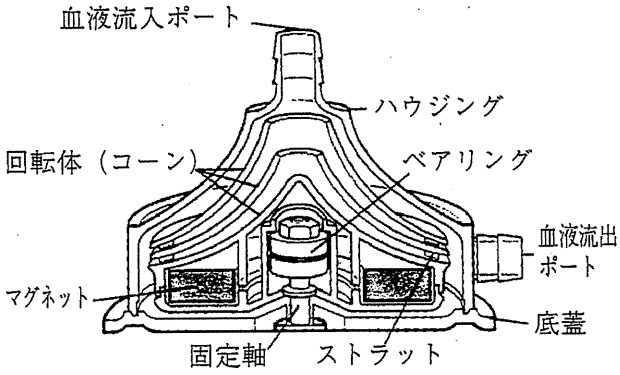

図3．バイオポンプ断面図（軸シール表示目的）

シールと呼ばれる部品を必要とする（図3では回転する部分と静止部分の境界に設置されるゴム製の部品であり回転部との境界で摩擦熱を生じる）。ポンプをディスポーザブルとするためにポンプ内部に磁石を配置し分離可能とした場合でも，磁石部分に血液が入り込まないようにするために同様の部品が必要となる。この部分では，シールと静止壁との間に摩擦熱が発生するため，血液成分が熱変性し凝固する。また，軸中心付近では血流が鬱滞しやすいため，血栓の形成・付着につながる。よって，この軸シール部を持たない回転方式の実現が長期間使用する左心補助人工心臓としてのターボ型ポンプにとっての最重要課題であり，現在実用化されている人工心臓は以下に述べる様々な方法によってこの問題を解決している。

1．機械的接触を残した方式

回転部分の両端（中心部）に，ちょうどコマの先端のようなピボットと呼ばれる部分を設け，静止部品に押しつけられながら回転する方式。機械的接触が存在することは長期耐久性の面からはデメリットとなり得る。

2．完全非接触方式

1）動圧軸受

高速で回転している羽根車とケーシング壁の隙間において，隙間が狭い部分には高い圧力が発生することを利用して回転体を非接触で支持する方式。

2）磁気軸受

回転する羽根車の位置を検出するセンサーと電磁石を組み合わせ，羽根車の位置を能動的に制御することによって羽根車が完全に非接触で浮上回転する方式。

Ⅱ．ターボ型血液ポンプに関する将来的課題

1．ターボ型ポンプ応用治療戦略の拡大

補助人工心臓を用いた治療法はデバイスの充実と共に急速に普及している。しかしながら，急性心筋梗塞の患者の場合には多臓器不全の併発等により，治療効果が期待できない場合も多く含まれており，長期間使用する高額な人工心臓を適用するかどうか決定するまでの短期使用デバイス，すなわち，患者の循環を維持し血行動態の安定を図りつつその後の治療方針を決定するためのbridge-to-decisionデバイスの重要性が高まっている。この目的のためにも，非接触駆動可能な体外設置用ターボ型ポンプを開発することが急務である。また，呼吸器疾患を併発している患者の救命に使用するための，数週間連続駆動可能な呼吸循環補助装置についても現在長期間連続使用可能なデバイスが存在しないことから，その実用化が急務である。

2．小児患者あるいは小柄患者に使用できる小型デバイスの開発

内科的治療では救命不可能な不可逆性重症心不全患者に対しては心臓移植が選択肢となるが，我が国では深刻なドナー不足で，特に渡航移植患者の60％以上を18歳未満が占め

る現状であり，小児心疾患患者のためにも小型補助人工心臓の開発が必要である。血液ポンプの流量が成人に比べ著しく低いため，成人用ポンプの流用は抗血栓性などから困難な状況である。さらに，乳幼児を含む小児用人工心臓はサイズの小ささに加えて成長の問題など，成人にはない多くの問題があり開発が難しいことに加えて対象となる患者数は多くないため，製品化という経済的観点からも実現が困難であると考えられている。米国では，National Heart, Lung and Blood Institute (NHLBI) が乳幼児のための循環補助デバイス開発プログラムを2004年以来継続して実施している。現在は，Pumps for Kids, Infants and Neonates (PumpKIN) と呼ばれる開発プログラムが実施中であり，超小型体内埋込VAD，超低充填量ECMO (extracorporeal membrane oxygenation) など，4種類のデバイスが開発されている。

Ⅲ．動圧軸受技術を応用した血液ポンプ開発

上記の課題を解決するために，現在著者らのグループが新エネルギー産業技術機構 (NEDO) によるプロジェクトとして取り組んでいる研究開発の内容について示す。

① 「基礎研究から臨床研究への橋渡し促進技術開発/橋渡し促進技術開発/次世代高機能血液ポンプシステムの研究開発」では，羽根車を非接触で支持しながら回転させる方式の一つである動圧軸受を応用し，幅広い心疾患の治療体系構築のため超急性期から慢性期をカバーすることができる2つの異なる連続流血液ポンプシステムの開発を行っている。これは当センターを中核拠点都市，日本中の多くの病院，研究機関，企業が参画する「先端医療開発特区（スーパー特区）」採択課題である「先端的循環器系治療機器の開発と臨床応用，製品化に関する横断的・統合的研究」の実施施設に対する事業として実施されている。

② 「次世代機能代替治療技術の研究開発/次世代心機能代替治療技術の研究開発」では，深刻なドナー不足により多くが渡航移植となっている小児心疾患患者，あるいは成人用の小型補助人工心臓の適用対象外となる体重35kg程度以下の患者に適用できる植込み型補助人工心臓の実現を目指し，小型補助人工心臓を小柄患者用まで適用領域拡大を行うための技術開発を行う。

1．1カ月の連続使用が可能な呼吸循環補助システム

人工肺と連続流血液ポンプを用いたPCPS (percutaneous cardiopulmonary support) あるいはECMOは，急性期の重症心不全や重症呼吸不全症例の一次救命を中心に有用性が高まりつつある。PCPS研究会のPCPSレジストリでは年間700～800例，膜型肺研究会のECMOレジストリでは年間300～400例の症例が報告されており，我が国におけるPCPS/ECMO症例の総数は優に1,000例を超える。しかしながら，従来のシステムは抗血栓性に乏しく使用期間も数日程度に限られており，臨床成績も良好とは言い難かった。PCPSシステム構成要素のうち人工肺については，①連続使用を行った場合，装置からの血漿リークが2～3日で発生する，②抗血栓性に乏しく抗凝固薬のヘパリン全身投与が必須で出血合併症を惹起する，という2つの大きな問題が存在した。これに対して当センターでは，20年以上の継続的研究の成果として，1カ月以上の連続使用が可能でヘパリン投与をほぼ必要としない，高い抗血栓性と長期耐久性を併せ持つ人工肺を開発し，その製品化を達成した。

一方，PCPS/ECMO用の血液ポンプについては，主に体外循環用に市販されているディ

スポーザブル遠心型血液ポンプを流用している。しかしながら，本来数時間以内の使用を前提として開発されたポンプであるため，すでに説明したように接触回転部分を有している。その結果，血栓形成が生じ耐久性も低く，2～3日から長くても1週間程度での交換を余儀なくされている。

本研究では，具体的開発目標の一つとして1カ月以上の長期耐久性を有するディスポーザブル遠心型血液ポンプを開発し，上記人工肺と組み合わせることにより，世界に類をみない1カ月以上の長期耐久性と高い抗血栓性を有するPCPS/ECMOシステムの構築を目的としている。かかるシステムが実用化されれば，現在数日の補助を限度として適用されている急性心不全/急性呼吸不全の1,000例超の症例に加えて，これまで適用困難であった出血合併症例や1週間以上の中～長期補助，すなわち慢性呼吸不全患者の急性増悪期の呼吸補助や新型インフルエンザなど感染症による重症呼吸不全患者の救命（肺炎：年間死亡約8.7万人，慢性呼吸不全：年間死亡約1.3万人），出血を伴う重症患者（脳出血：年間死亡約4.6万人，交通事故：年間死亡約1.2万人）の生命維持など，現行システムでは適用外となる多くの重篤な患者に対して用いることが可能となり，臨床的インパクトは極めて大きいものと考えている。

図4に開発中の動圧軸受型遠心ポンプシステムの外観を示す。血液ポンプ部の重量が駆動モータ部も含めて500gであり，これは体外設置型の血液ポンプとしては最軽量であり，システムの配置における自由度が高まるのみならず，救命救急の現場で使用可能な移動性に優れたシステムも実現しうると考えられる。

当センター人工臓器部では，大型動物（成ヤギ）を用いた慢性実験によって，上下大静脈脱血・頸動脈送血のPCPSシステム評価を実施しており，この遠心ポンプを用いたPCPSシステムを用いて，慢性動物実験において抗凝固薬剤を一切投与せずに30日間の連続運転に成功している。30日連続使用後のポンプには血栓が一切見られず，人工肺についても流路の閉塞やガス交換の低下は特に見られず，従来のシステムを凌駕する高い抗血栓性を持つシステムが実現できていることを証明している。また，PCPSシステムの装着による溶血量も許容範囲内であり，血液ポンプ自体はすでに製品化目前の段階に達していると考えている。

2. 体内埋込型補助人工心臓システム

体内埋込式VADに関しては，複数の遠心式または軸流式の連続流型システムが既に臨床応用・製品化されている。しかしながら，遠心式VADは容積・重量とも比較的大きく，小児症例を含む小体格の患者には埋め込むことが困難である。現行の遠心式VADは体表面積1.4m²以上の患者への適用が主流であり，本邦成人女性の平均体表面積が1.43～1.52m²であることを考えると，やや小柄な成人女性と同等の体格以下の患者や中学生以下の殆どの患者は適用対象外となる。一方，軸流式VADは遠心式と比べて小型化することが可能であるが，最大駆出流量が低い傾向にあり，また羽根車の回転数が毎分10,000回転以上となるため，回転部分に接触部位がある場合には長期耐久性や血栓形成が問題となる。これらの点を考慮すると，今後できる限り多くの患者に対して体内埋込式VADを用いたDT治療を普及させていくためには，まず良好な解剖学的適合性が得られる小型化が可能な軸流式で，さらに長期耐久性と抗血栓性が期待できる非接触型インペラ駆動機構を有するシステムの開発を目指す必要がある。本研究では，非接触型軸受のVADとしては世界最小最軽量クラスの軸流型ポンプを開発し，小児から

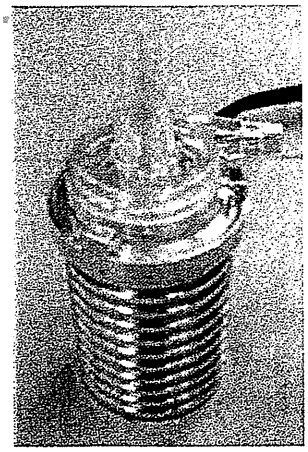

図4. 長期使用可能な呼吸循環補助システムとして開発中の遠心ポンプ

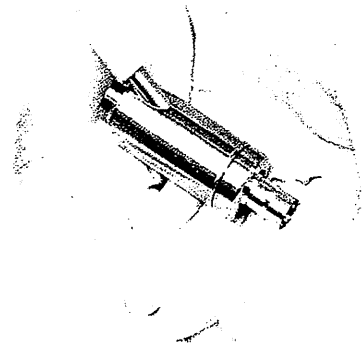

図5. 体内埋込型補助人工心臓として開発中の動圧軸受式軸流ポンプ

大人まで広い範囲の体格の患者に対しての適用を目標とする。非接触である動圧軸受採用により10年以上の連続使用可能な超高耐久性が期待できる。図5が開発中の体内埋込型補助人工心臓用軸流ポンプ(試作機)の外観である。ポンプは主にチタン合金製であり，血液ポンプの重量は約150gと軽量であり，回転数約9,000rpmにおいて平均的運転条件である流量5.0L/min，発生圧力100mmHgを達成している。これまでに，ポンプによる血球破壊量が許容範囲であること，3カ月の慢性動物実験(抗凝固剤，抗血漿板剤の使用によりプロトロンビン時間〔PT〕を術前の2～3倍に管理)においてポンプ内部に血栓を生じないことを確認しており，現在製品化に向けたシステム全体の最終設計に向け開発を続けている。

3. 小柄患者用補助人工心臓システムの開発

これまで成人用として設計，製作を進めてきた動圧軸受式植込み型軸流ポンプについて，小柄な患者にも適用できるように血液流路，翼形状の設計変更，改良を行い，流量1～4L/minの条件で循環補助可能なポンプを開発する。この運転条件において，以下の項目を検証しつつ開発を進める。

・モータ発熱による組織の損傷が発生しない
エネルギー効率，消費電力を維持。
・溶血，血栓等を発生しない。
・耐久試験，動物実験を含めた長期連続運転において動圧軸受による羽根車の安定浮上。

さらには駆動装置の設計，製作，動作検証についても行う。駆動装置の構成はドライバ，携帯バッテリ，携帯バッテリ用充電器，商用電源変換装置である。各機器は携帯バッテリを除き，成人用機器と共通化を図っている。各機器の仕様については院内使用および長期在宅治療での使用を想定した操作性，駆動状態の表示，警報機能等を設計に取り入れ，経済産業省・厚生労働省にて策定された「高機能人工心臓システム開発ガイドライン」および関連するISO，IEC規格に準拠し，駆動装置の電気的安全性試験の実施を行うこととする。設計が完了し，そのプロトタイプを用いて溶血量評価試験を実施，さらに本システムが想定する患者と同程度の体重を持つ実験動物の体内にプロトタイプ機を植え込み，慢性管理下で3カ月間システムを運転することによりトータルシステムとしての生体適合性ならびに信頼性・安全性の評価を行う予定である。

Ⅳ. まとめ

ターボ型血液ポンプは拍動型ポンプに比べ圧倒的に小型化することが可能であり，体内埋込型補助人工心臓をはじめとする各種血液ポンプとして広く普及した。今後もその特性を生かしてさらに広い範囲の治療への応用が期待されており，その具体例として著者らが取り組んでいる血液ポンプ開発について説明した。本稿では技術的な側面を中心に述べているが，血液ポンプを用いた治療体系の定着には，例えば人工心臓を装着して退院した患者に対する医学的・社会的サポートが持続的に行われる社会システムの構築も不可欠である。現在では関連学会を中心にして，例えば人工心臓管理技術士認定制度ができるなど，様々な活動が広がっていることを付け加えておく。

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Society For Biomaterials

Water-soluble argatroban for antithrombogenic surface coating of tissue-engineered cardiovascular tissues

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Abstract: Argatroban is a powerful synthetic anticoagulant, but due to its water-insoluble nature, it is unsuitable for use as a coating material to reduce the thrombogenic potential of natural or tissue-engineered blood-contacting cardiovascular tissues. On the other hand, anionic compounds could adsorb firmly onto connective tissues. Therefore, in this study, an anionic form of argatroban was prepared by neutralization from its alkaline solution, dialysis, and freeze-drying. The subsequently obtained argatroban derivative could be easily dissolved in water. Analysis of the surface chemical composition showed that the water-soluble argatroban (WSA) could be adsorbed on the entire surface of tissue-engineered connective tissue sheets composed mainly of collagen. Adsorption was achieved on immersion of the tissue-engineered connective tissue sheet in a saline/WSA solution for only 30 s without any change in the mechanical properties of the tissue-engineered sheets. Complete surface adsorption (ca., 1 mg/cm^2) was obtained at WSA concentrations of over 5 mg/mL. WSA adsorption was maintained for at least 7 days with rinsing. Blood coagulation was significantly prevented on the WSA-adsorbed surfaces in acute *in vitro* experiments. The coating was applied to *in vivo* tissue-engineered vascular grafts (biotubes) or tri-leaflet tissues (biovalves) under development, ensuring a high likelihood of nonthrombogenicity of their blood-contacting surfaces with high patency, at least in the subchronic phase. It appears that WSA satisfies the initial requirements for a biocompatible aqueous coating material for use in natural or tissue-engineered tissues. J Biomed Mater Res Part B: Appl Biomater 99B: 420–430, 2011.

Key Words: argatroban, antithrombogenic coating, aqueous coating, anionic, surface modification

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INTRODUCTION

Recently, a novel and practical concept in regenerative medicine known as in-body tissue architecture technology, based on the tissue-encapsulation phenomenon in living bodies, has been implemented for the development of autologous tissues for implantation.[1–6] This phenomenon is a biological defense mechanism and involves embedding synthetic foreign bodies in body tissues. We previously developed in-body tissue architecture-based, small-caliber vascular grafts termed "biotubes"[1–3] and tri-leaflet tissues termed "biovalves."[4–6] Unfortunately, the tissues formed using this technology consist mainly of collagen and fibroblasts, which can trigger severe thrombosis.[3] Therefore, the development of water-soluble, nonthrombogenic coating agents for safe implantation is imperative.

The other approach for cardiovascular replacements in regenerative medicine is the use of natural scaffolds with total removal of cellular antigens.[7,8] It is observed, however, that these acellular luminal surfaces without endothelial cell (EC) coverage carry a substantial risk for thrombosis when exposed to the blood directly.[9] Therefore, most recent studies have focused on designing tissue-engineered vascular grafts by using decellularized scaffolds and host vascular cells.[10–12] The innovative use of stem cells has opened up new avenues for designing fully engineered vascular grafts. However, such tissue-engineered vessels may only be appropriate for use in patients undergoing planned surgery but not in those who need immediate vascular reconstruction. This is because reseeding procedures cannot be completed in a short time. Therefore, it is clinically meaningful to

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produce off-the-shelf engineered vessels by using decellularized biological scaffolds.

An alternative way of exploring the nonthrombogenic potential of natural tissue-based implants is the engineering of these implants through surface coating with antithrombogenic agents.[13–15] Recently, we developed a novel aqueous antithrombogenic coating material—a heparin bioconjugate—containing a thermoresponsive cationic polymer that acts as a surfactant.[15] Because of the low critical solution temperature of the heparin bioconjugate (~35°C), it could be coated with an aqueous solution at room temperature. The coating showed excellent adsorptivity and high durability below 37°C on collagen sheets and rat skin tissues.

Argatroban, an arginine-derived synthetic low-molecular-weight compound that binds to thrombin, competitively inhibits fibrinogen cleavage and platelet activation stimulated by thrombin.[16,17] As a result, argatroban has been used for designing antithrombogenic surfaces in percutaneous transluminal angioplasty (PTA) devices, including balloon catheters and stents.[18–20] Previously, Imanishi et al.[18] reported that they developed hydrogel-coated balloon catheters loaded with an argatroban solution for reduction of intimal hyperplasia after PTA. Ikada and coworkers[19] demonstrated that direct delivery of argatroban can be achieved by adsorption of the drug particles onto ionically grafted polyethylene balloon surfaces. High concentrations of argatroban were found within the ballooned arterial segment immediately after angioplasty. Nishi et al.[20] reported that experimental aneurysms treated with argatroban-coated, polyurethane-covered stents were completely occluded without significant parent artery stenosis. However, owing to its water insolubility, argatroban is unsuitable for use as an antithrombogenic coating material in natural or tissue-engineered, blood-contacting cardiovascular tissues or organs.

The purpose of this study was to formulate water-soluble argatroban (WSA) for designing antithrombogenic surfaces of natural or tissue-engineered, blood-contacting cardiovascular tissues. Besides an *in vitro* evaluation of the adsorbability and antithrombogenic ability of WSA in tissues, the preliminary studies included evaluation of its efficiency in *in vivo* studies involving the "biotube" vascular grafts[1–3] and "biovalve" tri-leaflet heart valves[4–6] that we have developed with completely autologous connective tissues by using in-body tissue architecture technology.

EXPERIMENTS

Materials

WSA was prepared as follows: Argatroban (1 g; Mitsubishi Chemical Co., Tokyo, Japan) was dissolved into a 1*N* NaOH solution (4 mL). After 1 h of stirring, the solution was poured into a vessel containing 70 mL of deionized water, dialyzed with deionized water until a pH of about 7.4 was achieved using a seamless cellulose tube (Dialysis Membrane, size 36; Viskase Co., IL), and finally lyophilized using a freeze dryer (FRD-82M; Asahi Techno Glass Co., Chiba, Japan) to yield WSA (yield, 95%).

The connective tissue sheets and tubes were prepared using in-body tissue architecture technology. All animals were treated humanely according to the Principles of Laboratory Animal Care (National Institutes of Health Publication No. 56-23; received in 1985). The research protocol for this study (No. 9044) was approved by the ethics committee of the National Cerebral and Cardiovascular Center Research Institute. Silicone rods (with a diameter of 3 or 5 mm) were embedded into subcutaneous pouches of 1-year-old female beagle dogs (weight, ca., 10 kg). After 1 month of embedding, the implants with surrounding connective tissues were harvested. The connective tissue sheets were obtained by circumferentially cutting the tubular connective tissues formed around the 5-mm diameter silicone rods after trimming the peripheral connective tissues. The connective tissue tubes were obtained from the 3-mm diameter silicone rods after trimming the peripheral connective tissues.

Surface coating of ionic dyes on connective tissue sheets

A section of the connective tissue sheets (size, 10 × 10 mm; thickness, ca., 0.1 mm) was coated with 20 μL of toluidine blue O (cationic dye (Wako Co.) 10 mg/mL in deionized water) or 20 μL of eosin Y (anionic dye (Wako Co.) 10 mg/mL in deionized water) by the solvent evaporation method.

Adsorption of WSA on connective tissue sheets

The connective tissue sheets (size, 5 × 5 mm; thickness, ca., 0.1 mm) were immersed in 5 mL of a saline solution of WSA (concentration, up to 10 mg/mL) for a predetermined time period (10 s to 5 min), and then rinsed with saline solution for 30 min. The mass of WSA was calculated from the calibration curve between the concentration of WSA and fluorescence intensity (IX71; Olympus Co., Tokyo, Japan; excitation, 340 nm; emission, 405 nm).

Surface characterization

The chemical composition of the outermost surface layer of the connective tissue sheets, prepared using in-body tissue architecture technology, was determined before and after WSA adsorption, by X-ray photoelectron spectroscopy (XPS 3400; Shimadzu Co., Kyoto, Japan), using a magnesium anode (MgKα radiation) at room temperature under 5 × 10^{-6} Torr pressure (10 kV, 20 mA) at a take-off angle of 90°. The take-off angle is defined as the angle between the sample surface and the electron optics of the energy analyzer.

Mechanical properties

The mechanical properties of the connective tissue tubes (internal diameter, 3 mm; length, 20 mm; thickness, ca., 0.1 mm) were examined before and after WSA adsorption using the apparatus designed by Hayashi et al.[21,22] Saline solution was introduced into the connective tissue tubes at a rate of 5 mmHg/s. The luminal pressure, *P*, was measured with a pressure transducer (N5901; Nihon Denki Sanei, Tokyo, Japan), and the external diameter at the center of the biotube (*D*) was determined by an apparatus comprised of a video camera (C2400; Hamamatsu Photonics, Shizuoka,

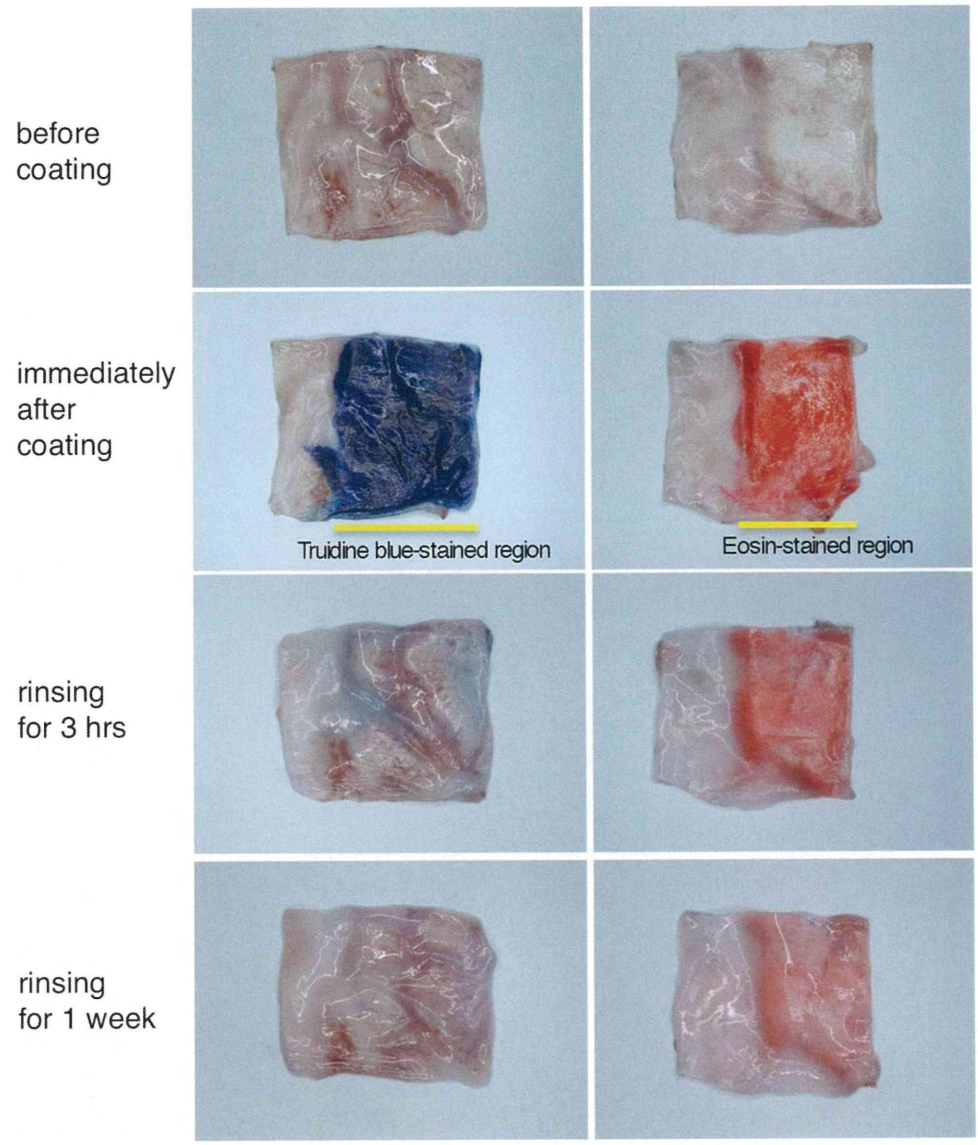

FIGURE 1. Comparison of adsorption of the cationic dye toluidine blue and anionic dye eosin. The dyes were coated on a section of the connective tissue sheets. The color changes were macroscopically observed for up to 1 week. A large amount of anionic dye remained even after rinsing for 1 week, whereas almost all the cationic dye was washed away within 3 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Japan), a TV monitor, and a width analyzer (C3160; Hamamatsu Photonics). The compliance of the graft ($n = 3$) was calculated as the stiffness parameter (β) defined by Hayashi et al., which is determined according to the following equation:

$$\ln(P/Ps) = \beta(D/Ds - 1)$$

where Ps and Ds denote standard pressure (100 mmHg in this study) and external diameter at pressure Ps, respectively. The logarithm of the normalized pressure [ln (P/Ps)] was plotted against the normalized external diameter (D/Ds). The value of β was determined as the approximate slope of the plot in the physiological blood pressure range of 60–140 mmHg. The tests were run in triplicate.

In vitro blood coagulation test

Whole blood (ca., 20 mL) sample from a 1-year-old female beagle dog (weight, ca., 10 kg) was collected in a conical tube, and 1-mL samples were immediately dropped on the connective tissue sheets (size, 10 × 10 mm; thickness, ca., 0.1 mm). After incubation at 37°C for a predetermined time, the sheets were rinsed three times with phosphate-buffered saline (PBS). The gross appearance of the sheets was photographed for macroscopic evaluation. This experiment was repeated four times.

In vivo evaluation

The feasibility of the nonthrombogenic property of the connective tissues (attributable to WSA adsorption) was determined using two different animal implantation models[1]

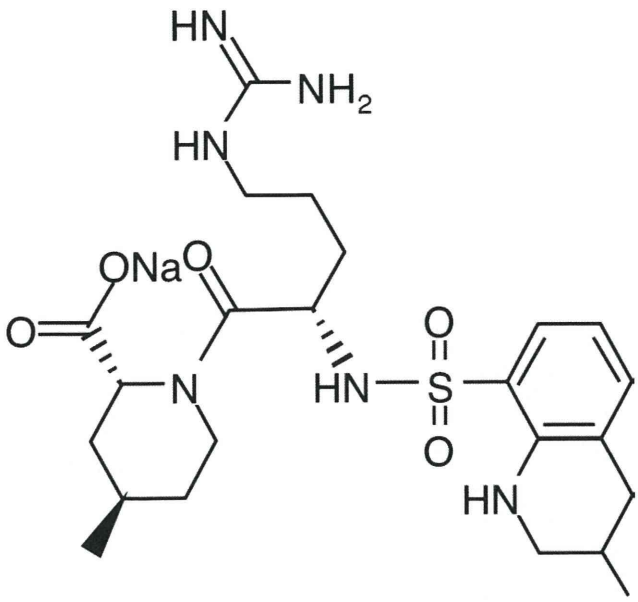

FIGURE 2. Chemical structure of WSA.

small caliber vascular tissue replacement in rats ($n = 6$ for 12 weeks) with autologous connective tissue tubes, termed "biotubes";[2] heart valve replacement in beagle dogs ($n = 3$ for each period, 2, 4, and 12 weeks) with allogeneic connective tri-leaflet tissues, termed "biovalves." Both biovalves and biotubes were prepared using in-body tissue architecture technology.

Silicone rods (diameter, 2 mm; length, 20 mm) were used as a mold for biotube preparation. The rods were embedded in dorsal subcutaneous pouches in rats (weight, 238 ± 58.6 g). After 4 weeks, the implants were harvested with surrounding connective tissues Biotubes were obtained from the implants after the rods were pulled out. Following the adsorption of WSA by immersion of the biotubes in a WSA/saline solution (volume, 4 mL; concentration, 5 mg/mL) for 10 min, the WSA-treated tubes were auto-implanted into the infrarenal abdominal aorta of the same rats by end-to-end anastomosis and a custom-designed sutureless vascular connecting system under microscopic guidance.[23] Patency was examined at the time of surgery by direct inspection. The wound was closed with 4-0 silk sutures. Neither antiplatelet nor anticoagulant agents were administered, except for the intraoperative heparin injection. Thereafter, the rats had free access to standard food and water. After 12 weeks of implantation, the luminal surface was observed.

A mold for biovalve organization was assembled using two types of silicone rods with a small aperture with a tri-leaflet shape between them.[4] The concave rods had three projections that resembled the protrusions of the sinus of Valsalva. The molds were placed in the dorsal subcutaneous spaces of beagle dogs. After 4 weeks of embedding, biovalves with three leaflets in the inner side of the conduit were obtained after removing the molds. The obtained biovalves were implanted in the main pulmonary artery of the beagles ($n = 4$; ca., 7 kg) as allogeneic conduit valves. Anesthesia was induced with 0.3 mg/kg of midazolam and

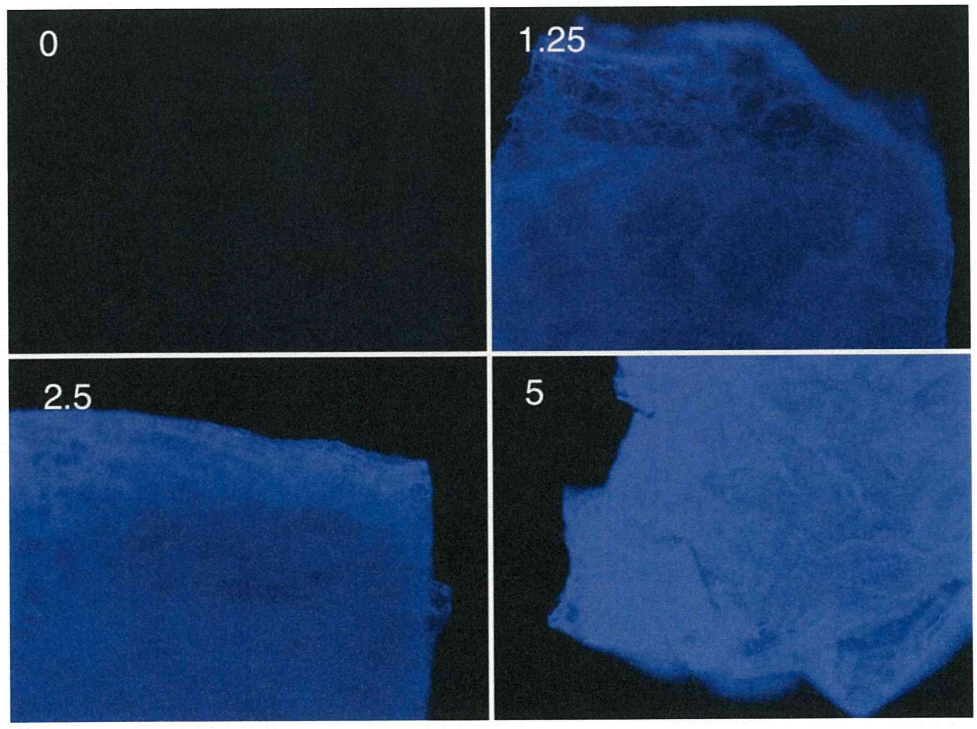

FIGURE 3. Fluorescent micrographs of the connective tissue sheets after immersion in PBS solutions of WSA with different concentrations of WSA up to 5 mg/mL. Blue fluorescence indicates WSA adsorption on the surfaces. The numbers show the concentration of WSA (mg/mL). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1. Surface Chemical Composition Change of Connective Tissue Sheets before and after Immersion into Argatroban Solution

	N/C	S/C
Connective tissue sheet	0.14	0.017
After immersion into argatroban solution	0.21	0.035
Argatroban	0.22 (0.26)	0.037 (0.044)

Determined by electron spectroscopy for chemical analysis.
Parentheses indicates the theoretical values of argatroban.

maintained with continuous infusion of 0.3 mg/(kg min) of propofol and 1% isoflurane. The heart was exposed through a left thoracotomy at the fourth intercostal space. Using common carotid artery and internal jugular vein cannulation, a moderately hypothermic cardiopulmonary bypass was established. The ascending aorta was clamped, and cardioplegic solution (10 mL/kg) (Miotecter; Mochida Pharmaceutical Co., Tokyo, Japan) was infused through the aortic root cannula to achieve cardiac arrest. Cardioplegia was repeated every 30 min. Afterward, the native pulmonary leaflets were excised. Biovalves with WSA adsorption (volume, 4 mL; concentration, 5 mg/mL; immersion time, 10 min) were implanted into the main pulmonary artery by end-to-end anastomosis with a 7-0 nylon suture. Then, the aorta was declamped, and normal heart rhythm was restored. Finally, the beagle was weaned off cardiopulmonary bypass. Systemic anticoagulation was achieved by subcutaneous injection of low-molecular-weight heparin [50 U/(kg·day)] for 1 week after implantation. Thereafter, the dogs had free access to standard food and water. After 12 weeks of implantation, the luminal surface of the biovalves was observed.

Histological evaluation

The rats and beagles were sacrificed at 12 weeks after implantation. Subsequently, the specimens were fixed with 10% formalin, embedded in paraffin, sliced into circumferential sections, and finally stained with hematoxylin-eosin and Masson's trichrome. In addition, few sections of biotubes were also stained for α-smooth muscle actin (α-SMA) and factor VIII by immunohistochemical techniques; these proteins were detected using monoclonal antibodies (Dako Japan, Kyoto, Japan).

RESULTS

Adsorbability of ionic dyes on connective tissues

The connective tissue sheets used as model substrates were prepared using in-body tissue architecture technology.[3] When a part of the tissues was coated with toluidine blue or eosin, the coated areas were stained in dark blue or orange, respectively (Figure 1). The stained samples were rinsed with deionized water. After 3 h of rinsing, most of the dark blue color disappeared while the orange color remained. The orange color was maintained even after 1 week of rinsing. Toluidine blue is a positively charged dye while eosin is a negatively charged dye. Therefore, it is considered that connective tissues have good affinity for anionic compounds.

Preparation of WSA

Argatroban is water insoluble under neutral and acidic conditions, but it is easily soluble in an alkaline solution containing sodium hydroxide, due to ionization of the carboxyl group in argatroban. After removal of the excess sodium hydroxide by dialysis and then freeze-drying, argatroban sodium salt was obtained as a neutral, white powder. The argatroban obtained (Figure 2) could easily dissolve in water with a neutral pH by the formation of an anionic form via dissociation of the sodium ion. Therefore, the anionic form of argatroban is WSA.

Adsorption of WSA on connective tissues

The connective tissue sheets obtained by in-body tissue architecture technology exhibited only slight fluorescence with an excitation wavelength of 340 nm and emission wavelength of 405 nm. After immersion in a saline solution of WSA, blue fluorescence was strongly observed on the connective tissues (Figure 3). The increase in blue

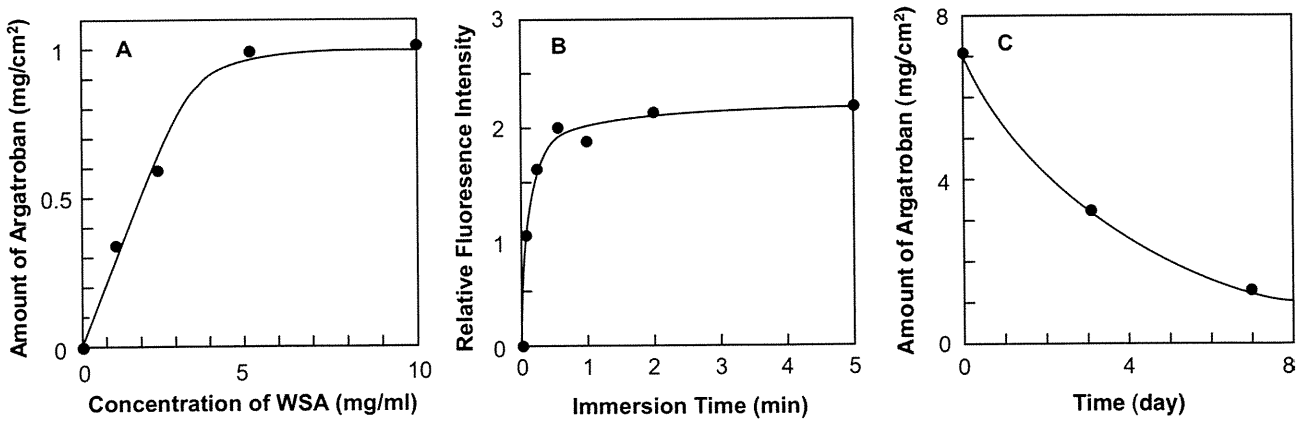

FIGURE 4. (A) Mass of WSA adsorbed on the connective tissue sheets after immersion in different concentrations of WSA, up to 10 mg/mL. (B) Relative fluorescence intensity of WSA adsorbed on the connective tissue sheets for different immersion times. The WSA concentration was set at 5 mg/mL. (C) Release of WSA coated on the connective tissue sheets. WSA was sustained and released for 7 days.

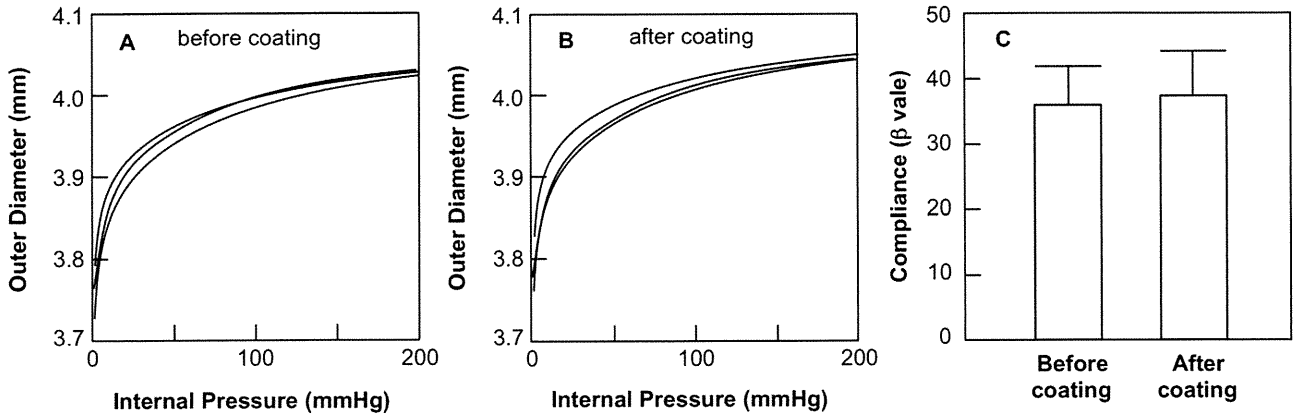

FIGURE 5. Internal pressure-induced outer diameter changes in 3-mm internal diameter connective tissue tubes before (A) and after (B) WSA adsorption ($n = 3$). (C) Comparison of compliance (β value) of the connective tissue tubes before and after WSA adsorption obtained from (A) and (B).

fluorescence of the tissue sheets was directly proportional to the mass of WSA. N1s and S2p signals in the XPS spectra of the connective tissue sheets also increased following immersion in the WSA solution. The N/C and S/C elemental ratios, determined from the peak areas of the C1s, N1s, and S2p signals, were 0.21 and 0.035, respectively (Table I). These values are very similar to those of argatroban. These results indicate that WSA was adsorbed entirely onto the connective tissues.

From the intensity of fluorescence in the connective tissue sheets, the mass of WSA adsorbed could be estimated. The mass adsorbed increased with the concentration of WSA. About 1 mg/cm^2 of WSA was adsorbed at a concentration of 5 mg/mL [Figure 4(A)]. This adsorption occurred within 30 s of immersion [Figure 4(B)]. When WSA was coated on the connective tissue sheets using a solvent evaporation method, a large mass of the coated WSA was released gradually over a period of 7 days [Figure 4(C)].

Mechanical changes caused by WSA adsorption

The mechanical properties of the connective tissue tubes before and after WSA adsorption were determined by noting outer diameter changes that occurred with increasing internal water pressure. The internal water pressure and outer diameter behaviors of the tissues were quite similar before and after WSA adsorption [Figure 5(A,B)]. Therefore, there is little difference in the compliances, determined as the approximate slope of the plot of internal water pressure against outer diameter in the physiological blood pressure range of 60–140 mmHg, before and after WSA adsorption [Figure 5(C)]. It was considered that, on adsorption of WSA, little tissue damage was observed.

In vitro antithrombotic activity

The connective tissue sheets were subjected to whole blood coagulation tests. The degree of coagulation of whole blood from beagles was preliminarily evaluated on the sheets with and without WSA adsorption. Figure 6 shows photographs of the time-dependent changes in blood coagulation. As expected, there was no blood coagulation on the WSA-adsorbed surfaces for up to 20 min, whereas blood gradually coagulated on both the nontreated tissue surfaces. The WSA-adsorbed surface therefore showed potent anticoagulant property.

In vivo application

Two different types of *in vivo* applications were performed as the preliminary procedure: (1) implantation of biotubes, which were autologous connective tissue tubes, into the abdominal aorta of six rats (Figure 7); (2) implantation of biovalves into the main pulmonary arteries of nine beagle dogs (Figure 8).

The biotubes, with a post-WSA adsorption diameter of 1.5 mm were auto-implanted successfully into the 1.3-mm diameter abdominal aorta of the rat by end-to-end anastomosis [Figure 7(A)]. There was little bleeding from either of the sites of anastomosis. Macroscopic observation revealed little thrombus formation with an extremely flat luminal surface and minimal neointimal hyperplasia in the biotube after 12 weeks of implantation [Figure 7(B)]. The whole body of biotube was mainly composed of collagen-rich tissue [Figure 7(D)]. The thickness of the formed neointima after 12 weeks of implantation was 235.8 ± 24.8 μm. The value was very closed to that of native vascular wall (220.4 ± 15.5 μm). The luminal surface was covered with ECs without infiltration of inflammation cells [Figure 7(C,E)]. In the wall, α-SMA-positive smooth muscle cells or myofibroblasts were significantly observed [Figure 7(F)].

The biovalves, after adsorption of WSA, were anastomosed as allogeneic conduit valves, while the native pulmonary leaflets were excised [Figure 8(A)]. After 2 weeks of implantation, the protrusions resembling those of the sinus of Valsalva did not change in size or shape [Figure 8(B)]. At 4 and 12 weeks after implantation, no thrombus was observed on the leaflets and the luminal surface of conduit of biovalves in macroscopic observation [Figure 8(C,D)]. The luminal surface was covered with neointimal tissue without thrombus formation and intimal hyperplasia [Figure 8(E,F)].

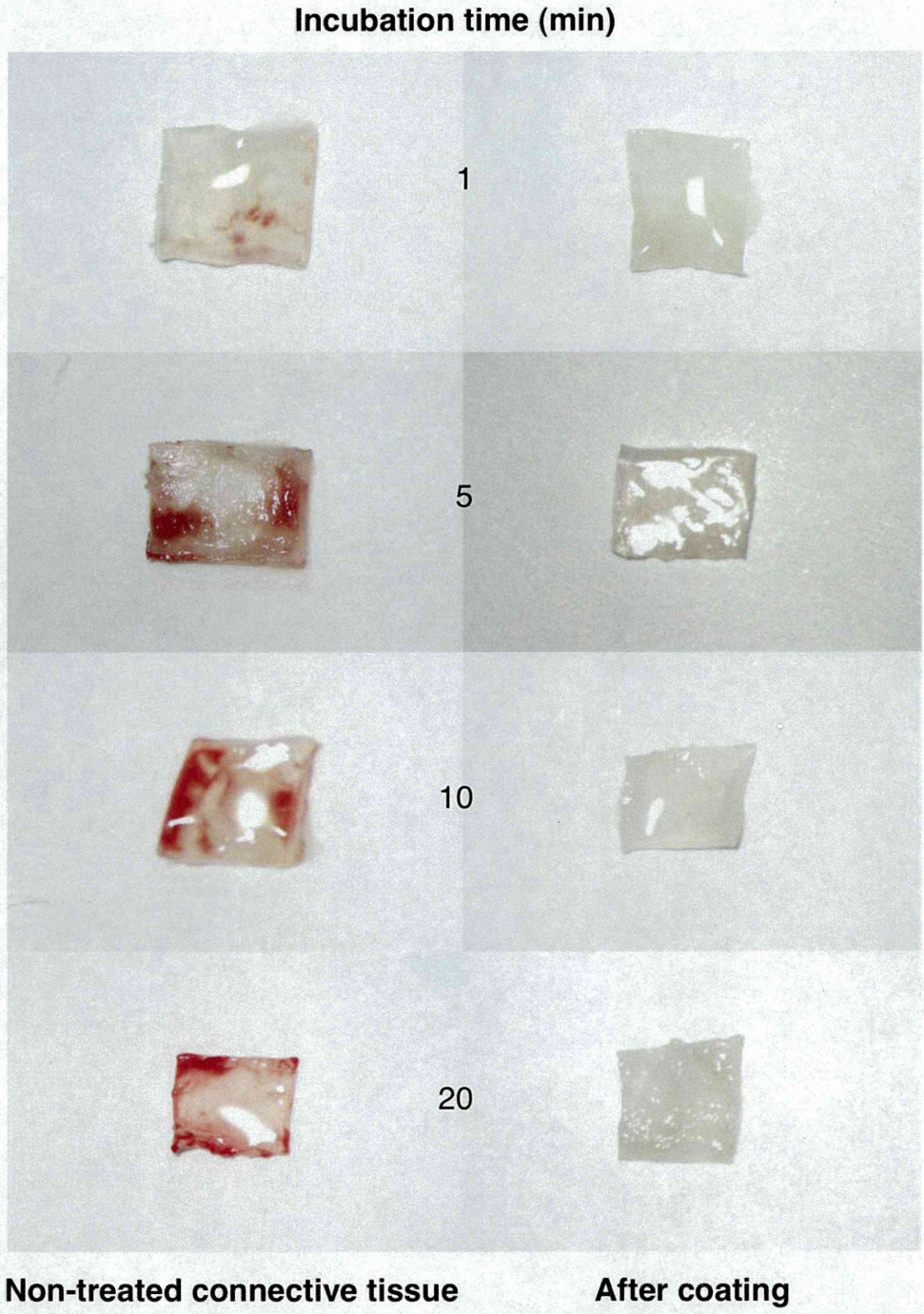

FIGURE 6. Photographs of the connective tissue sheets before and after WSA adsorption. Whole blood was applied to all the sheets and then rinsed with a saline solution after a predetermined period of incubation at 37°C. The left column is untreated control and right is WSA-treated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

Argatroban is commercially available as an aqueous solution for an intravenous, named SLONNON®. Argatroban is contained in the solution in the hydrate form, which is insoluble in water. A high concentration of glycerin is added for uniform mixing with the aqueous solution. Therefore, as argatroban has few ionic charges, it cannot be adsorbed on collagen-based tissues, as was observed with commercialized argatroban solution. In this study, we designed anionic argatroban as WSA for use as an aqueous coating material to reduce the thrombogenic potential of natural or tissue-engineered blood-contacting cardiovascular tissues. Although the molecular design was based on a very simple strategy for electrostatic interaction in coating, adequate performance with a high level of reliability was demonstrated in animal implantation studies using connective

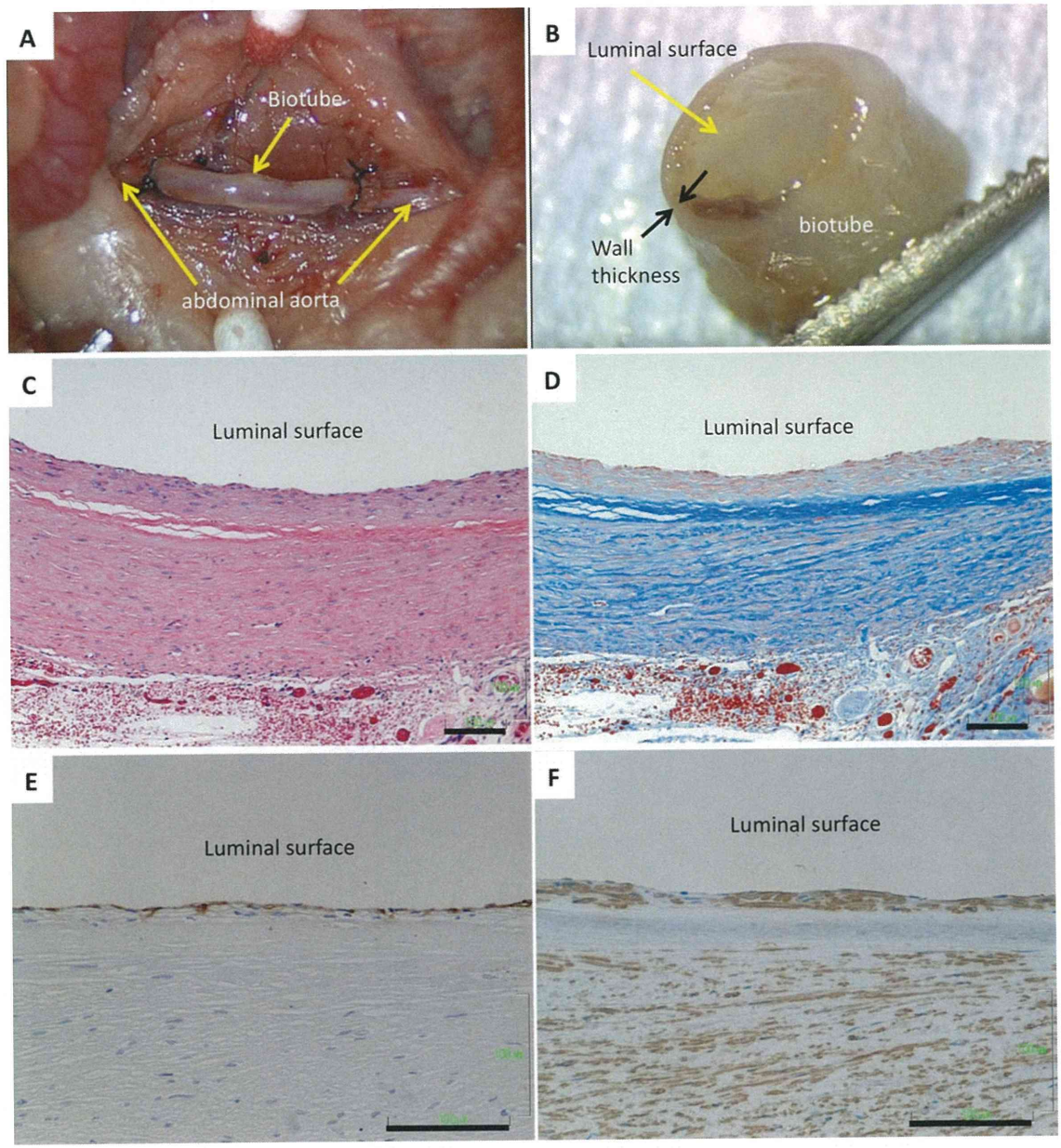

FIGURE 7. (A) Implantation of 1.5-mm internal diameter, biotube autologous vascular grafts into the rat abdominal aorta. (B) Macroscopic observation of the luminal surface of the biotube 12 weeks after implantation. Histology of the circumferential cross-section of the biotube at the midportion 12 weeks after implantation; (C: hematoxylin-eosin; D: Masson's trichrome; E: factor VIII; F: α-SMA). Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tissue-based replacements prepared using in-body tissue architecture technology. *In vitro* blood coagulation tests, clearly demonstrated that the anionic form of argatroban had high antithrombotic activity. For effective coating of WSA, no crosslinking agents or organic solvents were needed. Successful loading of WSA on collagen-based tissues was achieved following immersion of these tissues in an aqueous solution of WSA at a concentration of 5 mg/mL for as little as 30 s, which resulted in little mechanical damage. X-ray photoelectron spectroscopic examination showed that WSA could cover the tissue surface by adsorption alone.

The maximum loading mass by adsorption on the connective tissues was approximately 1 mg/cm^2. This mass is at least 10,000 times higher than that expected for a monolayer of argatroban. The substrates used in this study were connective tissue sheets prepared by in-body tissue architecture technology. The sheets have a multilayered mesh of collagen fibers.[3] Therefore, it was considered that WSA was also impregnated in the deep layers of the tissues. The mass of coated WSA was sustained and released over the course of 7 days.

The main components of the in-body engineered tissue used in this study were collagen fibers and fibroblasts, and it was considered that almost the entire outermost surface was covered with collagen fibers.[3] On the other hand, scaffolds with total depletion of cellular antigens were also

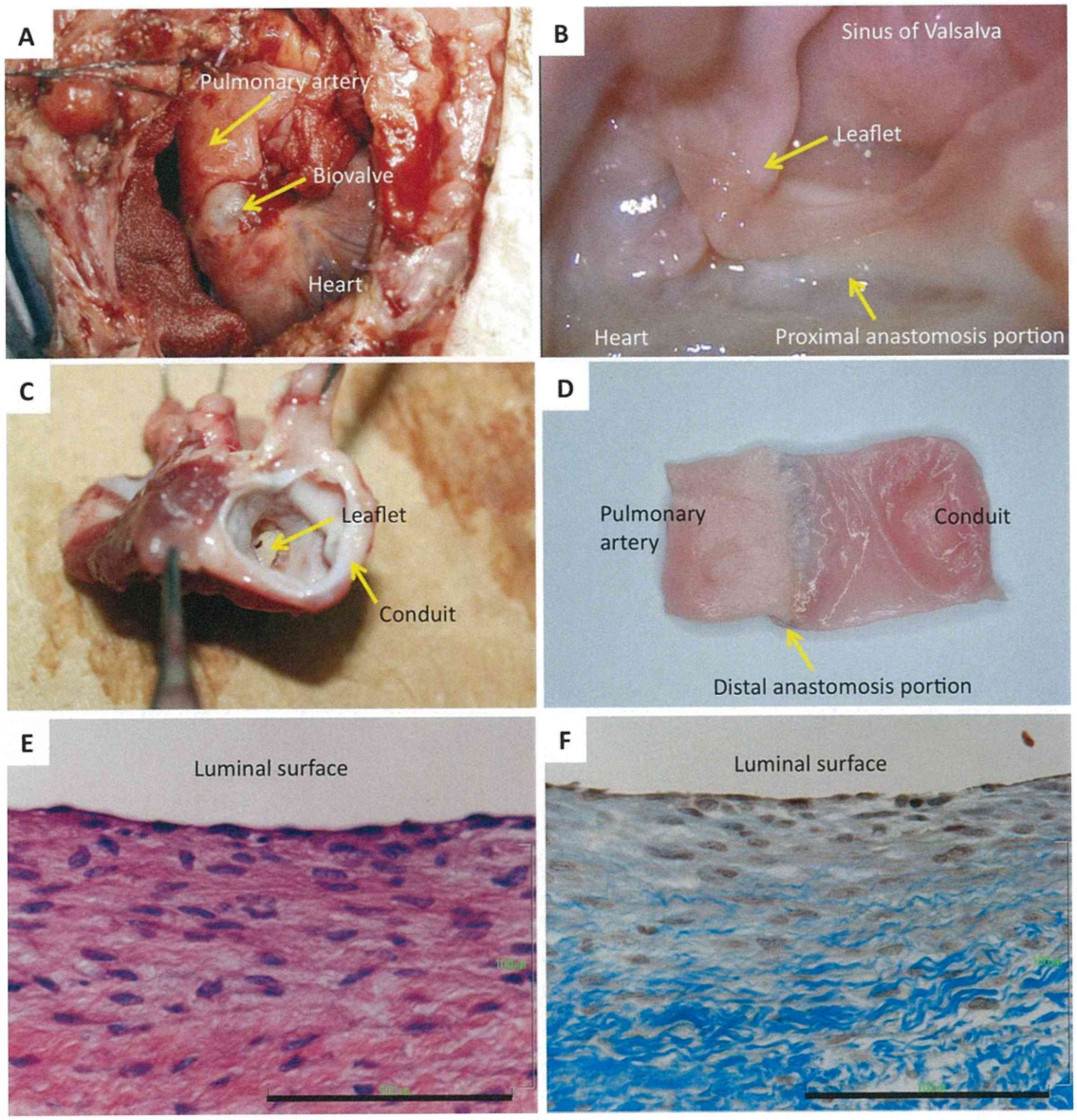

FIGURE 8. (A) Implantation of a biovalve allogeneic heart valve with tri-leaflets (14-mm internal diameter) into the main pulmonary artery of a beagle. Macroscopic photo of the biovalve after (B) 2 weeks, (C) 4 weeks, and (D) 12 weeks of implantation. There is no thrombus formation at the luminal surface of the biovalves. (E and F) Histology of the luminal surface of the biovalve after implantation for 12 weeks at the distal anastomosis portion. The entire luminal surface was covered with a thin neointimal layer containing ECs and exhibited little thrombus formation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mainly composed of collagen fibers. It is observed that acellular luminal surfaces without EC coverage carry a substantial risk for thrombosis when exposed to blood directly. In our previous implantation study of biotubes, all grafts without nonthrombogenic coating were completely occluded at 2 weeks after implantation ($n = 4$).[1] Strong antithrombogenicity, provided by complete endothelialization of the luminal surfaces, is highly desirable. For this reason, most recent studies have focused on the creation of tissue-engineered cardiovascular implants by using autologous cell seeding[10–12] or bioreactor culturing prior to implantation.[24,25] Seeding with autologous vascular cells on the luminal surface has provided a much higher patency rate than noncell-seeded grafts. However, such cell management and processing can be complicated, invasive, and will render the implants prone to infection. In addition, such a tissue-engineered approach cannot be used in emergencies.

In our study, thrombus formation was considerably prevented in *in vitro* and *in vivo* experiments. Two different animal implantation studies were performed. One was biotube vascular grafts. The compliance (β valve) of the biotubes was about 35 before and after argatroban coating. This is close to that of human coronary artery (39.8) and about two times of that of human femoral artery (19.8). None of the biotubes ruptured even after 200 mmHg inner pressure, showing pressure resistance. Therefore, biotubes

are adequate as vascular replacement in mechanical property. The developed vascular connecting device, which was assembled with a delivery rod, an introducing sheath, and a connecting device, was used for easy implantation of the biotubes with small caliber less than 2 mm in an internal diameter. This simple and "easy-to-acquire" technique will promote the development of small caliber arterial grafts, and furthermore, may provide potential ability for clinical application.

The other was biovalve heart valves. The biovalves with WSA coating harvested 12 weeks after implantation showed that the surface of the leaflet was covered with neointima including layering of ECs.[4] Therefore, it is considered that WSA displayed antithrombogenic potential at least until complete endothelialization after implantation. WSA may be effective for antithrombogenicity of other collagen-exposed implants including decellularized tissues.

Glycosaminoglycan heparin displays potent anticoagulant activity when complexed with antithrombin III. Consequently, heparin has gained long and widespread clinical use as an anticoagulant during extracorporeal circulation. Furthermore, various heparinization techniques have been proposed and developed for conferring anticoagulant properties to the blood-contacting surfaces of extracorporeal and implantable devices, including stents and catheters.[26–28] Because heparin is a sodium salt in its normal form, an anionic form is produced in water. Using this ionic property, heparin functions through ionic bonding onto surface of medical devices.[29,30] Therefore, heparin can also adsorb on the connective tissue-based tissues. However, its high water solubility induced easy delamination (data not shown). On the other hand, other low-molecular-weight antithrombogenic agents including antiplatelet agents such as aspirin, panaldine, plavix, and pletal, and anticoagulants such as warfarin, dabigatran, and ximelagatran, may be applied for surface antithrombogenic coating using the ionic adsorption concept used in this study.

The most important finding, in our opinion, is the inhibition of neointimal hyperplasia in WSA-treated tissues. In this study, a very thin neointima layer was formed after implantation. In other implantation studies that used biotubes, few cells showing active inflammation were observed even in the early stages of implantation. These results were observed in our endoscopic implantation study as well.[20] Argatroban coating of the self-expandable, covered stents was confirmed to effectively inhibit intimal thickening in the presence of few inflammatory cells. The anti-inflammatory role of argatroban may, therefore, be effective in the prevention of neointimal hyperplasia. Therefore, argatroban is an effective coating agent for blood-contacting cardiovascular devices including tissue-engineered tissues and artificial implants.

Finally, as WSA may be adsorbed by collagen-exposed surfaces of natural tissues, WSA coating is expected to produce highly reliable results in coronary artery bypass grafting, a procedure performed in many patients each year in the United States, using autologous conduits, internal thoracic arteries, radial arteries, and saphenous veins.

CONCLUSION

In our study, WSA was prepared as a novel, aqueous, antithrombogenic coating material and showed strong antithrombogenic potential on collagen-based tissue-engineered tissues by only simple immersion in a WSA aqueous solution for several tenths of a second with little mechanical damage. WSA coating will provide a highly reliable means of protecting tissue-engineered and natural tissues used for implantation.

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A completely autologous valved conduit prepared in the open form of trileaflets (type VI biovalve): Mold design and valve function *in vitro*

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Abstract: In-body tissue, architecture technology represents a promising approach for the development of living heart valve replacements and preparation of a series of biovalves. To reduce the degree of regurgitation and increase the orifice ratio, we designed a novel mold for a type VI biovalve. The mold had an outer diameter of 14 mm for implantation in beagles, and it was prepared by assembling two silicone rods with a small aperture (1 mm) between them. One rod had three protrusions of the sinus of Valsalva, whereas the other was almost cylindrical. When the molds were embedded in the subcutaneous pouches of beagles for 1 month, the native connective tissues that subsequently developed covered the entire outer surface of the molds and migrated into the aperture between the rods. The mold from both sides of the harvested cylindrical implant was removed, and homogenous well-balanced trileaflets were found to be separately formed in the open form with a small aperture at the three commissure parts inside the developed conduit, which had a thick homogenous wall even in the sinus of Valsalva. Exposure of the obtained biovalves to physiological aortic valve flow in beagles revealed proper opening motion with a wide orifice area. The closure dynamics were suboptimal, probably due to the reduction in the size of the sinus of Valsalva. The mechanical behavior of this biovalve might allow its use as a living aortic valve replacement. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 99B: 135–141, 2011.

Key Words: autologous valve, biovalve, *in vivo* tissue engineering, sinus of Valsalva

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INTRODUCTION

Valvular insufficiency is one of the major causes of morbidity and mortality in heart diseases. The homografts and replacement valves that are currently in use, such as mechanical valves and bioprosthetic xenograft valves, are efficacious; however, further development is still required. In the case of mechanical valves, lifetime anticoagulation therapy is required because thrombus formation around the valve can lead to insufficiency or serious embolisms,[1,2] whereas in the case of bioprosthetic valves, the structure of xenogenic tissues gradually deteriorates with calcification.[3,4] Moreover, owing to their inability to grow, both types of prosthetic valves are unsuitable for pediatric patients.[5] Although cryopreserved homograft valves contain viable fibroblasts, these valves may be rejected. In addition, the scarcity of homograft donors remains a key clinical limitation.[6,7]

Tissue engineering represents a novel scientific approach for overcoming these limitations. It is aimed at *in vitro* fabrication of living heart valves having a thromboresistant surface and a viable interstitium with both repair and remodeling capabilities. Several groups demonstrated the feasibility of creating living cardiovascular structures by seeding cells onto a synthetic polymer, collagen, or xenogenic scaffolds.[8–12] To develop autologous prosthetic tissues, we previously focused on the use of in-body tissue architecture technology, which is a novel concept in regenerative medicine that is based on the tissue encapsulation phenomenon of foreign materials in living bodies.[13,14] This technology involves the use of living bodies as a reactor, and it is simple, safe, and cost-effective. Since 2007, we have used in-body tissue architecture technology to develop a series of autologous trileaflet heart valves, named biovalves.[15–19] The most recent model is the type V biovalve, which is made of completely autologous tissues without any artificial materials.[19] It is designed to have the sinuses of Valsalva and was shown to successfully function as an allogeneic conduit valve in the pulmonary valve position in beagle models for up to 3 months.

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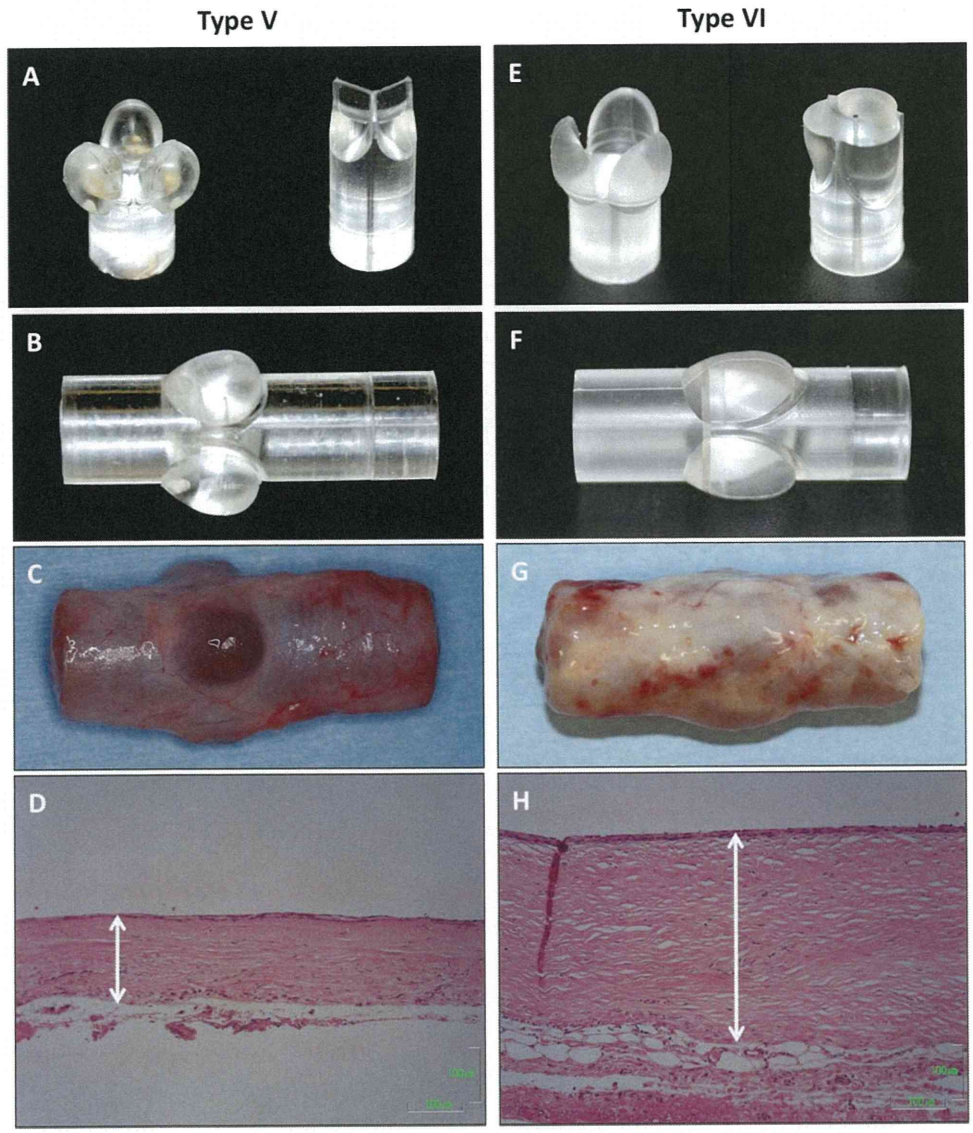

FIGURE 1. Convex and concave-shaped silicone rods (A for type V and E for type VI) and the assembled molds for biovalve preparation (B for type V and F for type VI). Biovalve types V (C) and VI (G) were formed by embedding their molds in dorsal subcutaneous pouches in beagle dogs for 1 month. Histological photos of the cross-sections in the circumferential direction at the top of the sinus of Valsalva parts of the conduits. The wall thickness of the type VI biovalve (H) was approximately twice that of the type V biovalve (D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

However, the process used to prepare type V biovalves has two major disadvantages. The first is in the structural design of the mold for the leaflets. Type V biovalves were prepared from cylindrical molds [Figure 1(B)] by assembling two types of specially designed silicone rods with a small aperture in the shape of a closed form of a trileaflet between them [Figure 1(A)]. By using in-body tissue architecture technology, the three leaflets were formed inside the conduit along with the inner shape of the concave rod as designed. Therefore, the three commissures between the leaflets had a small aperture that was at least 1 mm in size [Figure 2(B)]. To avoid regurgitation of the blood flow, it was necessary to suture the apertures prior to implantation [Figure 2(A)]. The second disadvantage is essentially due to the in-body tissue architecture technology itself. When the cylindrical molds were embedded into the subcutaneous pouches, the connective tissues that developed were almost homogeneous in thickness and completely covered the outer surface of the molds. However, with molds that had larger protrusions, the connective tissues that developed around the convex shape were generally very thin. Indeed, during the preparation of type V biovalves, we found that the top of the sinus of Valsalva had thin membrane tissues, although it had adequate burst pressure [Figure 1(C,D)].

In this study, to overcome these two drawbacks of the biovalve preparation process, we designed a novel mold for the preparation of the type VI biovalve, in which the trileaflet was in the open form and the sinus of Valsalva was relatively shallow. This provided the connecting leaflets with a small aperture at the commissure parts and allowed the formation of a thick conduit wall even in the sinus. The valve

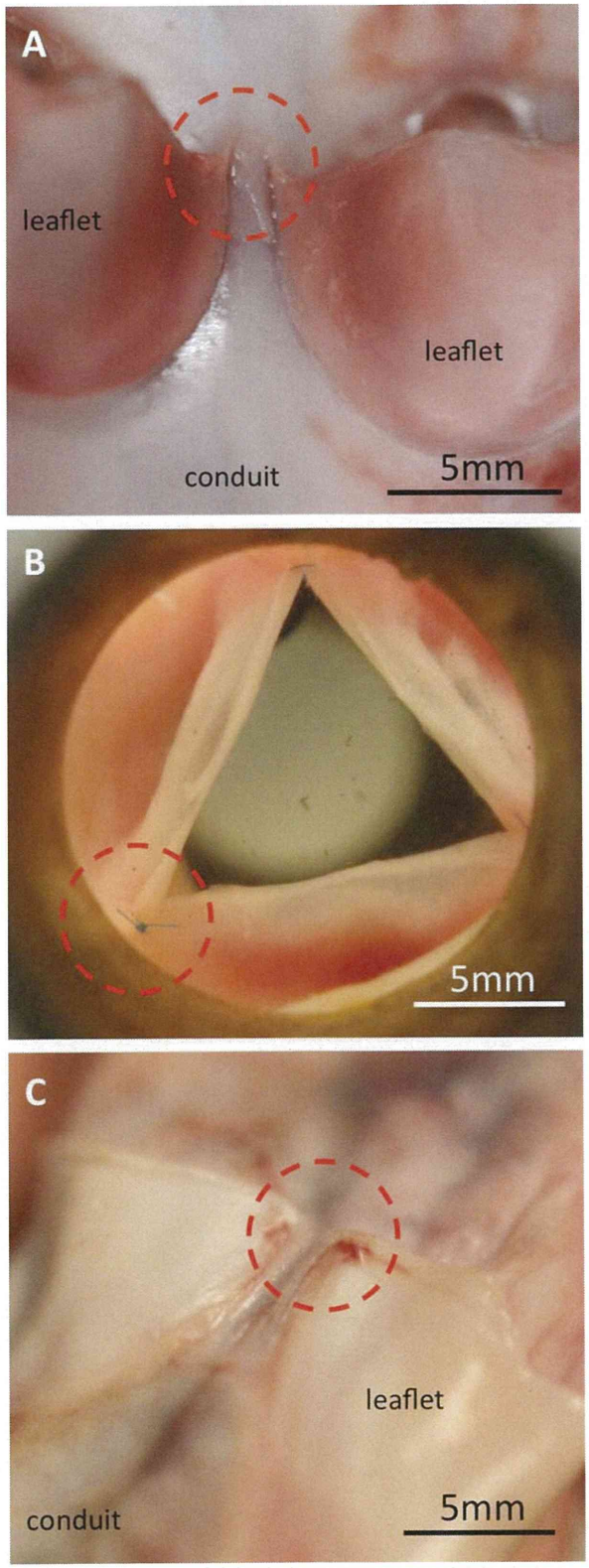

FIGURE 2. (A) The space formed between the leaflets in the type V biovalve. (B) The open form of the type V biovalve after suturing the edge between the leaflets. (C) In the type VI biovalve, the leaflets were in contact with each other with no space in between. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

function of the type VI biovalve was compared with that of the type V biovalve in a pulsatile flow circuit *in vitro*.

MATERIALS AND METHODS

Preparation of biovalves

Beagle dogs were used for the experiments. All animals received care according to the Principles of Laboratory Animal Care (formulated by the National Institutes of Health, Publication No. 56-23, received 1985), and the research protocol (no. 22-2-4) was approved by the ethics committee of the National Cardiovascular Center Research Institute.

Type VI biovalves were prepared as follows. A specially designed concave-shaped silicone rod with three projections that resembled the three protrusions of the sinus of Valsalva [diameter, 14 mm; length, 30 mm; rod on the right-hand side in Figure 1(E)] and a convex-shaped silicone rod [diameter, 14 mm; length, 30 mm; rod on the left-hand side in Figure 1(E)] were assembled to prepare a cylindrical mold for biovalve organization [Figure 1(F)]. The mold was designed such that the leaflets are separated from each other in the open form. The size of the biovalve was suitable for implantation in the native pulmonary valve (diameter, ca. 14 mm) of beagle dogs. We placed four molds per dog into the dorsal subcutaneous pouches of two beagle dogs (age, approximately 300 days; body weight, approximately 7 kg) under anesthesia induced by an intramuscular injection of ketamine (20 mg/kg). Sodium pentobarbital (20–30 mg/kg, Nembutal; Dainippon Sumitomo Pharma, Osaka, Japan) was used as a supplemental anesthetic. After 4 weeks of mold placement, we harvested the implants that were completely encapsulated with robust connective tissue [Figure 1(G)]. The rods from both ends of the developed cylindrical tissue were removed. Biovalves with three protrusions resembling the sinus of Valsalva were formed. Four biovalves were used for the *in vitro* evaluation of valve function and motion, and the remaining was used for macroscopic observation and histological examination.

Two type V biovalves were prepared by the method described in our previous report.[19] Specially, designed concave-shaped silicone rod [diameter, 14 mm; length, 25 mm; Figure 1(A)] and convex-shaped silicone rod [diameter, 14 mm; length, 30 mm; Figure 1(A)] were assembled with a small aperture of 1 mm to prepare a cylindrical mold for BIOVALVE organization. The mold [Figure 1(B)] was designed so the leaflets are separated from each other. The concave-shaped rod had three removable projections that resembled the three protrusions of the sinus of Valsalva. We placed four molds per dog into the dorsal subcutaneous pouches of two beagle dogs under anesthesia. After 4 weeks of mold placement, we harvested the implants that were completely encapsulated with robust connective tissue [Figure 1(C)]. When the rods from both ends of the developed tubular tissue were removed, type V biovalves were obtained.

The wall thickness of both types of biovalves was measured from histological sections that were prepared by the following steps: fixation in 10% formalin, embedding in paraffin, slicing in a circumferential direction, and final staining with hematoxylin–eosin.