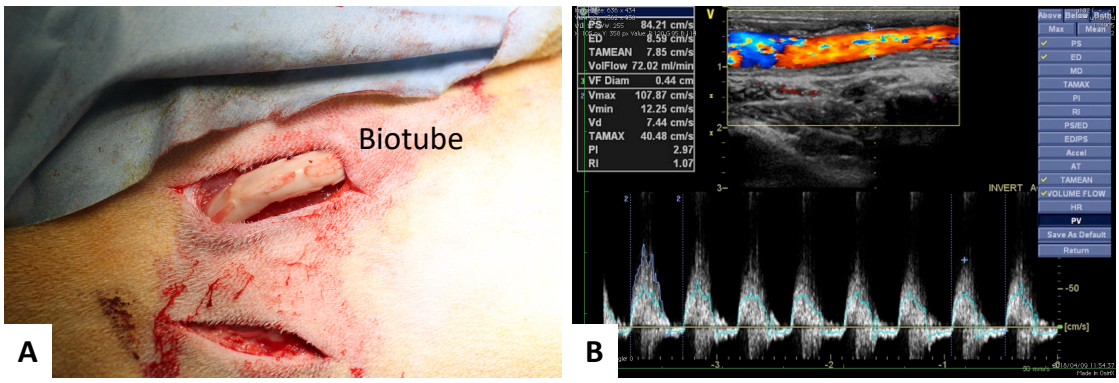

Figure 3. Photo of the straight Biotube that was implanted between the common carotid arteries of a goat **(A)**. Pulsed wave Doppler observation **(B)** 1 month following implantation of the straight Biotube.

2.4 Discussion

In vascular implantation, the shape of the vascular prosthesis must be maintained after implantation for it to fulfill its intended use. This study thus evaluated the Biotubes shape memory method.

When medical materials such as artificial vascular prostheses, are needed in a special shape, shape memory is induced using special metals, polymer materials, polyester fibers, or ceramics. Notably, in recent years, nitinol had been widely used in medical equipment. Nitinol is an elastic metal, but heat treatment renders it suitable to be used for shape memory [12, 13]. In the case of polyester fibers, shape memory is achieved using chemicals [14, 15]. It is possible to stabilize shapes by combining biomaterials with these artificial materials. However the inclusion of artificial materials may cause infection and foreign body reactions.

On the other hand, tissue fixation of biologically-derived materials is generally carried out by immersion in a fixative solution such as formalin or ethanol. There is a difference in the fixed tissue obtained depending upon the concentration and immersion time of each solution. Thus, the factors are modified depending on the intended outcome. In particular, glutaraldehyde fixation using a low concentration maintains shape memory and strength in autologous pericardial repair, which is often performed in cardiovascular surgery and used in aortic valve plasty [16, 17] and pulmonary artery reconstruction [18]. This treatment method has been used to treat many patients in recent years with favorable surgical outcomes. However, undesirable changes, such as the development of calcification, have been observed over time. In addition, tissues completely fixed with glutaraldehyde are prevented from regenerating.

This study focused on the fact that most of the components of Biotubes are collagen fibers,

and thus attempted a modified method of ethanol immersion. Biotubes can be shaped into a desired shape be simply inserting an axis that will take the desired shape and then immersing it in ethanol solution. Further, Biotubes formed from molds can be further modified into desired shapes. Thus, it is possible to apply shape memory without the need for heat treatment or intense chemical reactions.

As mentioned, Biotubes are composed mainly of collagen fibers, which serve a function as an extracellular matrix, and are interspersed with fibroblasts. In addition, a few cells that constitute blood vessels are included because angiogenesis occurs within the wall of the Biotube. However, in vascular prostheses, the extracellular matrix that makes up the blood vessel is important, whereas fibroblasts and other cells are not essential components. Therefore, even if the metabolism of these cells is deactivated on ethanol immersion, the composition of the vascular prostheses is not affected. In the ethanol solution, water molecules in the Biotube are replaced by alcohol, resulting in dehydration of the Biotube. This phenomenon is thought to cause collagen fibers to aggregate. Further the actual shape of the Biotube after immersion in ethanol solution is stronger than that before immersion, thus facilitating handling during implantation. In addition, ethanol immersion enables the preservation of Biotubes. Significantly dehydrated Biotubes can be hydrated to some extent by washing with saline before implantation to regain flexibility.

On the other hand, Biotubes, which are almost devoid of living cells and exist as aggregates of collagen matrix, show infiltration of host-derived vascular cells and reconstruction of the vessel wall after implantation. Collagen, in this way, thus serves as a scaffold in the body [9, 10]. Hence, after implantation, tissue remodeling occurs in the body. Further, reconstruction into a vascular-like structure is evident, and regeneration as part of a blood vessel can be expected.

In this study Biotubes were changed from their original shapes to the desired shape using the ethanol immersion method. Then an arteriovenous or arterial implantation model using these Biotubes were prepared. Finally, these Biotube implantation models were evaluated the performance during the acute phase of one month. It was confirmed that the Biotubes with shape memory retained their respective shapes and maintained their patency during the one-month observation period. Even in the model where the spiral-shaped Biotube was stretched in a straight shape and implanted between arteries, the Biotube was able to withstand arterial pressure and maintain blood flow without evidence of kinking in the middle. Furthermore, in the previous study, a 25-cm Biotube, which was implanted between the carotid arteries of beagle dogs, achieved patency for three months [19]. Based on these results, regardless of length, Biotubes that are implanted between arteries can be expected to provide at least a few months. or more, of patency.

This study did not note any long-term effects of ethanol immersion on the Biotube tissue. This was a pilot study aiming to describe the results of implantation for a limited observation period. In the previous study, Biotubes immersed in 70% ethanol solution were implanted into rat femoral arteries of rats and long-term observation was conducted for one year [10]. In that study, histological evaluation showed no calcification in the Biotubes after one year implantation. It has been reported that immersion in ethanol kills the cells contained in the tissue, suggesting that subsequent cellular metabolism does not occur and that calcification may be inhibited [20]. Therefore, the ethanol immersion method may yield the additional advantage of preventing calcification.

In the use of artificial vascular prostheses, long-term patency is a concern, and the prostheses being used are yet to demonstrate ideal outcome in today's clinical practice. In many cases, the patency of the prosthesis is hindered by thrombus formation and

intimal hyperplasia. The Biotubes used in this study maintained their shape for one month and did not show any geometrical abnormalities, such as stenosis or kinking. However, the study results did not lend an insight into long-term effects or any histological changes. Therefore, further studies are needed to explore these factors.

The number of implantations conducted in this study for arterial-venous implantation models was small. Further, the interpretation of the results was limited by the short observation period. Implantation of vascular prostheses between arterial-venous vessels is an essential surgical procedure for patient hemodialysis. A vascular prosthesis that can maintain constant blood flow and long-term patency is needed. The next chapter focuses on implantation performance following arterial-venous Biotube implantation.

2.5 Conclusion

By simply storing Biotubes in 70% ethanol solution, the shape of the Biotube tissue was permanently set. The Biotube with a curved shape was implanted without kinking. In addition, a spirally-prepared Biotube was implanted in a straight form and showed complete patency. To obtain tissues with a specialized desired shape, using ethanol immersion as a pre-implantation shape memory method, it is unnecessary to align the shapes of the molds.

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Chapter 3

Effects of ethanol treatment alone or additional glutaraldehyde treatment of Biotubes on implantation performance

3.1 Introduction

Vascular implantation connecting arteries and veins allows blood flow that is not naturally present in the living body; this specific vascular implantation is an important treatment for patients who require hemodialysis. Hemodialysis requires multiple dialysis procedures per week after placing the vascular prosthesis in the body, such as in the forearms. In dialysis, a needle is inserted percutaneously into a vascular prosthesis to remove blood, clean it, and then return it to the body. Therefore, it is necessary to maintain blood flow and patency for a long duration and ensure hemostasis and infection resistance against repeated punctures. However, when using artificial vascular prostheses, many factors can cause prosthesis failure, such as infection, the tendency to develop stenosis due to thrombus formation, and intimal hyperplasia, rendering long-term patency difficult [1]. Therefore, there are high expectations for the development of vascular prostheses to replace artificial vascular prostheses, especially for hemodialysis [2–4].

The purpose of this study was to evaluate whether Biotubes could be applied as vascular access for hemodialysis, and to evaluate the performance of arterial-venous implantation using animal models. First, I used ethanol-treated Biotubes to prepare animal implantation models with three types of anastomosis methods, followed by observation. However, during one month of observation, deformation of the Biotube was confirmed in one case. In addition, problems related to prosthesis deformation have been reported as adverse events after implantation in various vascular prostheses used for hemodialysis, even when the patient's own blood vessel is used as the vascular prosthesis. Therefore, a method for more effectively maintaining the tissue shape may be necessary for arterial-venous applications.

Next, I performed chemical treatment of the Biotubes using glutaraldehyde.

Glutaraldehyde treatment is often used medically to fix a tissue shape [5, 6]. Comparing the respective implantation models of ethanol- and glutaraldehyde-treated Biotubes, I evaluated the effects of these chemical treatments on the shape and patency of Biotubes during the six-month implantation period using angiographic imaging.

3.2 Methods

3.2.1 Ethical approval

In all experiments, all animals were treated humanely by according to the Guide for the *Care and Use of Laboratory Animals*, published by the United State National Institutes of Health (NIH Publication No. 85-23, received 1996). The animal experiments were approved by the National Cerebral and Cardiovascular Center Research Institute Committee (No. 17013).

3.2.2 Biotube preparation

To prepare the Biotubes, two types of molds were used in this study; one was constructed from a silicone tube as an axis (4 mm in diameter and 5 cm in length of the long axis) and a cylindrical cover made of stainless steel as an outer cover (caliber 6 mm and 5 cm in length of the long axis), and the other was constructed from a spiral shaft as an internal part (4 mm in thickness of the shaft and 15 cm in overall length of the entire spiral shaft) and a spiral shell as an outer parts (6 mm in inner diameter and 15 cm length of overall diameter of the part). These molds were inserted into the subcutaneous spaces on the back of the beagle dogs (1year old, female, ca. 10 kg). Post-surgery, all animals were subjected to IM injection of atropine (25 μg/kg), IV injection of buprenorphine sulfate (0.02 mg/kg), and IM of ketamine (5 mg/kg) for pre-anesthesia, followed by IV

injection of pentobarbital (15 mg/kg) to induce anesthesia. Anesthesia was maintained by administering 2–3% of the inhalant sevoflurane. After eight weeks, the Biotubes were harvested, and the outer cover parts were removed. Straight-shaped (caliber, 4 mm; length. 5 cm) and spiral-shaped (caliber, 4 mm; length, 15 cm) Biotubes were formed on the internal silicon tube and outer cover, respectively. All Biotubes were immersed in 70% ethanol solution and stored until implantation. The spiral Biotubes were cut to a length of approximately 7 cm, the internal surfaces were treated with 0.6% glutaraldehyde solution for 2 min, and the Biotubes were rinsed with saline solution.

3.2.3 Preparation of animal implantation models for arterial-venous implantation of Biotubes by three anastomosis methods

Biotubes obtained from straight molds were used for model preparation and treated only by ethanol immersion. Three anastomosis types of arteriovenous shunt models were prepared: type 1 (n = 4), the Biotubes and native vessels were anastomosed with lateral anastomosis (in) and end-to-end (out); type 2 (n = 4), the Biotubes and native vessels were anastomosed bilaterally with lateral anastomosis; and type 3 (n = 1), two Biotubes were directly connected to form a single tube (10 cm in length), and the inflow side was anastomosed by lateral anastomosis, and the outflow side was anastomosed by end-to-end anastomosis. After the animals were anesthetized and stabilized, the carotid artery and the jugular vein were secured through a midline neck incision, and 200 units/kg of heparin was intravenously administered; three minutes later, the jugular vein was clamped on the proximal side. Thereafter, the Biotube was anastomosed with 7-0 polypropylene sutures using the anastomosis method described above. Next, the carotid artery on the distal side was clamped and the Biotube and the artery were anastomosed in

the same manner to create the Biotube implantation between the artery and the vein as an arteriovenous shunt. Postoperative medications included subcutaneous low-molecular-weight heparin at 100 units/kg twice a day for one week only and oral clopidogrel at 25 mg/head once a day for one month.

3.2.4 Simulation of hemodialysis access

Four weeks after Biotube implantation, hemodialysis was simulated, and an 18-gauge indwelling needle was percutaneously cannulated into the Biotube to perform rapid blood withdrawal and return. This procedure was conducted every other day for a week and repeated for two weeks.

3.2.5 Preparation of Biotube implantation models with ethanol immersion only and additional glutaraldehyde treatment for long-term observation

Four models of Biotube implantation into the carotid artery and the jugular vein of beagle dogs were prepared. Biotubes were bilaterally anastomosed between Biotubes and native vessels using lateral anastomosis. Before implantation, 0.6% glutaraldehyde was added to the luminal side of the Biotubes for two minutes, following which the Biotubes were thoroughly rinsed with saline. These beagle models also received the postoperative medication with low-molecular-weight heparin and clopidogrel, as indicated in the previous section. Follow-up angiography was performed one month after implantation, and then repeatedly performed almost every month until six months after implantation or when the Biotube occlusion occurred. For comparison, a similar procedure was followed for the four Biotube implantation models with only ethanol immersion, described in the previous section.

3.2.6 Histological examination

Histological evaluation was carried out on Biotubes maintained patency for six months with immersed ethanol only and glutaraldehyde addition, respectively. 10% formalin fixation was followed by paraffin embedding to prepare tissue sections. Hematoxylin and eosin staining, Masson's trichrome staining and Elastica van Gieson staining were carried out respectively.

3.3 Results

3.3.1 Preparation of animal models by three type anastomosis methods and acute phase observation

For vascular access for hemodialysis, Biotube implantation models were created using three different anastomosis methods (Figure 1). In all models, blood flowing through the Biotubes after anastomosis was perfused without stagnation, and the thrill of continuity was detected via palpation. Controllable minimal bleeding was confirmed at the anastomosis sites. All implantation models maintained patency throughout a four-week observation period, and a palpable thrill was confirmed during this period.

Angiographic images did not indicate post-implant complications, such as stenosis, aneurysm formation, or the blooding of Biotubes, one week after implantation; however, a narrowing of the native artery occurred just before the anastomosis in type 1. Additionally, four weeks after implantation, angiographic images showed a kink in a type 1 model, and a marked deformation at the proximal side was confirmed in a type 2 Biotube model. However, ultrasonography showed turbulent blood flow through the entire length of the Biotube in all models, and adequate blood flow was maintained (Figure 2). Ultrasound images showed no evidence of luminal narrowing, such as

thrombus formation or intimal hyperplasia.

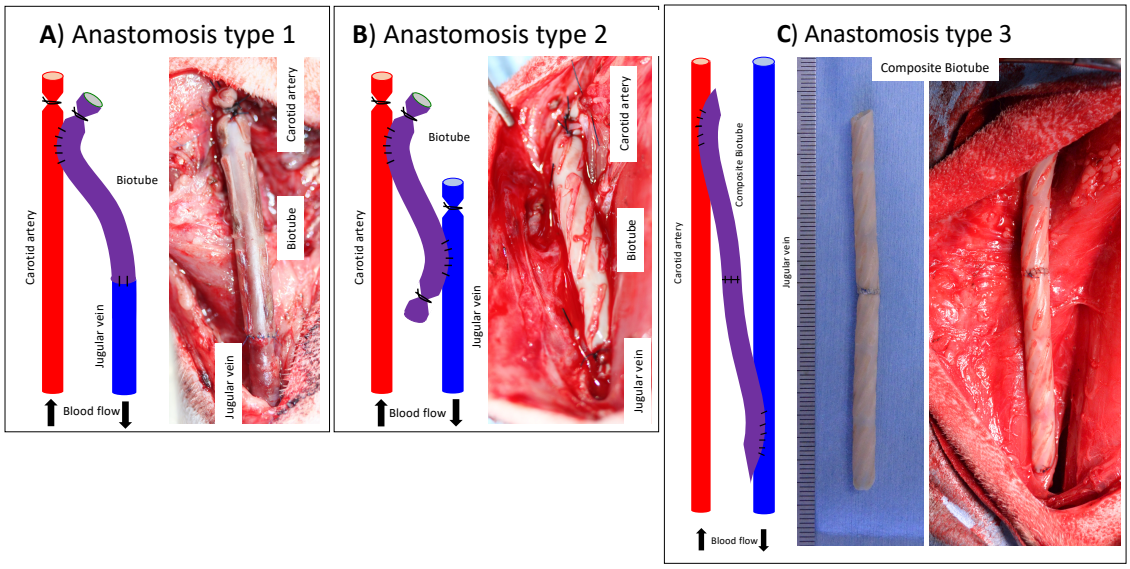

Figure 1. Three anastomosis types used in this study. Type 1: lateral anastomosis (in) - end-to-end (out) **(A)**; type 2: lateral anastomosis on both sides **(B)**; and type 3: beveled anastomosis on both sides **(C)** using a composite Biotube.

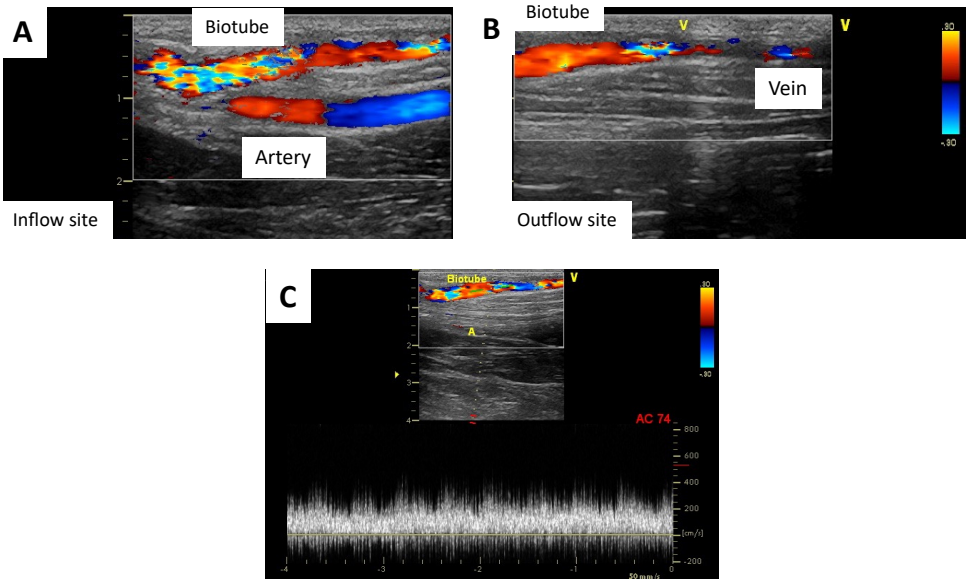

Figure 2. Ultrasound image of the Biotube implanted between the carotid artery and jugular vein **(A, B)**. Turbulent blood flow and adequate blood volume through the Biotube were confirmed **(C)**.

3.3.2 Simulation of hemodialysis access

Simulation of hemodialysis was carried out four weeks after implantation. An 18-gauge indwelling needle was canulated into the implanted Biotube percutaneously. Blood withdrawal could be carried out smoothly (Figure 3A), and then blood could be returned through the same needle into the Biotube with ease. This simulation was repeatedly carried out every 2 days for 2 weeks. During this period, there was no observation of aneurysm formation, symptom of infection and subcutaneous hemorrhage (Figure 3B).

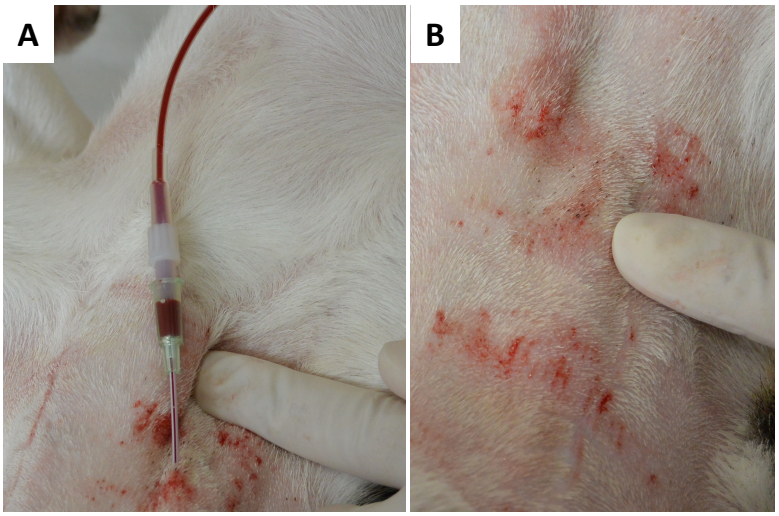

Figure 3. Simulation of hemodialysis access. It was capable of cannulation using an 18-gauge indwelling needle into the implanted Biotube subcutaneously. Smooth blood withdrawal and return from the Biotube via the indwelling needle were possible **(A)**. Bleeding could be stopped completely at the site of cannulation by manual compression within five minutes after removing an indwelling needle **(B)**.

3.3.3 Implantation of Biotubes with two different pre-implantation treatments

During the surgeries, regardless of whether glutaraldehyde treatment was used, the Biotubes were not influenced by anastomosis handling and bleeding from the

anastomosis site immediately after implantation. Immediately after surgery, no animal model showed stenosis, dilation, or uncontrollable hemorrhage, and good continuous turbulent flow was detected via palpation.

3.3.4 Evaluation of Biotubes for long-term implantation

Follow-up angiogram was performed in all cases after implantation almost every month from one to six months or until occlusion of the Biotube was confirmed. Figure 4 shows the patency period and the changes in each model. Models Nos. 1–4 were models of implanted ethanol-immersion-only Biotubes, and models Nos. 5–8 were models of implantations of glutaraldehyde-treated Biotubes.

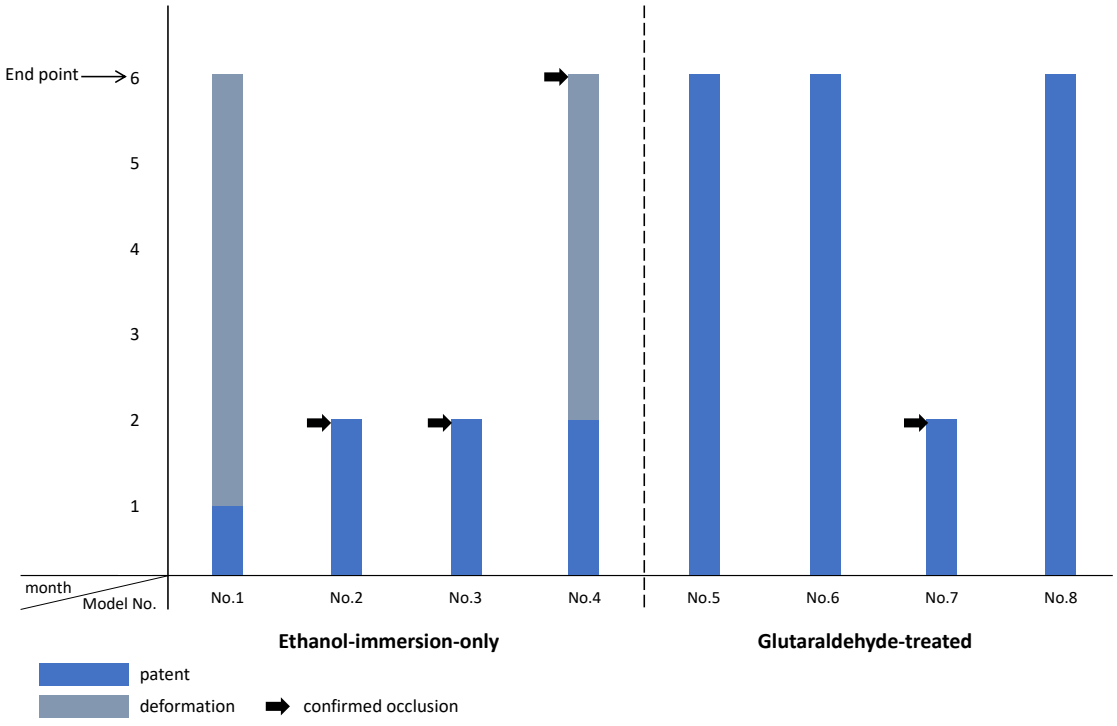

Figure 4. Time-series graph of all implantation models showing the Biotube patency (as seen in angiography) and the point at which deformation occurred.

Model No. 1 maintained patency for six months. However, the angiogram after two months revealed bending at the midgraft of the Biotube and stenosis. The length of the Biotube shrank by approximately 60% in the interim six months. In models Nos. 2 and 3, occlusion was confirmed at two months after implantation because of thrombus formations and clear stenosis. In model No. 4, stenosis was confirmed after one month; however, patency was maintained for five months, followed by subsequent occlusion (Figure 5A). In models Nos. 5–8, established using glutaraldehyde-treated Biotubes, only one case (No. 7) showed occlusion one month after implantation, whereas the other models maintained patency without stenosis or thrombus formation, and the angiographic images did not suggest a decrease in length (Figure 5B). Table 1 summarizes the results of each Biotubes obtained from the angiographic images.

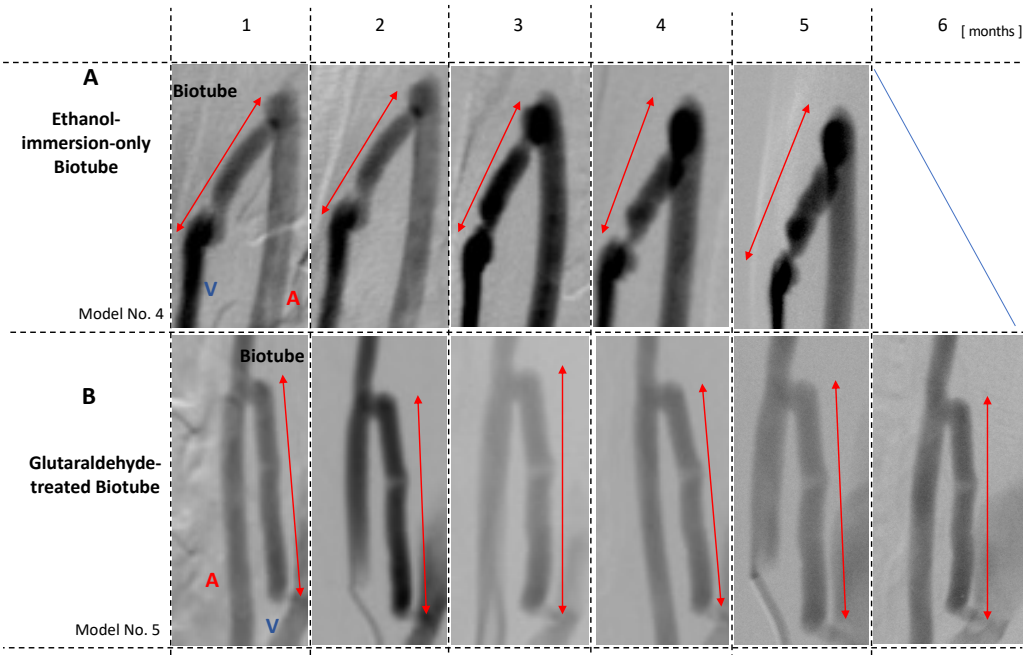

Figure 5. Time series of angiograms in two Biotube implantation models. In his model, stenosis was confirmed in the outflow site at one month after implantation **(A)**. In model No. 5, in which the glutaraldehyde-treated Biotube was implanted **(B)**, there was almost no change in Biotube shape and length in all observation periods.

Table 1. Patency rates and events during the observation period in all implantation models.

Treatment	Model No.	Deformation	Patency
Ethanol only	1	Stenosis and shrank	More than 6 months
	2	-	2 months
	3	-	2 months
	4	Stenosis	6 months
Glutaraldehyde treatment	5	No	More than 6 months
	6	No	More than 6 months
	7	No	2 months
	8	No	More than 6 months

3.3.5 Macroscopic and histological observation

The appearance of the Biotubes following the two treatments was different. The ethanol immersion-only Biotubes shrank relative to their shape before implantation and felt hard on palpation, the lumen surface was rough, and thrombi adhered in its lumen (Figure 6A and B). In contrast, glutaraldehyde-treated Biotubes exhibited no change in shape, the view of the lumen was flat, and there was no thrombus formation (Figure 6C and D).

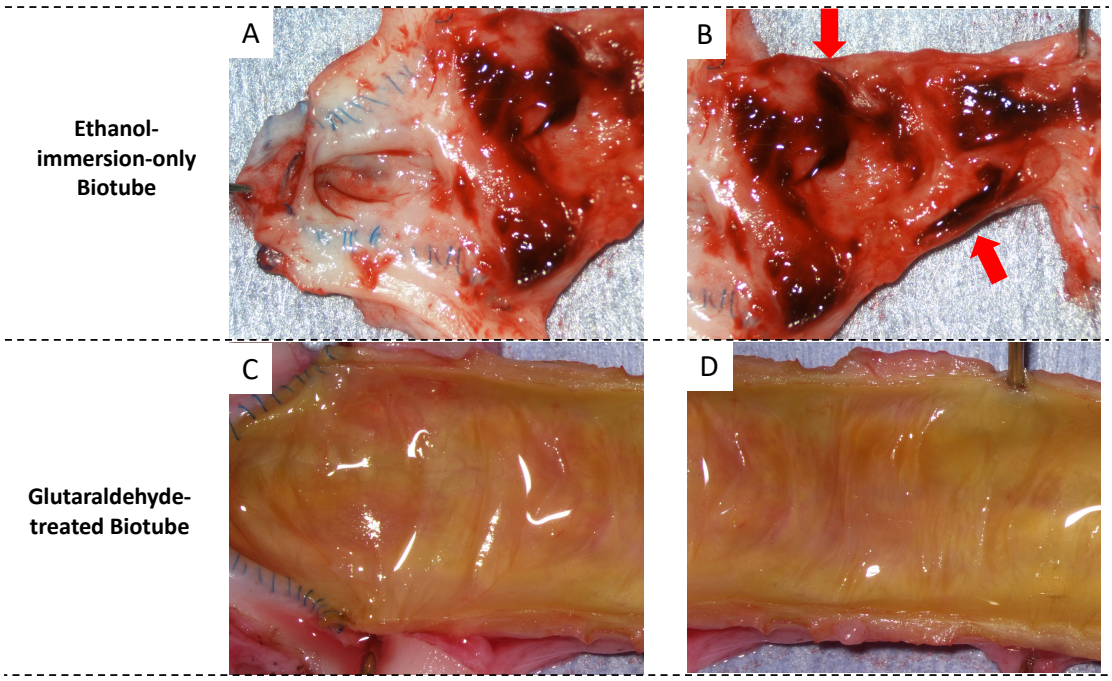

Figure 6. Photos of the Biotubes that were harvested after a six-month observation period. In ethanol-immersion-only Biotubes, the lumen was extremely uneven, and there were numerous adherent thrombi **(A and B)**. There was no change in the shape of glutaraldehyde-treated Biotubes, there was no thrombus formation, and its lumen was very flat **(C and D).**

The Biotube specimen obtained after ethanol immersion only exhibited intimal hyperplasia and thrombus formation at the lumen side, and many cells migrated to the tube walls (Figure 7A). The primary component of the Biotube component was mainly collagen fibers (Figure 7C), and no elastic fibers were observed (Figure 7D). No intimal hyperplasia was observed in the glutaraldehyde-treated Biotubes, there were few cells, and no elastic fibers were detected (Figure 7E, G, H). The outer sides of both Biotubes were surrounded by native connective tissues, and the presence of capillary vessels was confirmed (Figure 7B and F).

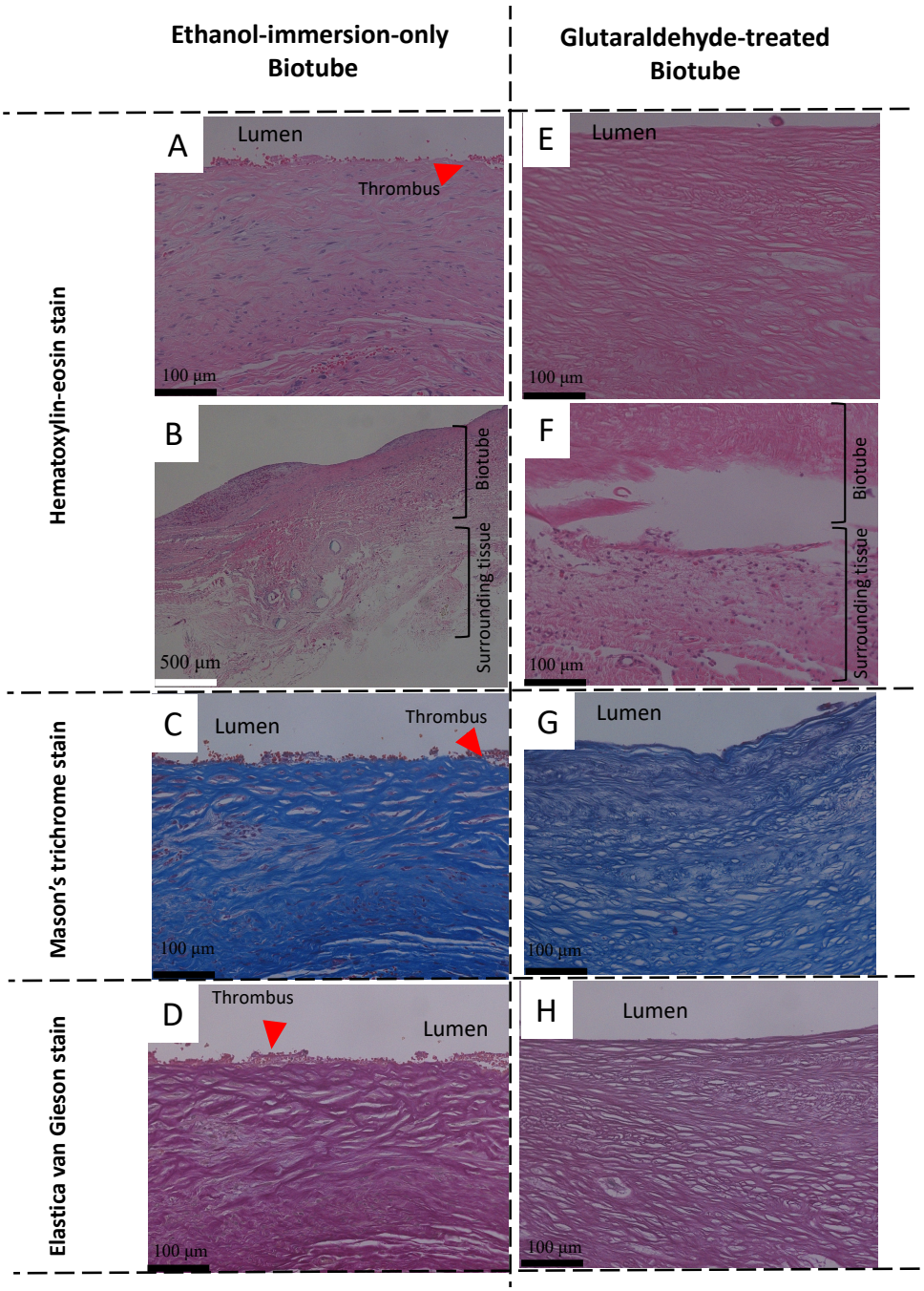

Figure 7. Images of the histological analysis six months after implantation. **(A, C, D, arrow heads)** Adhesion of thrombi was confirmed on the luminal surface of ethanol-immersion-only Biotubes. **(E, G, H)** In the glutaraldehyde-treated Biotube, the luminal side was devoid of cells and elastin fibers, indicating that the structure was still dominated by collagen fibers. **(B, F)** Both Biotubes were surrounded by connective tissues, and angiogenesis was confirmed in that region.

3.4 Discussion

In this chapter, the animal implantation models of Biotubes for application to the vascular access in hemodialysis were prepared, and their shape and patency were evaluated *in vivo* after implantation. First, implantation models were created using three different anastomosis methods with ethanol-immersion-only Biotubes, and short-term observations of the models were conducted.

After four weeks of implantation, to simulate hemodialysis, cannulation into the implanted Biotube using an indwelling needle was carried out repeatedly. The procedure of blood withdrawal and return could be carried out smoothly. In addition, after removing the indwelling needle, hemostasis could be completed within five minutes by astriction to provide the cannulation site. Although there was a limitation in this simulation owing to the shorter and less frequent preliminary test, this result shows that the Biotube was fully capable of responding to hemostasis after cannulation. Because the Biotube is composed of numerous collagen fibers that are randomly arranged. Therefore, it is thought that these collagen fibers will serve to seal the holes in the wall of the Biotube when the needle holes are made during cannulation.

In the above-mentioned acute phase assessments, some cases of Biotube deformation were observed, which may have been caused by external effects, such as active movement of the animals. However, shape deformation must be inhibited to maintain patency. I prepared the Biotube implantation model by performing tissue fixation using glutaraldehyde to enhance shape deformation and conducted long-term *in vivo* observation of this model and the ethanol immersion-only Biotube implantation model.

In the models implanted with the Biotube subjected to ethanol immersion only,

75% of the implanted Biotubes showed deformation with stenosis. For glutaraldehyde-treated Biotubes, although sudden occlusion was observed in one case, there was no deformation during the observation period, and patency was maintained. This suggests that cross-linking with glutaraldehyde is useful for improving patency in arterial-venous Biotube grafting. With the Biotube subjected to ethanol immersion only, collagen fibers constituting the Biotube aggregated because of dehydration and maintained the Biotube shape; thus, aggregation of each collagen fiber is expected to be alleviated upon hydration in the living body. Arteriovenous shunts cause non-physiological blood flow, increasing the blood flow rate and velocity in the veins and changing the blood flow pattern from laminar to turbulent [5–8]. Thus, the effects of abnormal hemodynamics, such as multidirectional wall shear stress and tissue vibration, may cause the Biotube to bend in the region exposed to the strongest blood jet flow.

The histological results differ from those obtained in previous studies. Previously, when Biotubes were implanted between native arteries or other iBTA-induced tissue bodies as prosthetic materials into various regions in living bodies, tissue remodeling of the Biotubes was observed with the organization structures similar to those of regional tissues [9–11]. However, tissue remodeling was not observed for any Biotube implanted in this study, regardless of the pre-implantation treatment. Biotubes subjected to ethanol immersion showed significant atrophy, and the luminal side was very rough with the adhesion of thrombi. The migration of endothelial cells and other vascular component cells to the Biotube may have been inhibited in tissues with adherent thrombi. Glutaraldehyde-treated Biotubes showed no deformation or adhesion of thrombi; however, there were a few cells on the lumen side, and the tissue component maintained almost the same structure as that observed pre-implantation (Figure 7G and H), possibly

because of cross-linking of collagen fibers that prevented cell migration. Liu et al. established an arteriovenous fistula model in which only the adventitia of rabbit jugular vein grafts was cross-linked using glutaraldehyde [12]. They reported that glutaraldehyde fixation of the adventitia inhibited intimal hyperplasia and dilation. Thus, differences in patency and luminal histological changes in the Biotube, which are caused by altering the fixation site, should be studied.

This study demonstrated that shape fixation with glutaraldehyde inhibited deformation. However, it remains unclear whether glutaraldehyde has harmful effects on the issue or if it can maintain its patency over a prolonged period. If deformity is caused largely by non-physiological hemodynamics generated by the arteriovenous shunt, it may be useful to reduce excessive hemodynamic changes by varying the vascular prostheses diameter, as reported by Fillinger et al [13, 14]. Given that Biotubes can be fabricated in any desired shape depending on the mold design, this method can be further developed for clinical application and exploited for its stable shape and patency over a longer period *in vivo*.

3.5 Conclusion

Biotubes treated with glutaraldehyde for strong tissue fixation did not show any deformation when implanted between the arteries and veins and maintained their patency for at least six months post-implantation. Further studies are needed to evaluate the patency on a yearly basis and determine whether the addition of glutaraldehyde has disadvantages. The glutaraldehyde concentration and soaking time during fixation must also be further optimized. However, these findings can be used as a guide for the implantation of Biotubes between arteries and veins.

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Chapter 4

Conclusion

This thesis describes the development of implantable tissue bodies and their functional evaluation in vivo using animal implantation models based on tissue engineering technology. The research work was conducted at the Division of Artificial Organs, or the Division of Regenerative Medicine and Tissue Engineering of National Cerebral and Cardiovascular Center Research Institute.

Chapter 2

In this study, I used a conventional method of treatment with 70% ethanol immersion for Biotubes before implantation. However, unlike the previous method, before ethanol treatment, straight shaped, U-shaped, and spiral-shaped Biotubes prepared by inserting mandrels retained their respective shapes without kinking even after removing the mandrels. When a vascular graft is placed at a joint region or near a strong curvature, if the shape of a vascular prostheses is easily deformable, even a slight bending of the lumen at the strongest point of the curvature is expected to narrow the lumen and adversely affect hemodynamics. The Biotube can be changed from one shape to another shape by performing ethanol treatment.

Chapter 3

In this study, I prepared animal models of arterial-venous implantation using ethanol-treated Biotubes and additional glutaraldehyde-treated Biotubes, and then evaluated respective shape deformation and the patency rate.

The results showed that the Biotube treated with ethanol alone was deformed at more than one month after implantation. There was a high probability of Biotube deformation within two months of implantation, and half of the models subsequently showed to occlusion. However, Biotubes subjected to additional glutaraldehyde treatment did not deform and maintained their patency for the 6-month observation period.

From these results, at least for arterial-venous Biotube implantation, it is difficult to maintain shape over the long term by performing ethanol treatment alone, leading to reduce patency. In contrast, glutaraldehyde treatment can fix the shape of the Biotube for implantation between the arteries and veins.

Summary

While the need for small-diameter vascular prostheses had increased in recent years in the medical field, existing treatments mainly using artificial vascular prostheses have not achieved satisfactory results, particularly over the long-term. Although many excellent medical materials can be applied in tissue engineering technology, iBTA technology is also highly useful because it can be used to produce safe vascular prostheses of requires sizes and shape at a relatively low cost. Vascular prostheses must maintain blood flow in the living body for a long period and not cause blood flow problems.

In this study, I evaluated the effects of chemical treatments on the shape and patency of Biotubes. Ethanol treatment is a conventional treatment used in Biotubes

studies; however, this study suggested that ethanol treatment can change the shape of Biotube from original shape to a different desired shape.

In animal implantation, ethanol-treatment-only Biotubes showed the potential to maintain patency when implanted between the arteries, regardless of their length. However, when Biotubes were implanted between the arteries and veins, ethanol treatment alone led to deformation of the Biotube within a short period. Therefore, when Biotubes are implanted between arteries and veins, the desired shape can be formed by ethanol treatment, and further glutaraldehyde treatment can be conducted before implantation to may maintain the shape and long-term patency.

Chemical treatments of Biotubes before implantation improved implantation performance when used appropriately for the intended purpose.

Future prospective

In patients who require vascular prostheses, the vascular prosthesis must be available for immediately treatment in an emergency situation or must be resistant to occlusion due to thrombosis formation or intimal hyperplasia and provide stable patency for many years. It is necessary to overcome these conditions to provide options for actual clinical use and to ensure that they are as safe as the artificial vascular prostheses currently used for clinical treatment.

As observed for many other TEVGs, the preparation time of Biotubes is at least 1–2 months, and thus they cannot be used in emergency situations. Development of strategies to prepare Biotubes in a shorter time would help improve their suitability for clinical use.

In the animal implantation models used for vascular access in hemodialysis in

this study, performing the two chemical treatments maintained patency for at least six months. However, no tissue-remodeling in response to implantation of a vascular-like structure was observed. Remodeling of a vascular-like structure, such as endothelialization and elastin layer formation on the luminal side of Biotubes, is a more desirable function for vascular prostheses because it confers antithrombotic properties and enhances bleeding control against repeated needle punctures during hemodialysis. Although collagen cross-linking associated with glutaraldehyde treatment improved patency, histologically it may have made the Biotubes susceptible to tissue remodeling. In recent years, attention has been focused on development of novel chemical treatment methods involving biomaterials to overcome the problems of glutaraldehyde toxicity in the body and calcification after implantation. Further studies aiming at combining this novel processing method with Biotubes are needed to achieve tissue-remodeling regardless of the implantation environment and to improve the performance as ideal vascular prostheses.

List of Publications

The contents of this thesis are composed of the following papers.

Chapter 2

Shape memory of in-body tissue-engineered Biotube ® vascular grafts and the preliminary evaluation in animal implantation experiments.

Nakayama Y, Furukoshi M, Tatsumi E.

J Cardiovasc Surg. 2020; 61(2): 208–213.

Chapter 3

Application of in-body tissue architecture-induced Biotube vascular grafts for vascular access: Proof of concept in a beagle dog model.

Furukoshi M, Tatsumi E, Nakayama Y.

J Vasc Access. 2020 ; 21(3) : 314–321.

Other Related Papers

1. Development of long in vivo tissue-engineered “Biotube” vascular grafts.
   Nakayama Y, Furukoshi M, Terazawa T, Iwai R.
   Biomaterials. 2018 ; 185 : 232–239.

2. Development of an in vivo tissue-engineered vascular graft with designed wall thickness (biotube type C) based on a novel caged mold.
   Furukoshi M, Moriwaki T, Nakayama Y.
   J Artif Organs. 2016; 19(1): 54–61.

3. Nakayama Y, Furukoshi M. Feasibility of in-body tissue architecture in pediatric cardiovascular surgery: development of regenerative autologous tissues with growth potential. *J Pediatr Cardiovasc Surg*. 2018; 2: 28–36.

4. Long-term observation of the patency and shape of in-body tissue architecture (iBTA)-induced Biotube vascular grafts with different processing methods in animal vascular access models.
   Furukoshi M, Nakayama Y.
   J Vasc Access. 2021 (Under article submission)

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