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Evaluation of biocompatibility of intra-arterial poly-L-lactic acid stent by tantalum ion implantation

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Abstract

Objective

Biodegradable poly-L-lactic acid (PLLA) with a highly biocompatible surface via tantalum (Ta) ion implantation can be an innovative solution for the problems associated with current biodegradable stents. The purpose of this study is to develop a Ta-implanted PLLA stent for clinical use and to investigate its biological performance capabilities.

Methods

The effectiveness of tantalum ion implantation on PLLA materials was evaluated by histological examinations, immunohistochemistry, and *in vitro* tests. The re-endothelialization ability and thrombogenicity were examined through *in vitro* endothelial cell and platelet adhesion tests. An *in vivo* swine model was used to evaluate the effects of Ta ion implantation on subacute restenosis and thrombosis. Nitinol self-expandable stents were also deployed for comparison. Angiographic and histologic evaluations were conducted at one, two and three months post-treatment. All parent artery diameters were measured to evaluate the effects of this parameter on the experimental results.

Results

The Ta-implanted PLLA stent was successfully fabricated, exhibiting a smooth surface morphology and modified layer integration. After Ta ion implantation, the surface properties were more favorable for rapid endothelialization and for less platelet attachment compared to the bare PLLA stent. In an *in vivo* animal test, follow-up angiography showed no evidence of in-stent stenosis in either group. In a microscopic histologic examination, luminal thrombus formation was significantly suppressed in the Ta-implanted PLLA stent group according to the two-month follow-up assessment (21.2% versus 63.9%, p=0.005). A large parent artery diameter was related to less thrombogenicity (p=0.015 at two months) and neointimal hyperplasia (p=0.020 at one month). However, less thrombogenicity at two months appeared to be more related to Ta ion implantation (p=0.066) than a large parent artery diameter (p=0.204). The Nitinol self-expandable stent was superior in terms of neointimal hyperplasia, inflammation and thrombogenicity compared to the PLLA stent groups.

Conclusion

The Ta-implant PLLA stents appear to be advantageous in terms of re-endothelialization and anti-thrombogenicity compared to the bare PLLA stents.

Keywords : Biodegradable, Ion-implantation, Poly-L-lactic acid, Stent, Tantalum

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목 차

Table 목차

Figure 목차

제 1 장 Introduction

Intra-arterial stenting is one of the most important treatment options for cerebral aneurysms. Although the use of bare metal stents in complex aneurysms has rapidly increased due to their superior mechanical strength and supportability, they remain permanently in the treated vessel and act as a foreign body, which may cause severe complications such as thrombus formation and arterial restenosis [9]. A biodegradable stent that dissolves naturally within the blood vessel after implantation can be a potential solution for these problems, and many studies have been conducted to develop clinically relevant biodegradable stents, especially in the cardiovascular field [8, 27].

Various materials have been used in research on biodegradable stents [7]. They can be roughly divided into two types: metals and polymers. Biodegradable metal stents are made of materials such as magnesium and iron, and biodegradable polymer stents are made of materials such as poly-L-lactic Acid (PLLA), poly glycolic acid (PGA), and polyanhydride [7]. Among them, PLLA is a widely utilized biodegradable polymer in biomedical applications because it can hydrolyze to a natural byproduct under physiological conditions [27]. With regard to stents and the use of PLLA, however, several critical

issues must be addressed [7, 19]. In particular, its strong hydrophobicity and innate hypersensitivity with an inflammatory response are regarded as the major causes of poor re-endothelialization and thrombosis [22]. We have initiated studies to overcome these problems via plasma-based surface modification techniques, especially Tantalum (Ta) ion implantation. Ta is well known for its excellent corrosion resistance, biocompatibility, and osteogenesis-osseointegration ability [16]. During this surface treatment, biocompatible Ta is directly implanted within the topmost surfaces of PLLA struts with a complex 3D geometry, forming a moderate hydrophilic surface and eliciting better cell-material interactions [11, 16].

In this study, we report for the first time a novel, biodegradable PLLA stent. This is realized by Ta ion implantation onto a laser cutting PLLA stent for intravascular implantation. The biological effectiveness of the Ta ion implantation treatment on re-endothelialization and anti-thrombogenicity was evaluated in *in vitro* and *in vivo* model systems.

제 2 장 Materials and Methods

제 1 절 Stent preparation

Figure 1 shows a schematic illustration of the Ta implantation treatment of a PLLA stent. PLLA tubes (PL 38, 3 mm OD, 2.75 mm ID, length: 10mm) were purchased from Biogeneral, Inc. (USA). Femtosecond laser processing (Spitfire Ace, Spectra Physics, USA) was utilized to manufacture a PLLA polymer stent under optimized conditions of the pulse energy, scan speed, and spot size. Before the surface treatment, the stent samples provided were ultra-sonicated in ethanol for 10 minutes. Using a DC magnetron sputtering device (Ultech Co. Ltd., Korea), Ta was implanted into the stent surface. The chamber was maintained in a vacuum state up to $5x10^{-4}$ Pa for 90 minutes and argon gas was then flowed until the inner pressure reached 0.6 Pa. Three different parts of the stent were marked at constant circumferential intervals for a uniform surface treatment. The three sections were attached to the substrate sequentially and sputtering onto the section was conducted for 20 seconds in each case. High negative voltage of 2000 V was applied to the substrate, and the Ta target power was 12 W.

For an *in vivo* animal experiment, a radio-opaque marker was adopted at the end of the stent using gold wire (0.1 mm thickness, Alfa Aesar, USA). The PLLA stent was then mounted onto a 4 x 20 mm non-compliant coronary balloon (Genoss, Korea) by means of a crimping process (Figure 2). Various sizes of balloons (3-5mm) and different pressure levels (8-12atm) were tested in order to deploy the stent successfully while maintaining its stable structure. The balloon size (4 mm) and pressure (10 atm) used in this study were selected as a result (Figure 3). The arterial coverage ratio by the stent was 27.3%. The control samples were a bare PLLA stent without a surface treatment.

제 2 절 *In vitro* test

The stents were observed via optical microscopy (OM) to confirm the maintenance of the structure after the surface treatment. The surface morphology and roughness of the bare and Ta-implanted PLLA stents were examined using a scanning electron microscope (SEM) and a confocal laser microscope (CLM), respectively. The wettability of the surface was evaluated by dropping 3.0 μl of distilled water onto it and fitting the droplet in a circle using a water contact angle (WCA) analyzer. For a WCA test of the luminal surface of a

Ta-implanted sample, a PLLA tube, only vertical half of which had undergone a laser cutting process, was treated via Ta implantation to mimic a luminal side of a Ta-implanted stent. Ta-implanted PLLA stents were fabricated as transmission electron microscope (TEM) specimens using a focused ion beam after carbon deposition to evaluate the content and depth of the implanted Ta on the surface with an energy-dispersive spectroscopy (EDS) line scan analysis by TEM. For a mechanical test, a flat plate test was conducted by referring to an earlier method [13]. The stent was placed between two parallel plates and compression force was applied until the gap distance reached 50% of the diameter of the stent.

An *in vitro* degradation test was conducted to investigate the degradation behavior of the Ta-implanted PLLA stent. This was done by immersing Ta-implanted PLLA tubes in phosphate-buffered saline (PBS) solution at 37 ℃ for eight weeks. After a predetermined time, they were gently rinsed with distilled water to examine the surface morphology and chemical composition via FE-SEM and the X-ray photoelectron spectra (XPS).

Human umbilical vein endothelial cells (HUVECs; CRL-1730), purchased from American Type Culture Collection (ATCC), were utilized to evaluate the endothelialization capabilities. All samples were sonicated in 70% ethanol for 10 minutes and then exposed to

ultraviolet light for one hour. The cells were seeded at a density of $5x10^4$ cells/mL in an endothelial cell basal medium-2 (Lonza, Walkersville, USA) with 10% fetal bovine serum and 1% penicillin-streptomycin. They were cultured in a humidified incubator containing 5% CO_2 at 37℃ and obtained after one, four, and seven days. To visualize the spreading morphology and area of the cells on each specimen through confocal laser scanning microscopy (CLSM), they were treated with 4% paraformaldehyde (Sigma-Aldrich, USA), 0.1% Triton X-100 (Sigma) and 1% bovine serum albumin (Sigma) in phosphate-buffered saline and then stained by phalloidin (Invitrogen, USA) and 4, 6-diamidino-2-phenylindole (Invitrogen) for the cytoplasm and nuclei, respectively. As a proliferation test, the seeding density was 3×10^4 cells/mL and the absorbance at 492 nm was measured according to a methoxyphenyl tetrazolium salt (MTS) assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

An *in vitro* platelet adhesion test was conducted as the sterilized stents were incubated in diluted platelet-rich plasma (PRP) for one hour. After washing out the remaining platelets, the numbers of platelets adhering to each stent were quantified using a lactate dehydrogenase (LDH) assay by Multiskan GO (Thermo, Finland). For a SEM analysis, the samples were placed into contact with PRP

at a higher concentration for one hour, washed, fixed with 2.5% glutaraldehyde, and then dehydrated.

제 3 절 *In vivo* test

In vivo test was conducted at the KBIO health facility. The animal experiment conducted here was approved by the Institutional Animal Care and Use Committee (KBIO-IACUC-2018-015) and followed the "the Guiding Principles in the Care and Use of Animals" approved by the American Physiological Society.

In total, four specific-pathogen-free male swine of about 30kg were used for the *in vivo* test. Antiplatelet drugs (aspirin 100mg and clopidogrel 75mg daily) were administered one week before stent placement and were maintained until the animals were sacrificed.

All procedures were performed under general anesthesia by a certified veterinarian. Anesthesia was induced with an intramuscular injection of Zoletil® (Virbac, French) 4mg/kg and Rompun® (Bayer, Germany) 2.2mg/kg. It was maintained using an inhaled agent (Sevoflurane) and oxygen during the procedures. Heparin (5000 IU) was intravenously infused before puncture of the femoral artery.

For stent deployment, a 6 Fr femoral sheath was inserted, and a 6 Fr guiding catheter was positioned in the common carotid artery (CCA)

using a 0.035-inch guide wire under angiographic guidance (Artis zee, Siemens, Germany). Two Ta-implanted PLLA stents were deployed in the right external carotid artery (ECA) and two bare PLLA stents were placed in the left ECA. The delivery of two bare stents failed due to stent damage caused by a rotating hemostatic valve. Ultimately, sixteen PLLA stents were successfully deployed in four swine. Four Nitinol self-expandable stents (Enterprise, Cerenovus, USA) with diameters of 4.5mm were deployed in the left CCA for comparison. All parent artery diameters were measured by a PACS (Picture archiving and communication system) to evaluate the effect of the parent artery diameter on the experimental results. Animals were sacrificed at one (n=2), two (n=1) and three (n=1) months after stent deployment by a potassium chloride injection. A follow-up angiography was performed to confirm arterial patency before sacrifice. One Ta-implanted stent at one-month follow-up was lost during the harvesting procedure and thus 19 stents were harvested (Figure 4). Three paraffin blocks (the proximal, middle, and distal parts) and one resin block were made from each stent sample. For a histologic analysis, hematoxylin and eosin (H&E) staining and immunohistochemical staining for CD 31, CD 34, CD 68, and anti-smooth muscle cell alpha actin were performed. One Ta-implanted PLLA stent specimen was partially harvested; therefore, a

total of 74 H&E staining slides were prepared for the histologic analysis (Ta-implanted PLLA stent group: 26, bare PLLA stent group: 32, Enterprise stent group: 16).

The histologic analysis included luminal thrombus (% = 100 x [luminal thrombus area/luminal area]), neointimal hyperplasia and stenosis, as suggested by Schwartz et al., and inflammation and fibrosis according to a quantitative method to evaluate local biological effects after the implantation of the prosthesis in this case [10, 17, 21]. Inflammation was measured by summing the scores of the following factors: inflammation polymorphonuclear, lymphocytes, plasma cells, macrophages, giant cells, necrosis, neorevascularization, fatty infiltrations, epithelium, leukocytes infiltration, arterial congestion, and edema. Fibrosis was evaluated by dividing histologic response into five grades (Grade 0: no inflammatory response, Grade 1: minimal, Grade 2: mild, Grade 3: moderate, Grade 4: severe).

All histologic analyses were performed by an independent, blinded, and certified pathologist. SPSS software (version 20, IBM corp., Armonk, New York, USA) and MedCalc for Windows (version 19, MedCalc Software, Ostend, Belgium) were used for the statistical analysis. A value of $P < .05$ was considered statistically significant.

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제 3 장 Results

제 1 절 Surface characterization

We observed the macroscopic morphology of the entire stent structure and its surface via optical microscopy (OM) and scanning electron microscopy (SEM) to determine whether any thermal damage was inflicted during the Ta-implantation process. After the Ta ion implantation treatment, the PLLA stent had an opaque and colorless appearance, and its complex shape was well maintained without any deformation or distortion (Figure 5). The implanted Ta layer was confirmed as an embedded layer on the outermost surface of PLLA using a transmission electron microscope (TEM). Despite the several manufactured tracks on the stent surfaces before and after the surface treatment, their average surface roughness (Ra) values were only approximately 0.028 ± 0.008 ㎛ and 0.028 ± 0.003 ㎛ for the bare and Ta-implanted PLLA stents, respectively, representing excellent micro-scale flatness on both surfaces (Figure

6).

As shown in Figure 7, the water contact angles (°) of the luminal and abluminal sides of the Ta-implanted samples were 60.0 ± 1.88° and 51.1 ± 9.12°, respectively, while it was 104.1 ± 0.44° on the bare PLLA (p=0.000); that is, the Ta treatment increased the hydrophilicity of the stent by introducing a tantalum oxide layer. As depicted in Figure 8, the Ta-implantation process on the surface did not affect the mechanical strength of the PLLA stent, indicating that good mechanical integrity remained.

Figure 9 shows that Ta ions were successfully implanted into both the luminal and abluminal surfaces of the PLLA stent, forming a Ta-implanted skin layer regardless of the location. Because the distance from the Ta target and physical hindrance could affect the efficiency of Ta implantation on the luminal surface, the atomic concentration of Ta was higher on the abluminal surface. However, even on the luminal surface, the Ta layer was clearly observed at a depth of 10 nm on the topmost surface with the highest atomic concentration of approximately 6%.

제 2 절 *In vitro* test

The degradation behavior of the Ta-implanted PLLA was assessed to ascertain whether the Ta-implanted PLLA stent is biodegradable regardless of the incorporated Ta layer. As the immersion time in PBS was increased, the samples exhibited small holes and wrinkles on the surface due to degradation (Figure. 10). In a chemical composition analysis, some elements (K, Na, Cl, and P) were detected, which originated from the immersing medium and PBS. The concentration of Ta gradually decreased with an increase in the immersion period, which reached half of the initial value after two months.

The effects of Ta ion implantation on the interaction between the HUVECs, platelets and the PLLA stent surface were examined by visualizing the corresponding adhesion morphologies on bare and Ta-implanted PLLA substrates. Figure 11a shows representative CLSM images of the adhered HUVECs after one, four, and seven days of culturing. On the Ta-implanted PLLA surface, a significant number of HUVECs were adhered and progressively spread in every direction, forming a typical cobblestone-like morphology with tight cell-cell interaction, whereas the bare PLLA exhibited fewer cells which were adhered individually with a lack of cell-cell junctions. The fractions of cell surface coverage with culturing times of one, four and seven days were 48%, 61% and 77% on the Ta-implanted PLLA,

respectively, and were 14%, 34% and 40% on the bare PLLA (Figure 11b). In addition, HUVECs tended to proliferate more actively on the Ta-implanted PLLA, showing a higher cell viability value when compared to those cultured on bare PLLA (Figure 11c).

The blood compatibility of each sample was investigated by platelet adhesion tests and the results were compared quantitatively. As shown in Figure 12a, significantly fewer platelets were found to have adhered when the PLLA surface was treated with Ta ion implantation; the density of the adhered platelets (number/mm^2) on the Ta-implanted PLLA was 92.2 ± 95.5, significantly lower than that on the bare PLLA (306.0 ± 10.2, p=0.046).

제 3 절 *In vivo* test

The follow-up angiographic images showed that all ECAs were patent with no evidence of in-stent stenosis (Figure 13a). However, an arterial lumen collapse was identified in the histologic analysis (Figure 13b). Therefore, an accurate analysis of percent area stenosis ([1-luminal area/internal elastic lamina] x 100) based on a histologic examination could not be done exactly.

Luminal thrombus formation was significantly suppressed in the Ta-implanted PLLA stent group at two months after stent placement

(21.2% versus 63.9%, p=0.005, Figure 14). The Enterprise stent showed low thrombogenicity one month after stent placement compared to the PLLA stents (p=0.000). However, thrombogenicity associated with all stents decreased after three months. Neointimal hyperplasia was similar in both PLLA stent groups (Figure 15). However, the Enterprise stent showed a low degree of neointimal hyperplasia. The inflammation score was high in both PLLA stent groups compared to the Enterprise stent at one month; however, the scores became similar over time (Figure 16). There were no differences in fibrosis between the groups.

CD 68(+) cells, which represent an inflammatory response, were more frequently identified around the bare PLLA stent during the one-month follow-up (Figures 17a and 17b), and the difference disappeared over time (Figure 17c). As an endothelial marker, the expression levels of CD 31 and CD 34 were evaluated. However, there were no significant differences between the groups (Figure 18). There was no definite evidence of endothelization of the stent. Anti-smooth muscle cell alpha actin was strongly expressed in the PLLA stent group (Figure 19). However, there was no significant difference between the Ta-implanted and the bare PLLA stent groups.

Sixteen balloon-mounted PLLA stents were deployed in the ECA

with diameters of 3.20 ± 0.55mm. Overall, there was no difference between the Ta-implanted PLLA stent group (3.29 ± 0.44mm) and the bare PLLA stent group (3.10 ± 0.66mm) in terms of the diameter of the parent artery (p=0.521). However, when the diameter of the parent artery was divided by the follow-up period and analyzed, it was identified that the Ta-implanted PLLA stents were deployed in the parent artery with a larger diameter in the one-month follow-up group (3.18 ± 0.30 versus 2.73 ± 0.30, p=0.001, Table 1). This difference was also observed in the two-month follow-up group, but statistical significance was marginal (p=0.055). Four Enterprise stents were deployed in the left CCA with diameters of 3.78 ± 0.55mm.

To evaluate the effect of the parent artery diameter on the experimental results, the PLLA stents were categorized into two groups (the small group ≤ 3mm, and the large group > 3mm) based on the parent artery diameter (Table 2). The large group showed less thrombogenicity (p=0.015 at two months, Figure 20) and less neointimal hyperplasia (p=0.020 at one month, Figure 21). However, all specimens in the small group at two-month follow-up were bare PLLA stents (Table 2), and the findings of less thrombogenicity at two months appeared to be related more to Ta ion implantation (p=0.066) than to the large parent artery diameter (p=0.204)

according to a two-way ANOVA test.

In the small group, the inflammatory response was relatively severe at one month, but this finding was not statistically significant (Figures 22 and 23).

제 4 장 Discussion

Our study aimed to investigate the biological effects of Ta ion implantation on the re-endothelialization and thrombogenicity of PLLA stents to assess whether this surface treatment technique can ameliorate the serious complications associated with bare PLLA stents. Although PLLA stents for cardiovascular applications have been approved by the Food and Drug Administration in the USA and have been used in clinical practice [27], they are currently being withdrawn from the market due to excessive inflammation and thrombus formation [4, 12, 19, 23]. Because these problems are caused by hypersensitive reactions to the PLLA surface in the vessel, biocompatible coatings have been demonstrated as an effective and suitable treatment capable of reducing the risk [25]. In particular, previous studies have revealed that the surface of Ta is hydrophilic

and biologically stable, enabling favorable interaction between endothelial cells and blood proteins [1, 15].

Tantalum (Ta) is a well-known biocompatible metal used in various medical applications, of which oxides enhance its hemocompatibility by preventing the charge transfer of fibrinogen in the blood, resulting in the restricted formation of thrombosis [3]. It was reported relatively few metal ions rarely cause cytotoxicity, allergies, or other biological side-effects [6]. Figure 10 implies that Ta implantation is applicable to biodegradable materials because fine Ta particles are released out and become partially dissolved and transported with normal circulation.

In this study, we confirmed that the surfaces of the PLLA stents were successfully modified with Ta ion implantation. Sputtered Ta ions from a target gun were implanted into the topmost surface of the PLLA stent, forming a Ta-implanted skin layer with a thickness of around 10 nm on its surface (Figure 9). After the Ta ion implantation treatment, the PLLA stent maintained its original 3D geometry and surface flatness without any micro-scale unevenness or defects (Figure 5), which should minimize turbulent intraluminal blood flows and provide a stable environment at the implantation site [26].

In vitro studies showed that Ta ion implantation substantially improved the endothelialization ability of the PLLA stent, whereas

platelet attachment was suppressed on its surface. These favorable interactions of the Ta-implanted PLLA stent with cells can enhance the *in vivo* biocompatibility.

Prior to a discussion with histological results, we would like to highlight a practical difficulty which arises during the preparation of histologic specimens. In general, resin embedding is used in intravascular stent experiments to maintain the integrity of the stent structure. However, in this study, relatively weak paraffin embedding was selected in most specimen preparation cases for the immunohistochemical analysis. In addition, removal of the stent strut was required for H&E staining and immunohistochemical analysis. Such a process inevitably leads to damage to normal arterial architecture [14]. Even when using resin embedding, it is important to use a tissue-preparation protocol optimized for stent specimens [5, 14]. Therefore, in order to obtain an appropriate specimen for a stent study, careful attention is required in the histological sample preparation process.

In addition, the relatively small size of the PLLA stent (3 x 10 mm) also complicated the maintenance of the original shape. However, vasoconstriction was also identified in the Enterprise stent specimens (Figure 16c). This phenomenon appears to be related to the use of potassium chloride when euthanizing the animals.

Potassium chloride is well known to induce vasoconstriction, and this may be another cause of the arterial collapse of the stent specimens in this study [18]. Therefore, when euthanizing animals in a stent experiment, it would be better to use other methods such as the inhalation of an anesthetic or carbon dioxide, which do not induce vasoconstriction.

Despite the fact that the histologic examination showed what appeared to be an arterial occlusion in a segment of the stent, the angiographic results clearly indicated that both stents were widely patent without any signs of in-stent stenosis. Therefore, it should be reasonable to conclude that the angiographic results are more reliable with respect to the long-term patency rate of the stent.

Anti-thrombogenicity is a crucial characteristic of a hydrophobic PLLA polymer stent, as it allows the suppression of platelet aggregation and prevents in-stent restenosis or late thrombosis, leading possibly to clinical failures. Although there were no differences found during the one-month follow-up assessment, less thrombogenicity was identified at the follow-up exam after two months in a histologic analysis. Interpretation of this result is described below.

A critical limitation of the *in vivo* test appears to be the use of a balloon to deploy the PLLA stent in the artery. We used coronary

balloons with a diameter of 4mm, greater by 1mm than the stent diameter used to reduce the recoil phenomenon of PLLA stents caused by the mechanical properties of PLLA. The diameter of the ECA was 3.20 ± 0.55mm; therefore, balloon inflation for the deployment of the PLLA stent may damage the arterial wall. As a result, the evaluation of thrombosis may have been affected by arterial damage during an acute period. However, over time, the arterial wall would have healed. The result of thrombogenicity in the two-month follow-up assessment appears to stem from the healing of the damaged arterial wall. Under such conditions, the Ta-implanted PLLA stent showed less thrombogenicity, in good agreement with the results of the *in vitro* experiment.

However, overall thrombogenicity in this experiment was higher than expected, which may be due to the characteristics of the experimental animals. In general, the platelet activity of swine is similar to that of humans, which is why swine are widely used in various medical device experiments. However, Dewanjee et al. reported that the platelet thrombogenicity of swine was much lower than that of the canine model but tended to be higher when compared to humans [5]. Therefore, it is necessary to consider the characteristics of experimental animals in future studies.

Rapid endothelialization after stent deployment is essential to prevent

intimal hyperplasia or restenosis. In the evaluation of endothelialization (CD 31, CD 34) and the tissue response (anti-smooth muscle cell alpha actin), there were no differences between the PLLA stent groups. It appears that the effect of arterial damage due to the inflation of the balloon is more prevalent than that of the PLLA stent itself. In addition, the longest follow-up duration after stent deployment was only three months in this study, which may be too short a period of time to assess endothelialization. The results of CD 68(+) cells, which represent inflammation, also appear to be strongly influenced by balloon-induced arterial wall damage. If we can increase the number of experimental animals for an acute-phase evaluation and minimize damage to blood vessels as caused by the balloons, more significant results can be gained.

In experiments involving balloon-mounted stents, the diameter of the parent artery can have a considerable influence on the experimental results. In this experiment, it was identified that Ta-implanted PLLA stents were deployed in the parent arteries with a larger diameter in the one-month and two-month follow-up groups. This is one of the important limitations of this study. Because these stents were deployed in an artery with a small diameter, it was difficult to maintain the diameter of the parent artery consistently during the experiment.

The fact that Ta-implanted PLLA stents were deployed in the parent artery with a relatively large diameter may have affected the outcomes of the thrombogenicity, neointimal hyperplasia, and inflammatory response. A significant ballooning effect according to the difference in the diameter of the parent artery can be expected to appear upon one-month follow-up, which is a relatively acute phase. Therefore, the differences in neointimal hyperplasia and the inflammatory response in the acute phase at one month appear to have been influenced more by the difference in the diameter of the parent artery than by Ta ion implantation.

However, less thrombogenicity at two months was noted after the acute phase had passed, and it is judged that the effect of Ta ion implantation was greater than the effect of the diameter of the parent artery. Of course, these results suggest that it is difficult completely to exclude effects caused by the difference in the parent artery diameter, making it necessary for future researchers to pay attention to the parent artery diameter to obtain accurate results.

Compared to the Enterprise stent, which is a widely used Nitinol self-expandable stent, the PLLA stent showed poor mechanical properties and poor biocompatibility, even after Ta-ion implantation. The Enterprise stent was superior in terms of neointimal hyperplasia, inflammation and thrombogenicity. It appears that Ta-ion

implantation cannot sufficiently improve the intrinsic physical and chemical properties of the PLLA. Of course, there are critical limitations associated with an immunohistochemical analysis. The PLLA stent was deployed by balloon inflation and the Enterprise stent was self-expandable. Therefore, it is possible that an arterial injury caused by balloon inflation affected the results of the immunohistochemical analysis.

This preclinical study of Ta-implanted PLLA stents presents some implications for future research on biodegradable PLLA stents in the cerebrovascular field. In a swine model, the balloon-mounted PLLA stent, which has a similar navigability compared to a conventional balloon-mounted coronary stent, was easily navigated to the external carotid artery with a 6 Fr guiding catheter. Of course, the arterial anatomy of the swine was straight, and the navigability of the stent was not an issue. More flexibility may be needed for application to the cerebral arteries to overcome the tortuosity of the internal carotid artery. Further research is therefore needed to improve the navigability of the stent.

The use of balloons may not be suitable, especially for the treatment of an intracranial aneurysm patient owing to the high risk of arterial damage during the deployment of the stent. Therefore, it is necessary to create a type of self-expandable stent to minimize

intraoperative vascular injuries. In order to make a biocompatible and self-expandable stent with PLLA, it is necessary to modify not only the surface biological properties but also the bulk mechanical strength of the PLLA, including the elastic modulus, toughness, and strain-to-failure levels. In previous studies, blending with several biopolymers such as cellulose, abaca fibers, chitosan, polyvinyl acetate, and low-density polyethylene has shown the ability to enhance the mechanical properties of PLLA [2, 20, 24]. We expect that a better understanding of the biological performance capabilities of Ta-implanted PLLA stents will be achieved by developing these devices in a self-expandable form, followed by testing in an *in vivo* model in the future.

제 5 장 Conclusion

A novel strategy for developing a more biocompatible and less thrombogenic PLLA stent has been demonstrated. The Ta ion implantation technique was able to produce a Ta-implanted surface layer without any deformation or distortion of the PLLA stent. The biological properties of the PLLA stent became remarkably more

favorable by enhancing endothelial cell responses and suppressing platelet adhesion. The swine *in vivo* study conducted here confirmed the lower level of thrombogenicity of the Ta-implanted PLLA stent as compared to that with a bare stent. However, the Ta-implanted PLLA stent is judged to be still insufficient in terms of biocompatibility than Nitinol self-expandable stent.

참고 문헌

1. Barbarash LS, Bolbasov EN, Antonova LV, Matveeva VG, Velikanova EA, Shesterikov EV, et al.: Surface modification of poly-ε-caprolactone electrospun fibrous scaffolds using plasma discharge with sputter deposition of a titanium target. **Materials Letters** 171: 87-90, 2016.
2. Bledzki AK, Jaszkiewicz A, Scherzer D: Mechanical properties of PLA composites with man-made cellulose and abaca fibres. **Composites Part A: Applied Science and Manufacturing** 40: 404-412, 2009.
3. Chen JY, Leng YX, Tian XB, Wang LP, Huang N, Chu PK, et al.: Antithrombogenic investigation of surface energy and optical bandgap and hemocompatibility mechanism of Ti(Ta(+5))O2 thin films. **Biomaterials** 23: 2545-2552, 2002.
4. Collet C, Asano T, Miyazaki Y, Tenekecioglu E, Katagiri Y, Sotomi Y, et al.: Late thrombotic events after bioresorbable scaffold implantation: a systematic review and meta-analysis of randomized clinical trials. **Eur Heart J** 38: 2559-2566, 2017.
5. Dewanjee MK, Kapadvanjwala M, Sanchez A, Elson R, Serafini AN, Zilleruelo GE, et al.: Quantitation of comparative thrombogenicity of dog, pig, and human platelets in a

hemodialyzer. ASAIO J 38: 88-90, 1992.

6. Hanawa T: Metal ion release from metal implants. Mat Sci Eng C-Bio S 24: 745-752, 2004.

7. Hou L-D, Li Z, Pan Y, Sabir M, Zheng Y-F, Li L: A review on biodegradable materials for cardiovascular stent application. Frontiers of Materials Science 10: 238-259, 2016.

8. Hu T, Yang C, Lin S, Yu Q, Wang G: Biodegradable stents for coronary artery disease treatment: Recent advances and future perspectives. Mater Sci Eng C Mater Biol Appl 91: 163-178, 2018.

9. Hwang G, Kim JG, Song KS, Lee YJ, Villavicencio JB, Suroto NS, et al.: Delayed ischemic stroke after stent-assisted coil placement in cerebral aneurysm: characteristics and optimal duration of preventative dual antiplatelet therapy. Radiology 273: 194-201, 2014.

10. Ikarashi Y, Toyoda K, Ohsawa N, Uchima T, Tsuchiya T, Kaniwa M, et al.: Comparative studies by cell culture and in vivo implantation test on the toxicity of natural rubber latex materials. J Biomed Mater Res 26: 339-356, 1992.

11. Jin W, Wang G, Lin Z, Feng H, Li W, Peng X, et al.: Corrosion resistance and cytocompatibility of tantalum-surface-functionalized biomedical ZK60 Mg alloy. Corrosion Science

114: 45-56, 2017.

12. Kereiakes DJ, Ellis SG, Metzger C, Caputo RP, Rizik DG, Teirstein PS, et al.: 3-Year Clinical Outcomes With Everolimus-Eluting Bioresorbable Coronary Scaffolds: The ABSORB III Trial. J Am Coll Cardiol 70: 2852-2862, 2017.
13. Krischek O, Miloslavski E, Fischer S, Shrivastava S, Henkes H: A comparison of functional and physical properties of self-expanding intracranial stents [Neuroform3, Wingspan, Solitaire, Leo+, Enterprise]. Minim Invasive Neurosurg 54: 21-28, 2011.
14. Malik N, Gunn J, Holt CM, Shepherd L, Francis SE, Newman CM, et al.: Intravascular stents: a new technique for tissue processing for histology, immunohistochemistry, and transmission electron microscopy. Heart 80: 509-516, 1998.
15. Nie FL, Zheng YF, Wang Y, Wang JT: Microstructures, mechanical behavior, cellular response, and hemocompatibility of bulk ultrafine-grained pure tantalum. Journal of Biomedical Materials Research - Part B Applied Biomaterials 102: 221-230, 2014.
16. Park C, Seong YJ, Kang IG, Song EH, Lee H, Kim J, et al.: Enhanced Osseointegration Ability of Poly(lactic acid) via Tantalum Sputtering-Based Plasma Immersion Ion

Implantation. ACS Appl Mater Interfaces 11: 10492-10504, 2019.

17. Pizzoferrato A, Ciapetti G, Savarino L, Stea S, Tarabusi C: Results of histological grading on 100 cases of hip prosthesis failure. Biomaterials 9: 314-318, 1988.

18. Qin GQ, Chen LW, Sjoberg T, Steen S: How to avoid severe coronary vasoconstriction in potassium induced cardioplegia. Scand Cardiovasc J 52: 344-347, 2018.

19. Raber L, Brugaletta S, Yamaji K, O'Sullivan CJ, Otsuki S, Koppara T, et al.: Very Late Scaffold Thrombosis: Intracoronary Imaging and Histopathological and Spectroscopic Findings. J Am Coll Cardiol 66: 1901-1914, 2015.

20. Rasal RM, Janorkar AV, Hirt DE: Poly(lactic acid) modifications. Progress in Polymer Science 35: 338-356, 2010.

21. Schwartz RS, Huber KC, Murphy JG, Edwards WD, Camrud AR, Vlietstra RE, et al.: Restenosis and the proportional neointimal response to coronary artery injury: results in a porcine model. J Am Coll Cardiol 19: 267-274, 1992.

22. Sharkawi T, Cornhill F, Lafont A, Sabaria P, Vert M: Intravascular bioresorbable polymeric stents: A potential

alternative to current drug eluting metal stents. **Journal of Pharmaceutical Sciences** 96: 2829-2837, 2007.

23. Stone GW, Ellis SG, Gori T, Metzger DC, Stein B, Erickson M, et al.: Blinded outcomes and angina assessment of coronary bioresorbable scaffolds: 30-day and 1-year results from the ABSORB IV randomised trial. **Lancet** 392: 1530-1540, 2018.

24. Suyatma NE, Copinet A, Tighzert L, Coma V: Mechanical and Barrier Properties of Biodegradable Films Made from Chitosan and Poly (Lactic Acid) Blends. **Journal of Polymers and the Environment** 12: 1-6, 2004.

25. Tverdokhlebov SI, Bolbasov EN, Shesterikov EV, Antonova LV, Golovkin AS, Matveeva VG, et al.: Modification of polylactic acid surface using RF plasma discharge with sputter deposition of a hydroxyapatite target for increased biocompatibility. **Applied Surface Science** 329: 32-39, 2015.

26. Wang J, Jin X, Huang Y, Ran X, Luo D, Yang D, et al.: Endovascular stent-induced alterations in host artery mechanical environments and their roles in stent restenosis and late thrombosis. **Regenerative Biomaterials** 5: 177-187, 2018.

27. Wiebe J, Nef HM, Hamm CW: Current status of bioresorbable scaffolds in the treatment of coronary artery disease. **J Am**

Coll Cardiol 64: 2541-2551, 2014.

초 록

탄탈럼 이온 주입을 통한 동맥내 폴리-L-젖산 스텐트의 생체적합성 평가

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목적

탄탈럼(Ta) 이온 주입을 통해 생체 적합성이 높은 표면을 가지게 된 생분해성 폴리-L-젖산(PLLA)은 현재 생분해성 스텐트가 가진 문제에 대한 혁신적인 해결 방안이 될 수 있다. 이 연구의 목적은 임상용 Ta-주입 PLLA 스텐트를 개발하고 생물학적 성능을 조사하는 것이다.

방법

PLLA 재료에 대한 Ta 이온 주입의 효과를 조직학적 검사, 면역조직화학 및 체외 실험들을 통해 평가하였다. 체외 환경에서 내피세포 및 혈소판 접착 테스트를 통해 재내피화 능력과 혈전형성도를 실험하였다. 체내 환경에서 Ta 이온 주입이 아급성 재협착 및 혈전형성에 미치는 영향을 평가하기 위해 돼지 모델을 사용하였다. 비교를 위해 Nitinol 자체 확장형 스텐트도 삽입되었다. 혈관 조영술 및 조직학적 평가는 시술 후 1, 2, 3개월에서 이루어졌다. 실험 결과에 미치는 영향을 평가하기 위해 모든

모동맥의 크기가 측정되었다.

결과

Ta-주입 PLLA 스텐트가 성공적으로 제작되었다. 스텐트의 매끄러운 표면 형태와 Ta주입을 통해 수정된 층이 잘 결합되어 있음을 확인하였다. Ta 이온 주입 후, 스텐트의 표면 특성은 생 PLLA 스텐트에 비해 빠른 내피화 및 혈소판이 덜 부착하는 성질을 보였다. 체내 동물 실험에서, 추적 혈관 조영술은 두 그룹 모두에서 스텐트내 협착의 증거를 보여주지 않았다. 스텐트 삽입 두 달 후 시행한 현미경 조직학적 검사에서 Ta-주입 PLLA 스텐트 그룹에서 혈관내 혈전 형성이 유의하게 억제되었다 (21.2% 대 63.9% p=0.005). 큰 모동맥 직경은 혈전 형성 억제 (p=0.015, 2개월 추적 관찰) 및 신내막증식 억제 (p=0.020, 1개월 추적 관찰)와 연관이 있었다. 그러나 2개월째 나타난 혈전 형성 억제는 큰 모동맥 직경 (p=0.204)보다 Ta 이온 주입 (p=0.066)과 더 관련이 있는 것으로 나타났다. Nitinol 자체 확장형 스텐트는 PLLA 스텐트 그룹에 비해 신생내막 증식, 염증 및 혈전 형성 측면에서 우수했다.

결론

Ta-주입 PLLA 스텐트는 생 PLLA 스텐트에 비해 재내피화 및 항혈전 능력 측면에서 유리한 것으로 보인다.

주요어 : 생분해, 이온주입, 폴리-L-젖산, 스텐트, 탄탈럼

학 번 : 2017-38238

Table 1. Parent artery diameter according to the follow-up period.

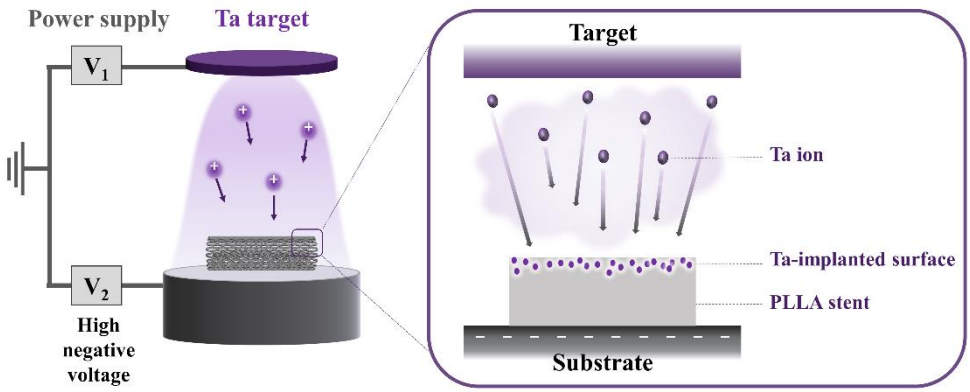
Follow-up period (n = number of slides)	Parent artery diameter
One month	p=0.001
Ta-implanted PLLA stent (n=12)	3.18 ± 0.30mm
Bare PLLA stent (n=16)	2.73 ± 0.30mm
Two months	p=0.055
Ta-implanted PLLA stent (n=8)	3.69 ± 0.19mm
Bare PLLA stent (n=8)	3.32 ± 0.44mm
Three months	p=0.147
Ta-implanted PLLA stent (n=8)	3.08 ± 0.52mm
Bare PLLA stent (n=6)	3.64 ± 0.80mm

Table 2. PLLA stents and the parent artery diameter. The numbers in the table indicate the number of tissue specimen slides in each group.

	Bare PLLA stent	Ta-implanted PLLA stent
Small group* (n=28)	20	8
One month	12	4
Two months	4	0
Three months	4	4
Large group† (n=30)	12	18
One month	4	8
Two months	4	8
Three months	4	2

The parent artery diameter is less than 3mm* and more than 3mm†.

Figure 1. Schematic illustration of the Ta implantation treatment on a PLLA stent. Ta ions were accelerated toward the surface of the PLLA stent, where high negative voltage was applied, forming a Ta-implanted polymer layer.①



① This figure is made by co-researcher (Park Suhyung, Department of Material Science and Engineering, Seoul National University).

Figure 2. PLLA stent manufactured by laser cutting. The PLLA stent is mounted on a coronary balloon with a gold marker, which is identified in the fluoroscopic image.

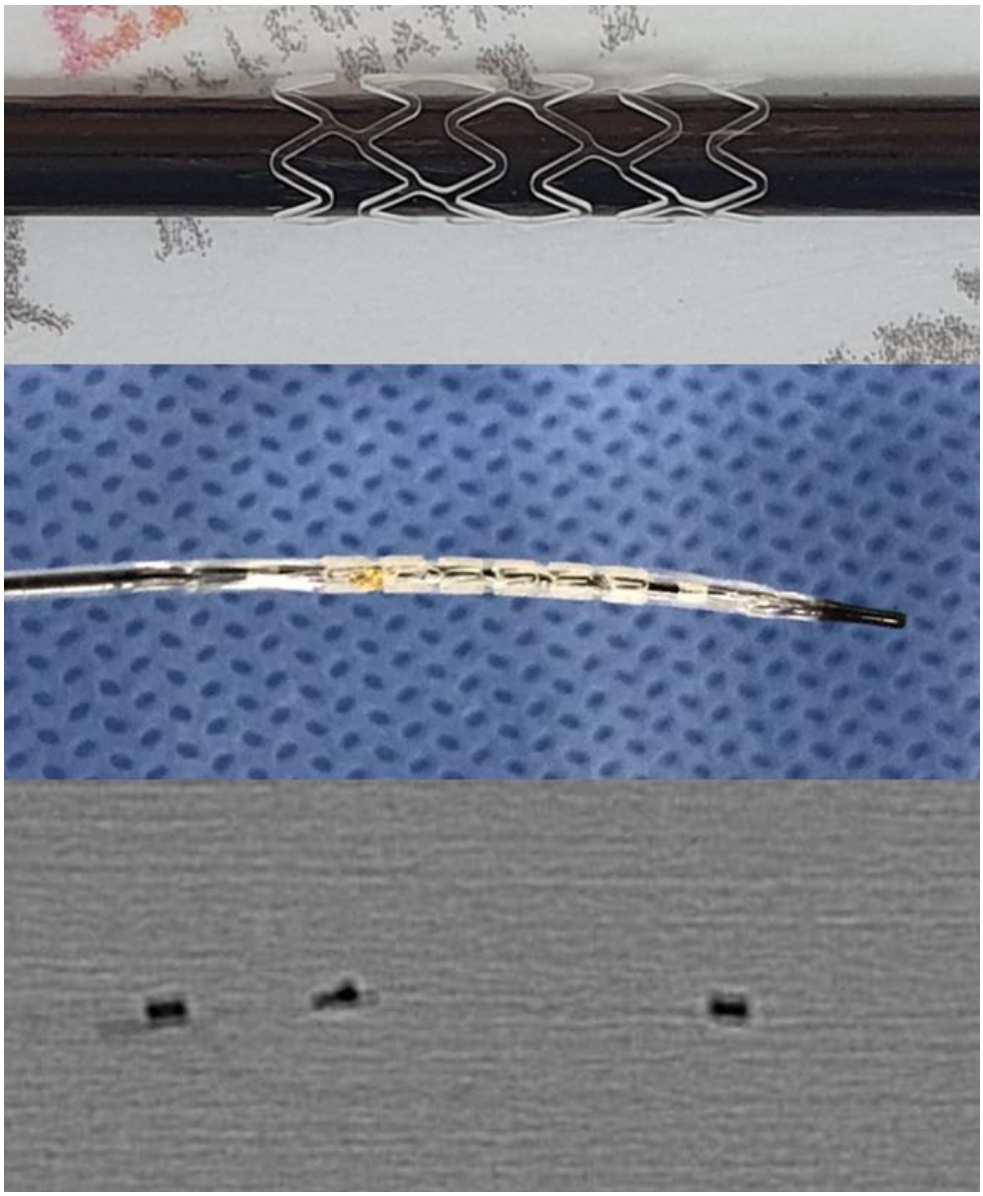


Figure 3. Selection of the size and inflation pressure of the balloon. The balloon size (4 mm) and pressure (10 atm) were selected by repetitive *in vitro* experiments.

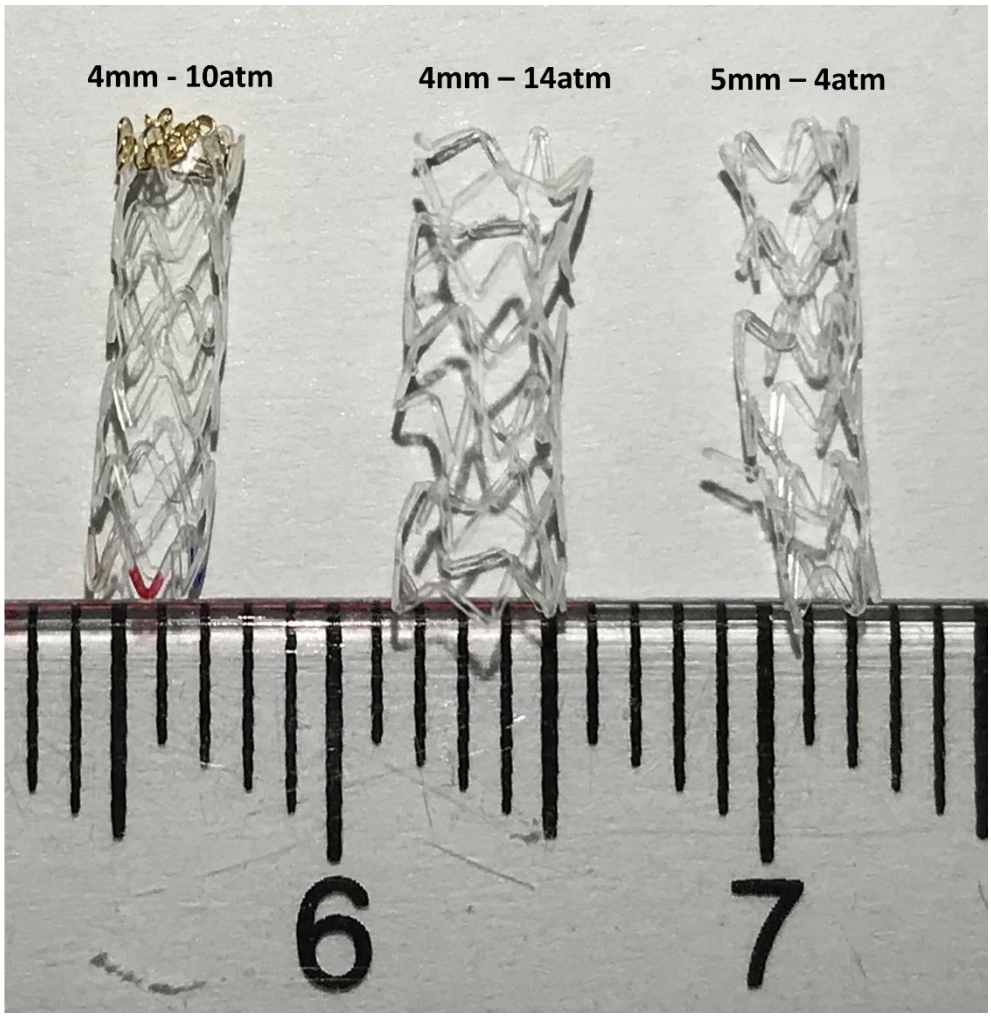


Figure 4. The carotid artery of the swine was exposed after sacrifice. One Ta-implanted stent at one-month follow-up was lost during the harvesting procedure.

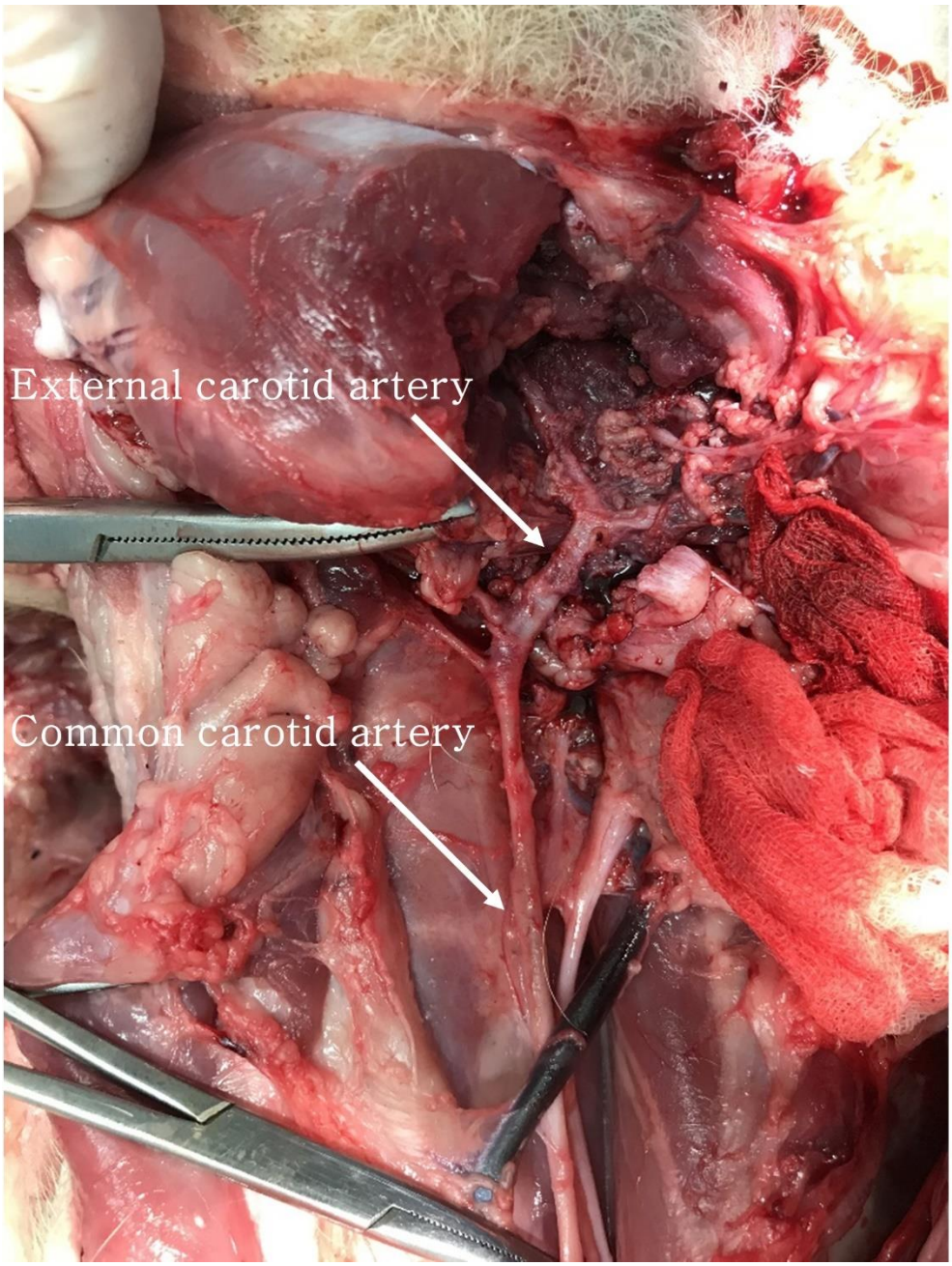
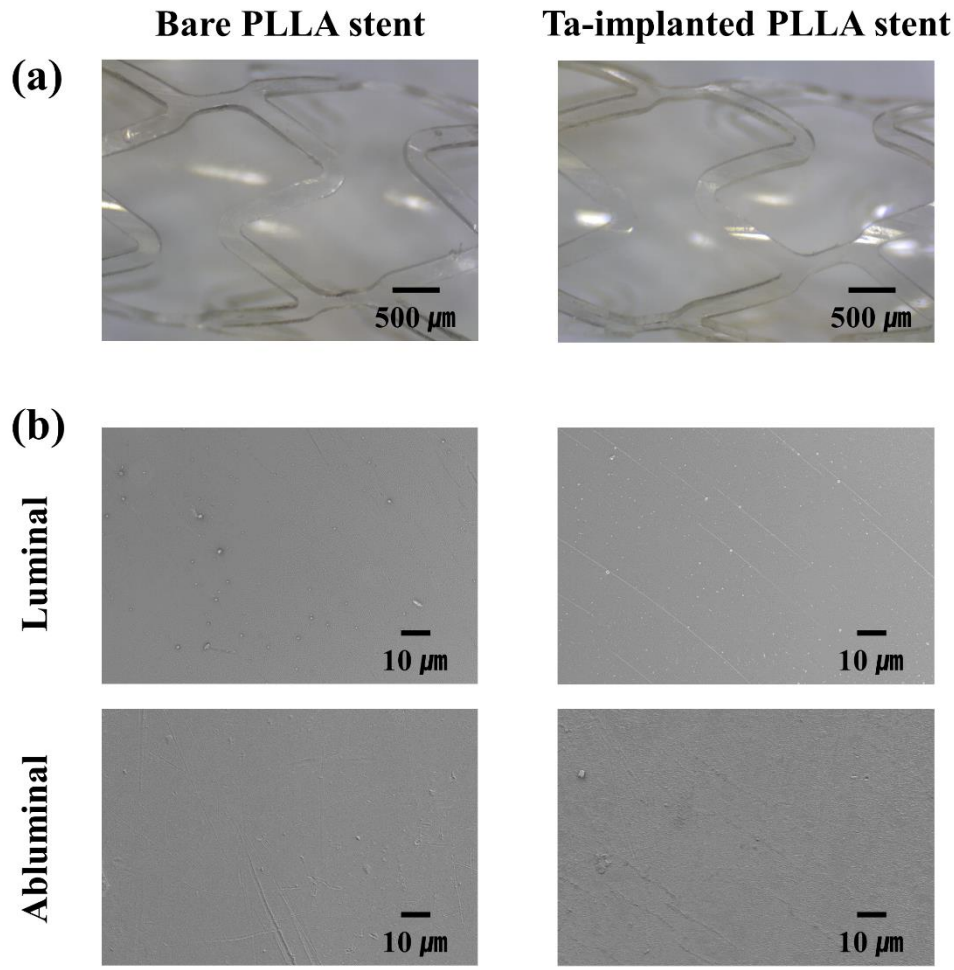


Figure 5. Surface morphology of the stent.[②]

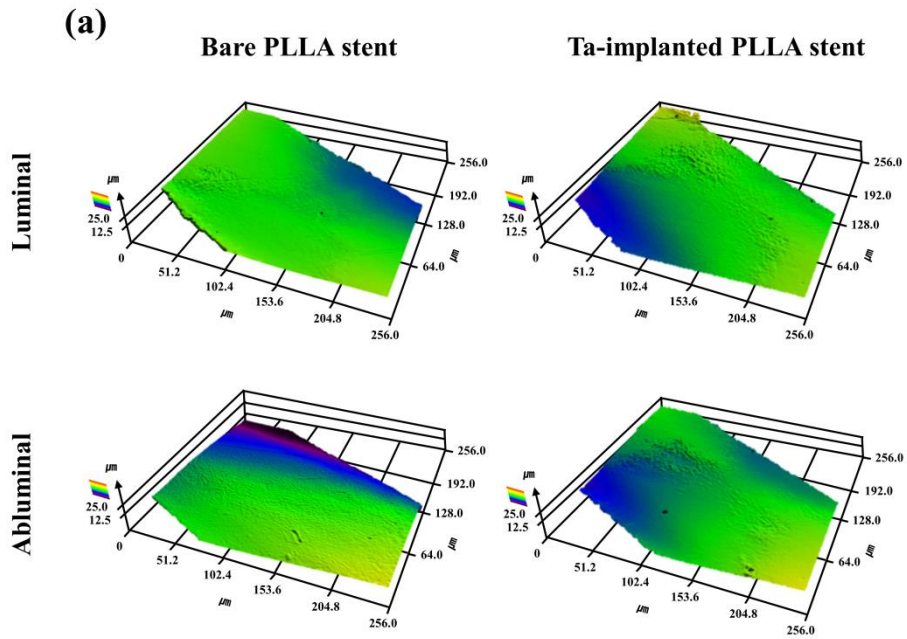
(a) Optical and (b) SEM images of bare and Ta-implanted PLLA stents.



[②] This figure is made by co-researcher (Park Suhyung, Department of Material Science and Engineering, Seoul National University).

Figure 6. Roughness of the stent.[③]

(a) Surface topography 3D maps of bare and Ta-implanted PLLA stents.



[③] This experiment was conducted by co-researcher (Park Suhyung, Department of Material Science and Engineering, Seoul National University).

(b) There is no significant difference in the calculated average roughness on each surface.

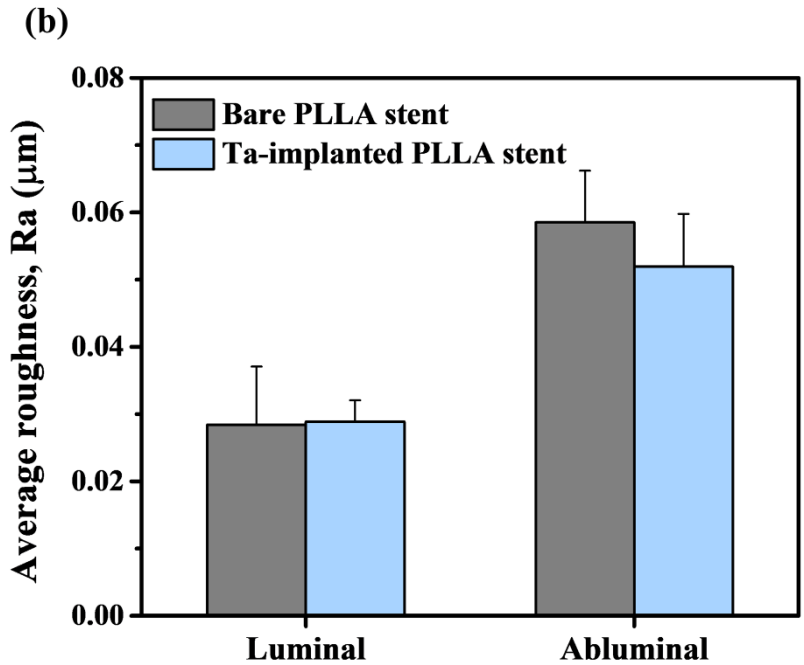
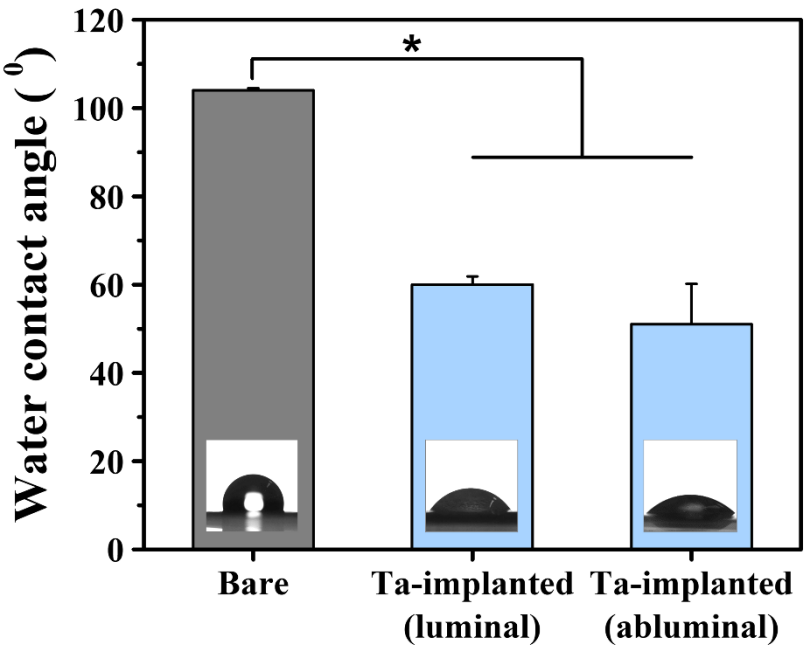


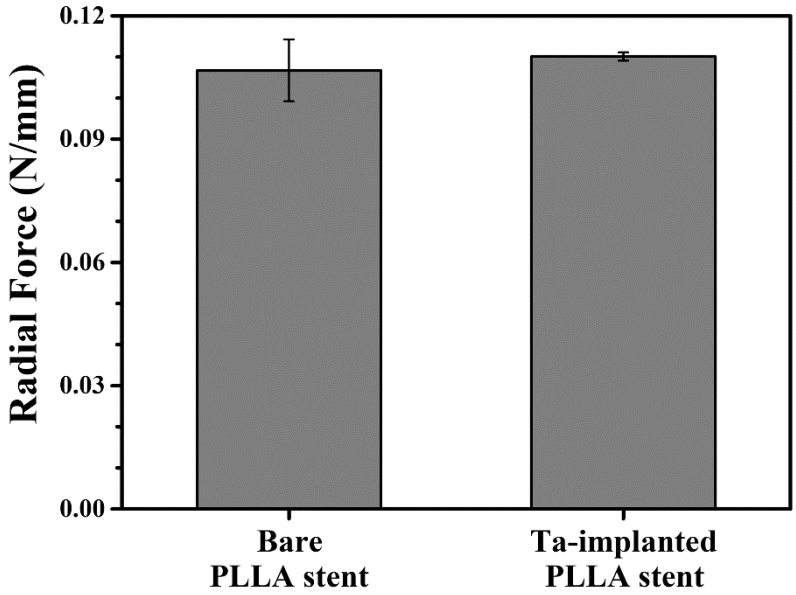
Figure 7. Water contact angle test.[④]

The Ta treatment increased the wettability of the stent (*p < 0.05).



[④] This experiment was conducted by co-researcher (Park Suhyung, Department of Material Science and Engineering, Seoul National University).

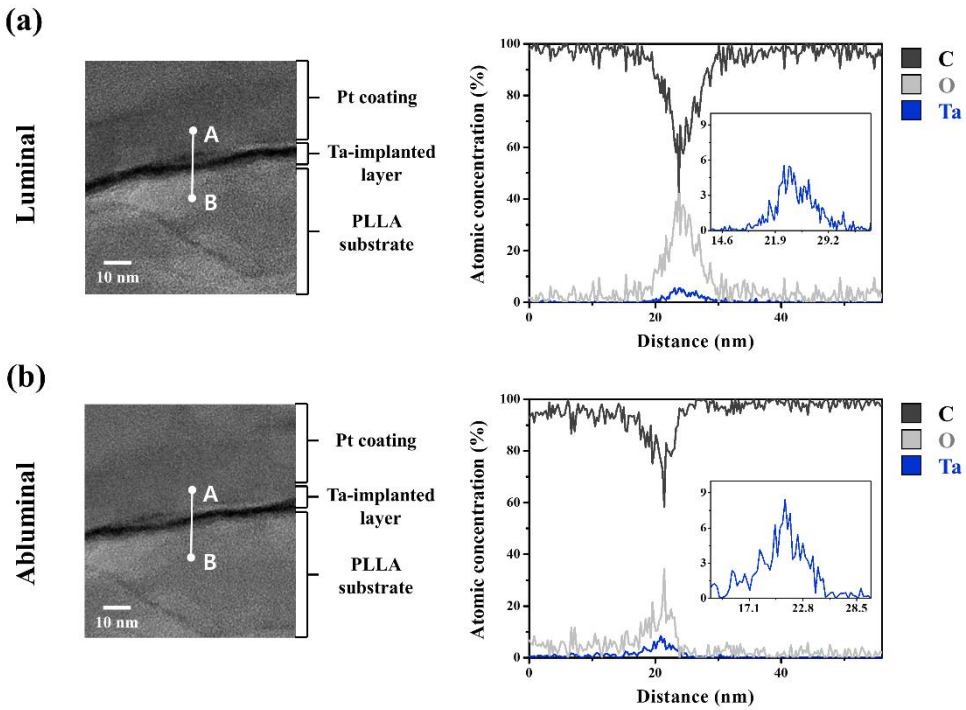
Figure 8. Radial force of the PLLA stent (No significant difference).[⑤]



[⑤] This experiment was conducted by co-researcher (Park Suhyung, Department of Material Science and Engineering, Seoul National University).

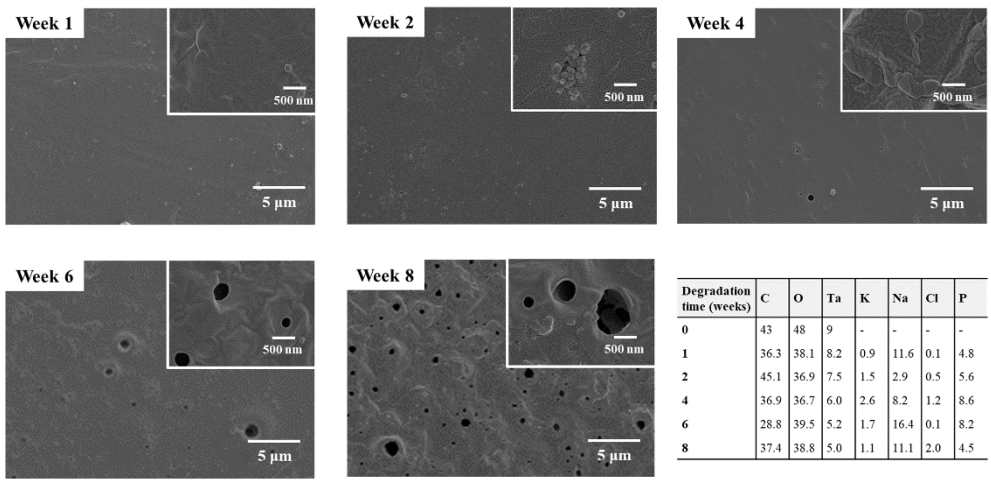
Figure 9. Tantalum layer of the PLLA stent.[⑥]

Representative cross-sectional TEM image and the TEM/EDS line profile with C, O, and Ta along the white line from points A to B on the (a) luminal surface and (b) the abluminal surface of the Ta-implanted PLLA stent.



[⑥] This experiment was conducted by co-researcher (Park Suhyung, Department of Material Science and Engineering, Seoul National University).

Figure 10. Representative SEM images and XPS surface chemical composition of Ta-implanted PLLA surfaces after degradation at different times.⑦

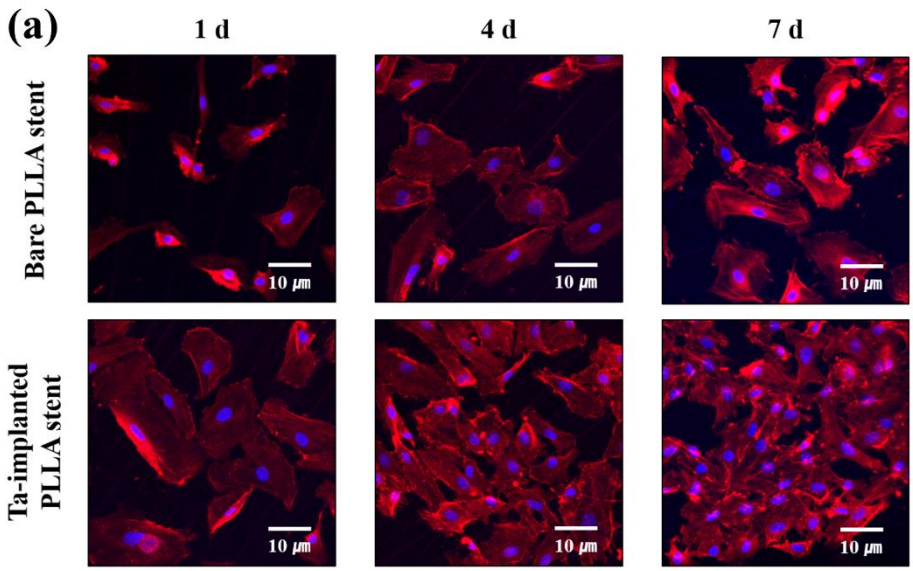


Degradation time (weeks)	C	O	Ta	K	Na	Cl	P
0	43	48	9	-	-	-	-
1	36.3	38.1	8.2	0.9	11.6	0.1	4.8
2	45.1	36.9	7.5	1.5	2.9	0.5	5.6
4	36.9	36.7	6.0	2.6	8.2	1.2	8.6
6	28.8	39.5	5.2	1.7	16.4	0.1	8.2
8	37.4	38.8	5.0	1.1	11.1	2.0	4.5

⑦ This experiment was conducted by co-researcher (Park Suhyung, Department of Material Science and Engineering, Seoul National University).

Figure 11. Endothelialization test.[⑧]

(a) CLSM images of adhered endothelial cells.



[⑧] This experiment was conducted by co-researcher (Park Suhyung, Department of Material Science and Engineering, Seoul National University).

(b) Surface coverage of endothelial cells and (c) cell viability on bare and Ta-implanted PLLA surfaces after one, four, and seven days of culturing, respectively (*p < 0.05, **p < 0.01).

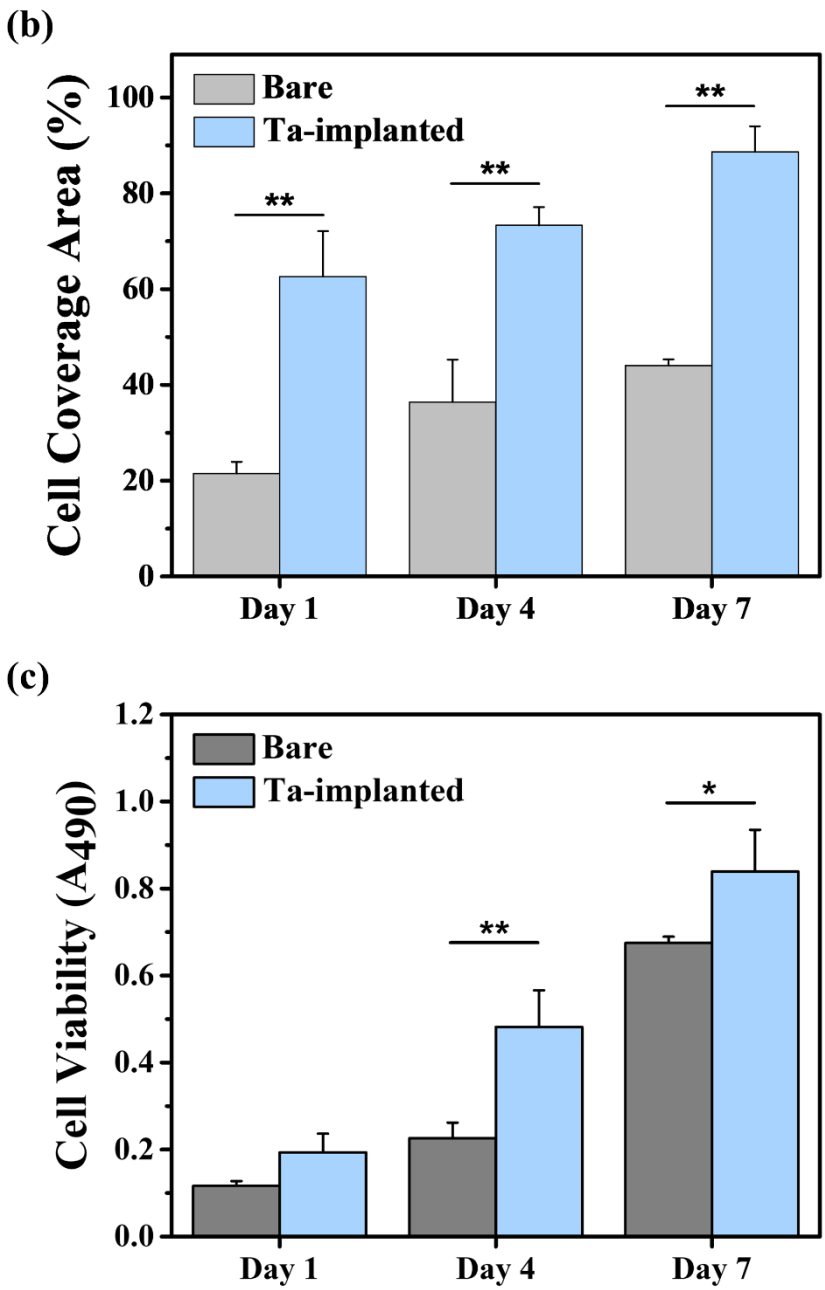
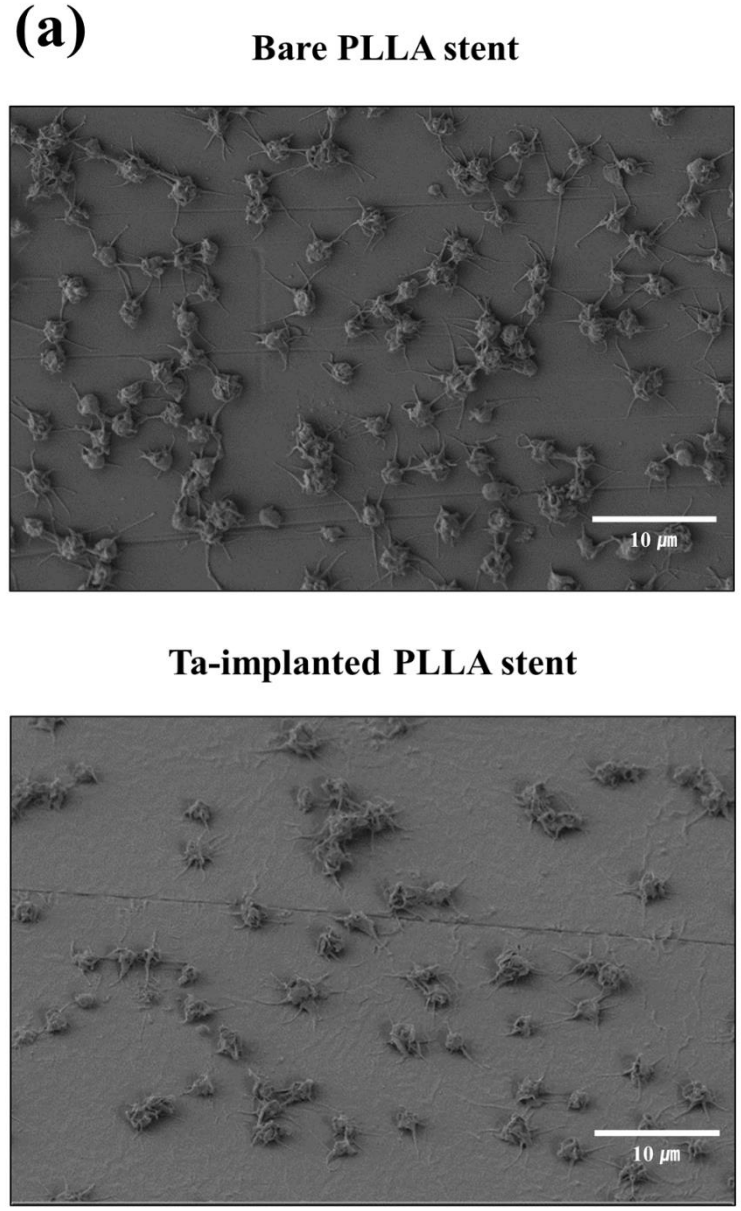


Figure 12. Platelet adhesion test.

(a) SEM images of the adhered platelet morphology on bare and Ta-implanted PLLA stents.[⑨]



[⑨] The SEM image was made by co-researcher (Park Suhyung, Department of Material Science and Engineering, Seoul National University).

(b) Absorbance difference between 492 nm and 690 nm of a platelet-lysed solution and (c) the number of adhered platelets on each PLLA stent from the LDH assay (*p < 0.05).

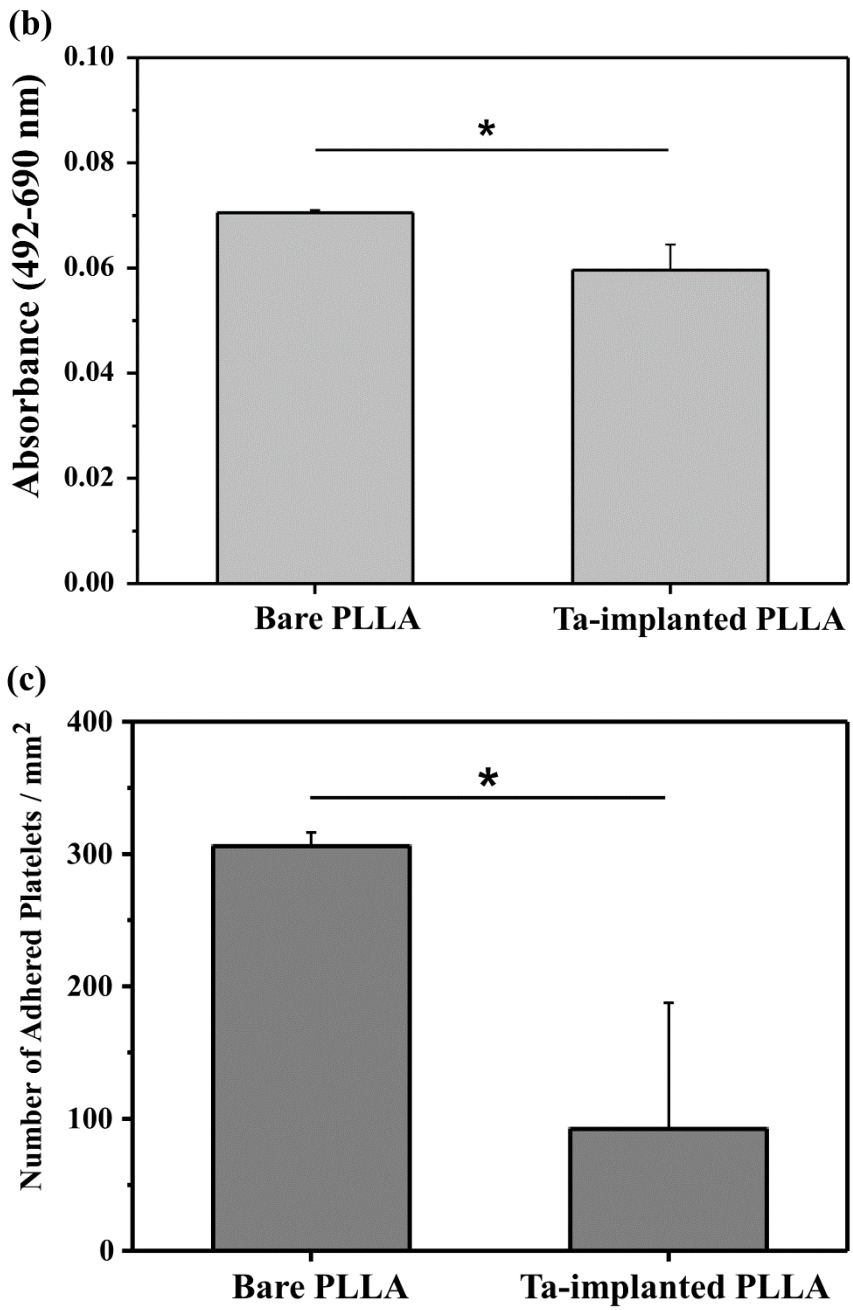


Figure 13. Angiographic and histologic results (x40 magnification) three months after stent deployment.

(a) All parent arteries are patent in the angiographic images without in-stent stenosis (right: Ta-implanted PLLA stent, left: bare PLLA stent).

(b) After tissue preparation and H&E staining with identical specimens, many specimens demonstrate what appears to be an arterial lumen collapse despite the lack of evidence of stenosis in the angiographic images. The discrepancy between the angiographic and histological results may be due to issues that arose during the tissue preparation step with paraffin embedding for the immunohistochemical analysis and stent characteristics such as the small diameter and weak radial force.

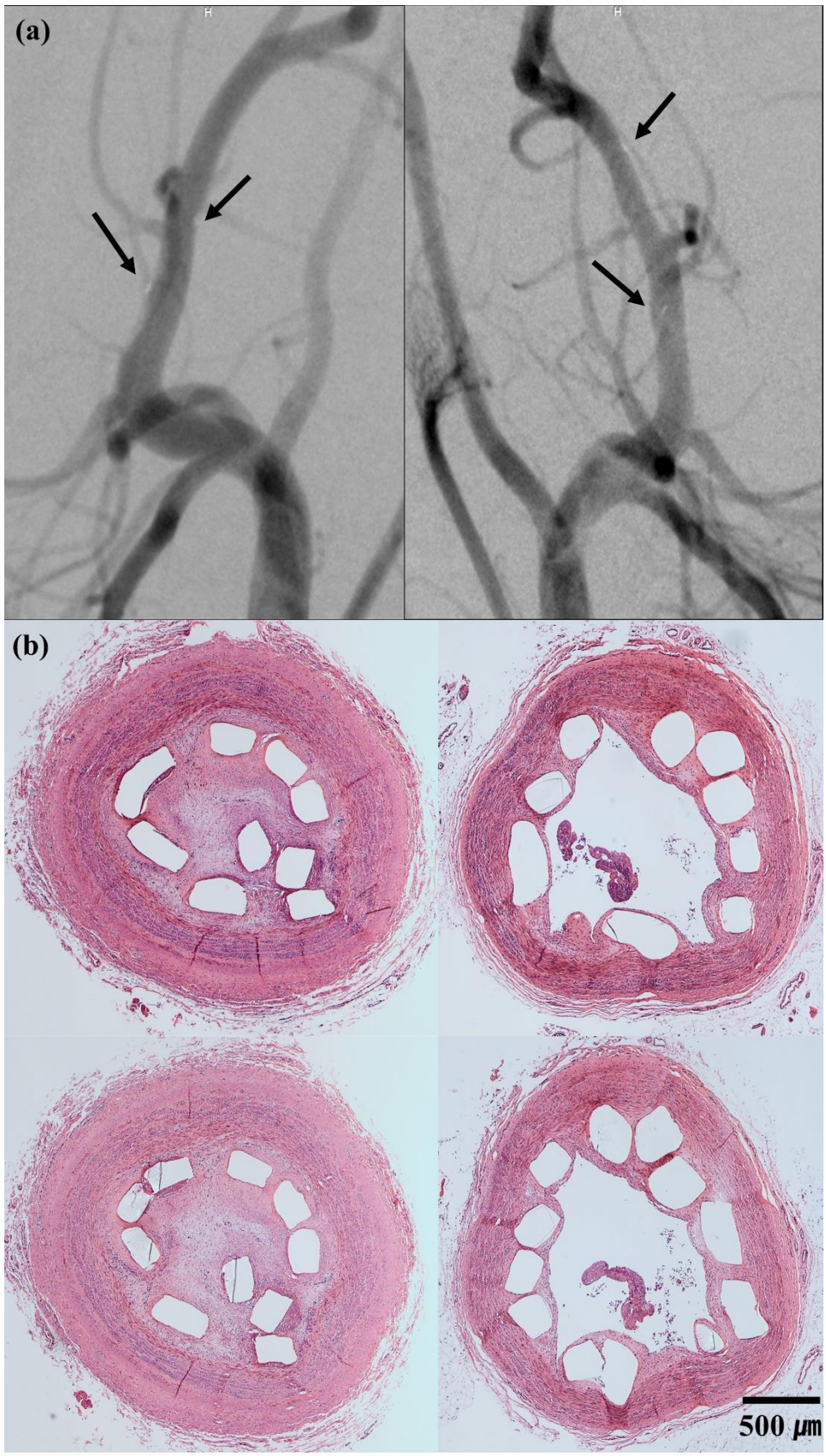

(a)
(b)
500 μm

Figure 14. *In vivo* thrombogenicity.

The Ta-implanted PLLA stent group shows less thrombus formation according to the two-month follow-up exam (21.2% versus 63.9%, p=0.005). The Enterprise stent showed low thrombogenicity one month after stent placement compared to the PLLA stents (p=0.000).

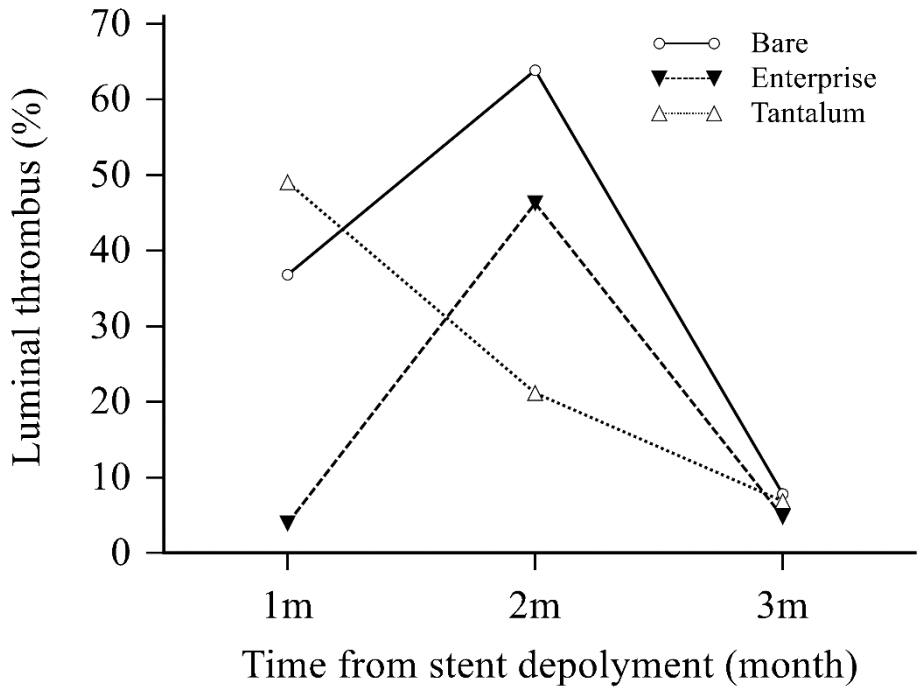


Figure 15. Neointimal hyperplasia.

The Enterprise stent shows a low degree of neointimal hyperplasia compared to that in the PLLA stent groups (p=0.001 and p=0.031 compared to the bare PLLA stent, p=0.000 and p=0.006 compared to the Ta-implanted PLLA stent at one and two-month follow-up, respectively)

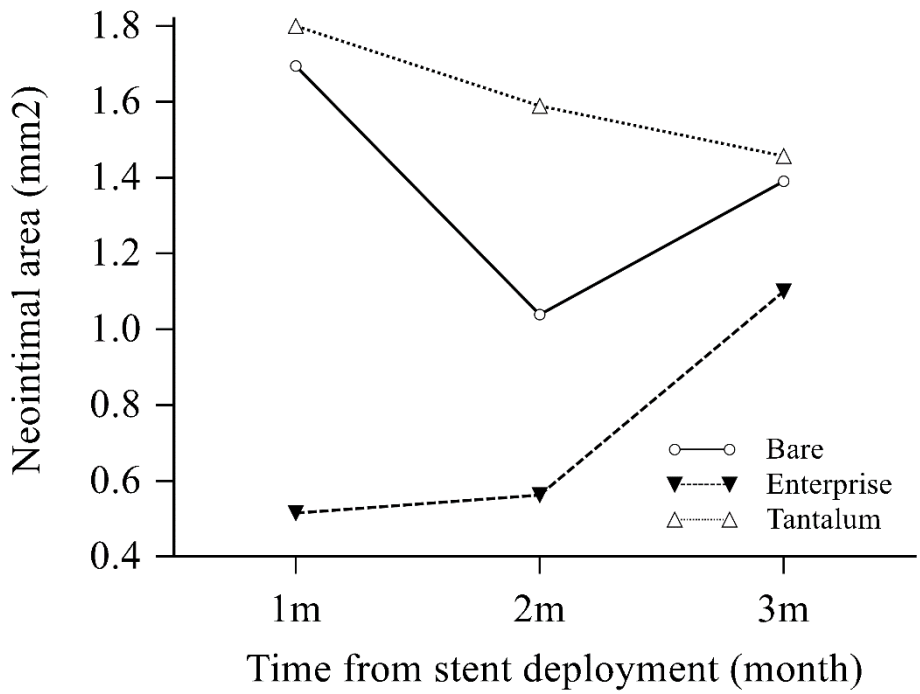
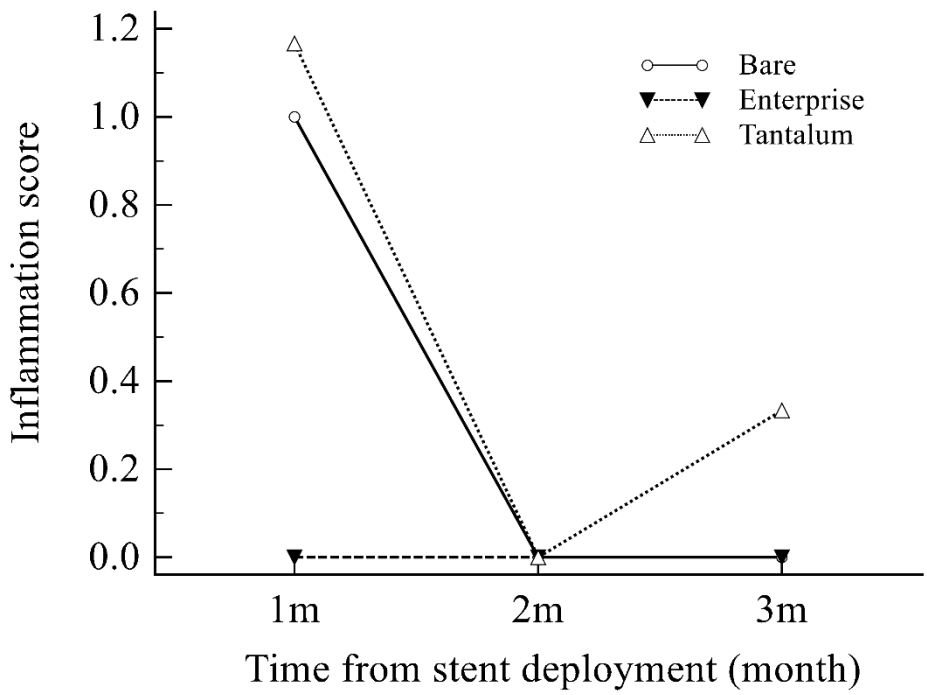
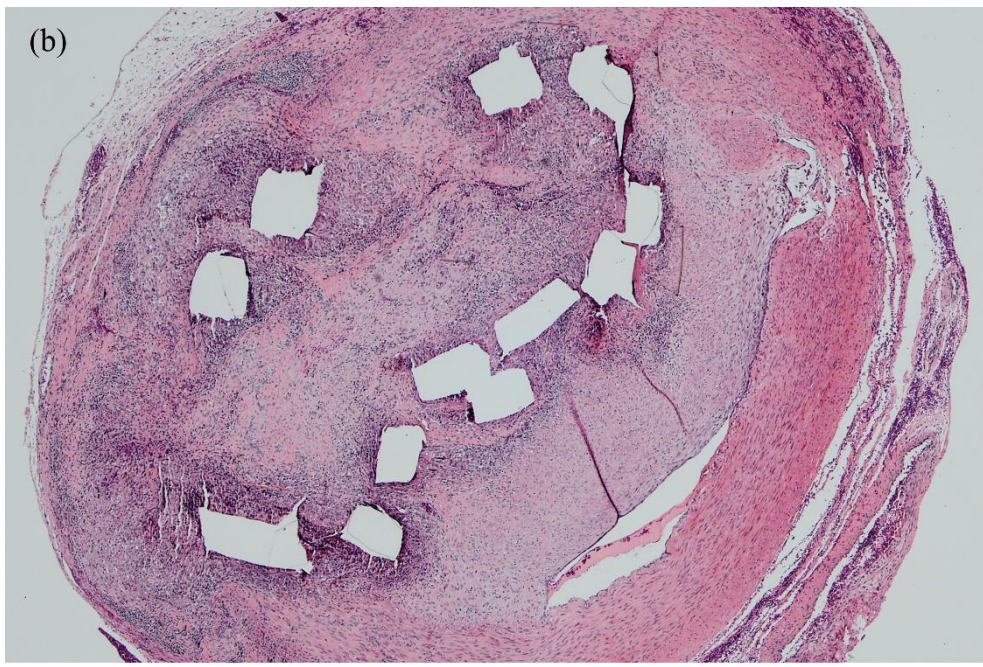


Figure 16. The inflammation score.

(a) The inflammation scores were found to be severe in both PLLA stent groups compared to the Enterprise stent group at one-month follow-up (p=0.006).

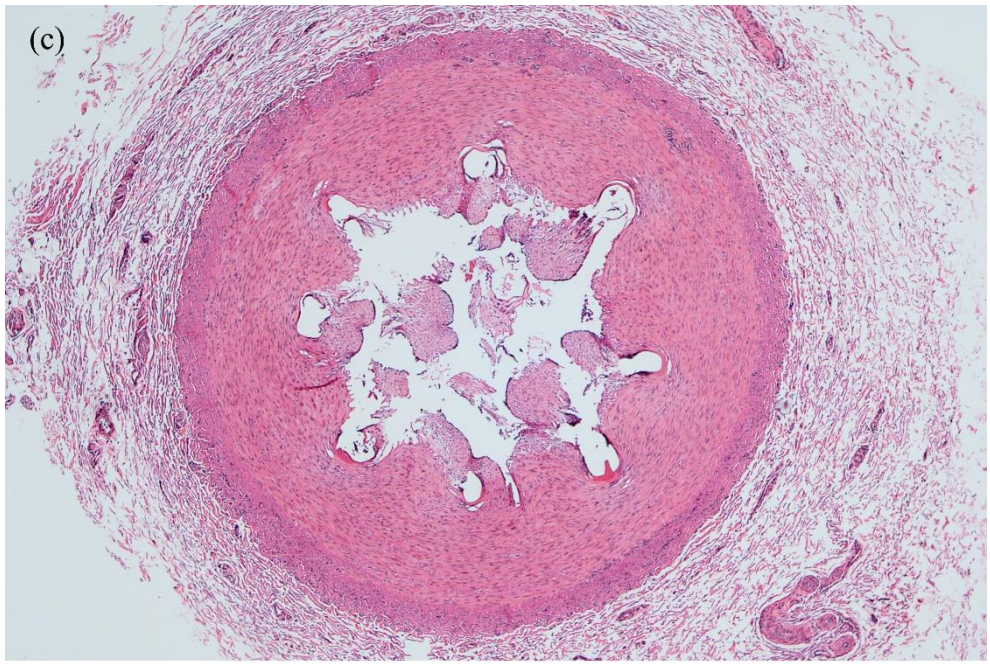


(b) Severe leukocyte infiltration is identified around the bare PLLA stent (one-month follow-up, x40 magnification).



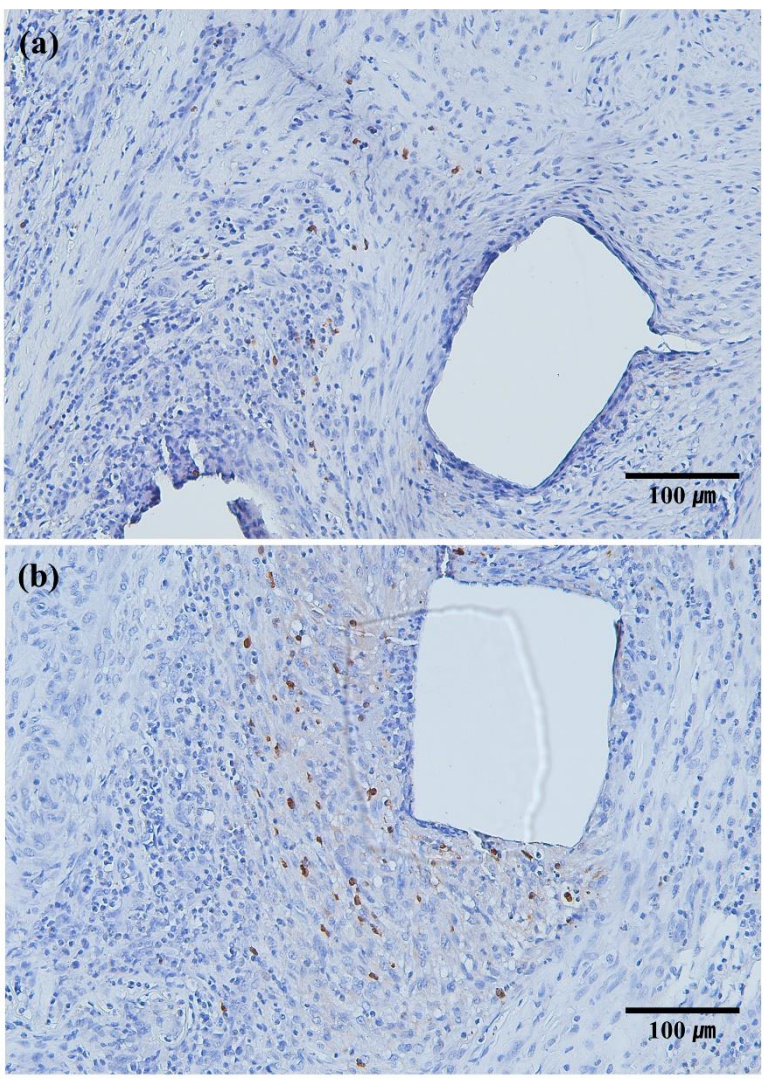
Inflammation, Bare PLLA stent

(c) On the other hand, there is no histological evidence of inflammation around the Enterprise stent (one-month follow-up, x40 magnification).



Inflammation, Enterprise stent

Figure 17. Immunohistochemical analysis of *in vivo* inflammation. Less of an inflammatory reaction is identified in the Ta-implanted PLLA stent group at one-month follow-up. The numbers of CD 68(+) cells / 400 HPF are 5.2±7.3 in the Ta-implanted PLLA stent group (a) and 9.0±16.2 in the bare PLLA stent group (b) (x200 magnification). However, these outcomes lack statistical significance (p=0.601).



(c) The inflammatory response decreases with time.

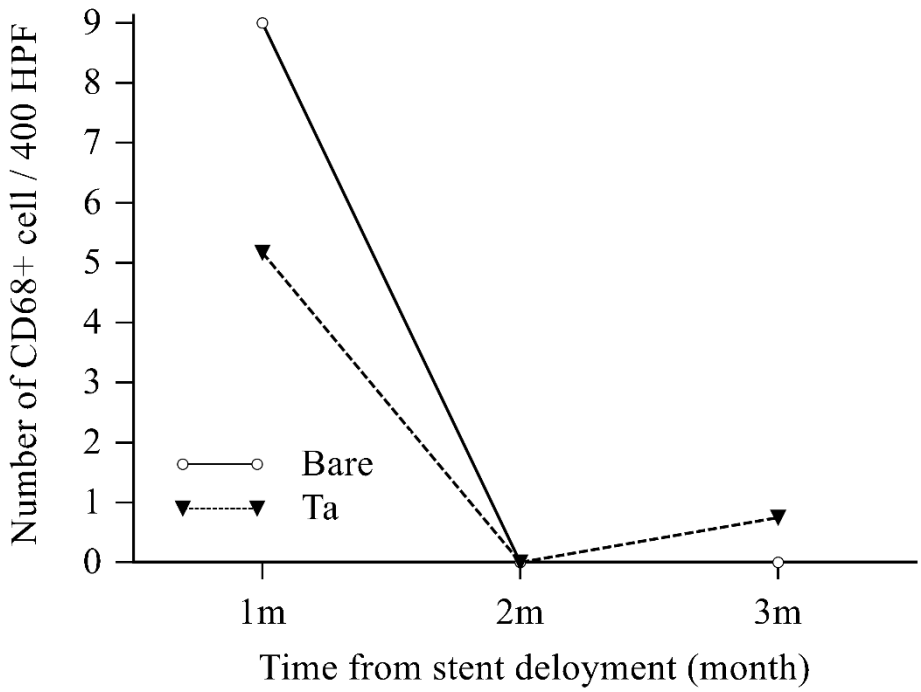
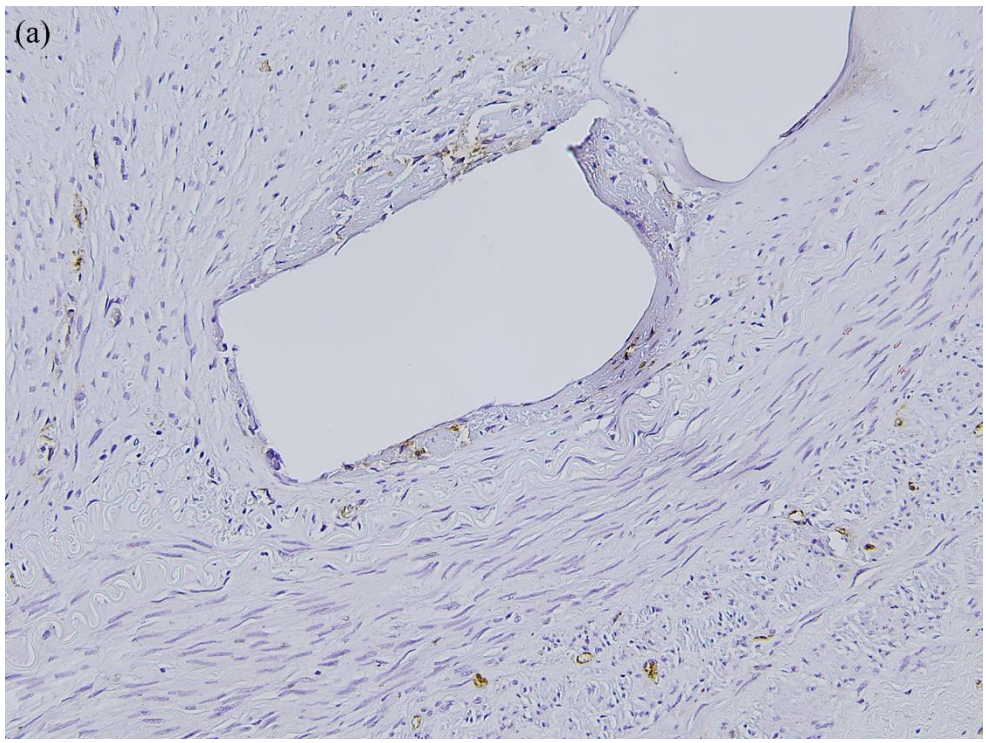


Figure 18. The expression of (a) CD 31 and (b) CD 34 around the Ta-implanted PLLA stent (three-month follow-up, x200 magnification). There was no definite evidence of endothelization of the stent. The result was similar in the bare PLLA and the Enterprise stent group.



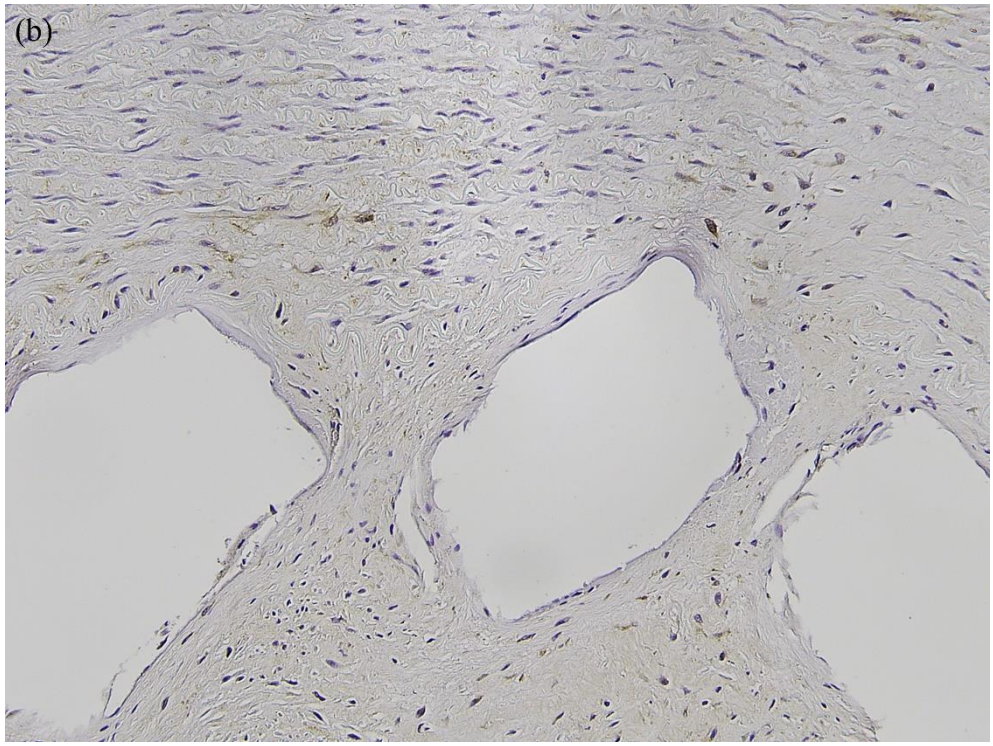
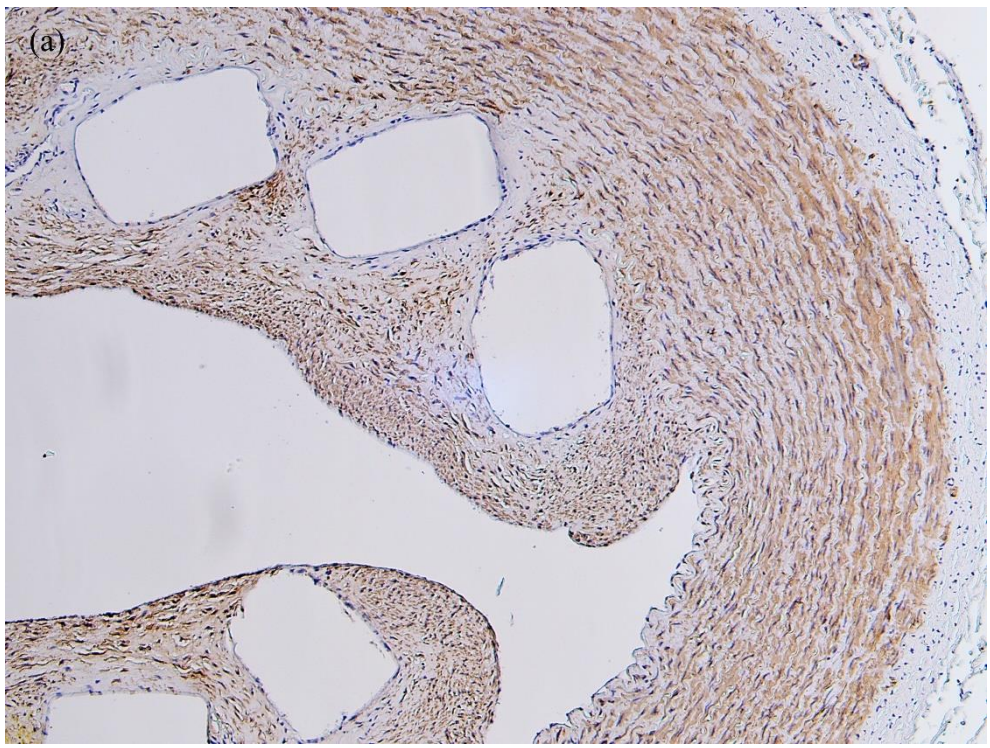
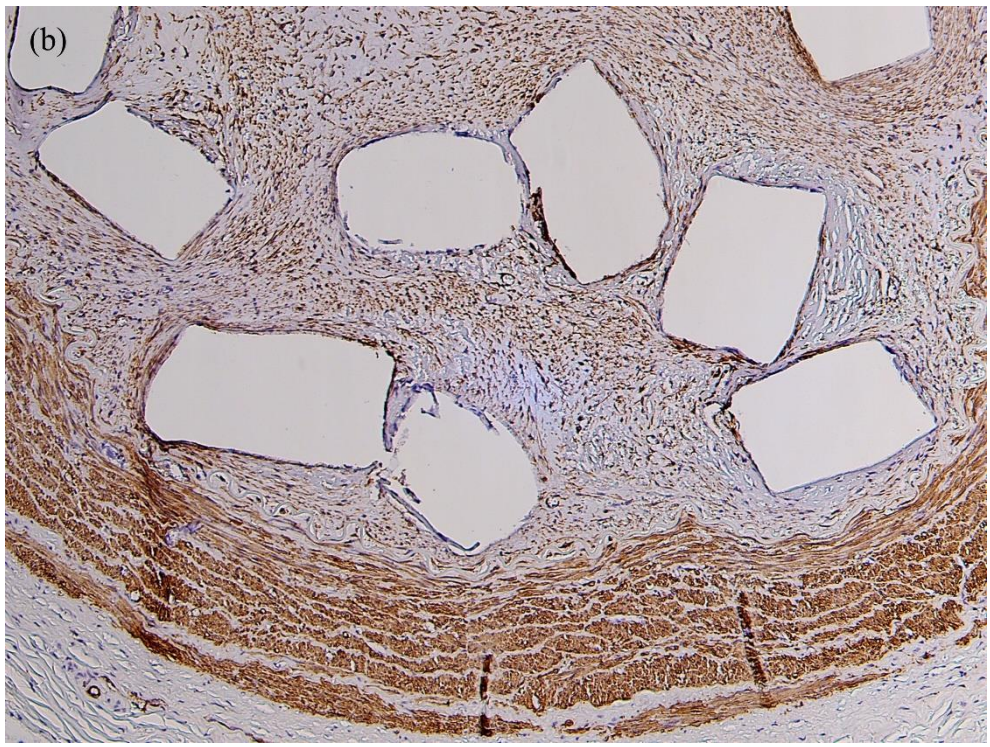

(b)

Figure 19. The expression of anti-smooth muscle cell alpha actin (three-month follow-up, x100 magnification). (a) bare PLLA stent (b) Ta-implanted stent and (c) Enterprise stent.




(b)

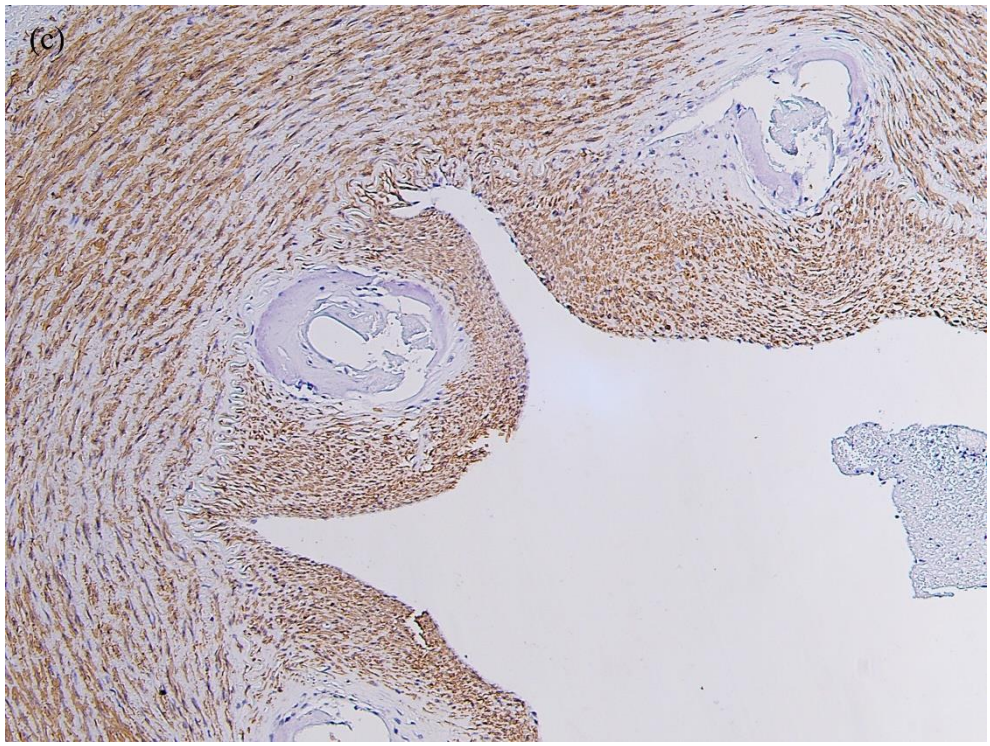

(c)

Figure 20. *In vivo* thrombogenicity and the parent artery diameter. The large group shows less thrombogenicity according to the two-month follow-up exam (31.4% versus 75.7%, p=0.015). However, all PLLA stents in the small group were bare PLLA stents. In a two-way ANOVA test, Ta ion implantation showed marginal significance for less thrombogenicity (p=0.066) compared to the large (>3mm) parent artery diameter (p=0.204).

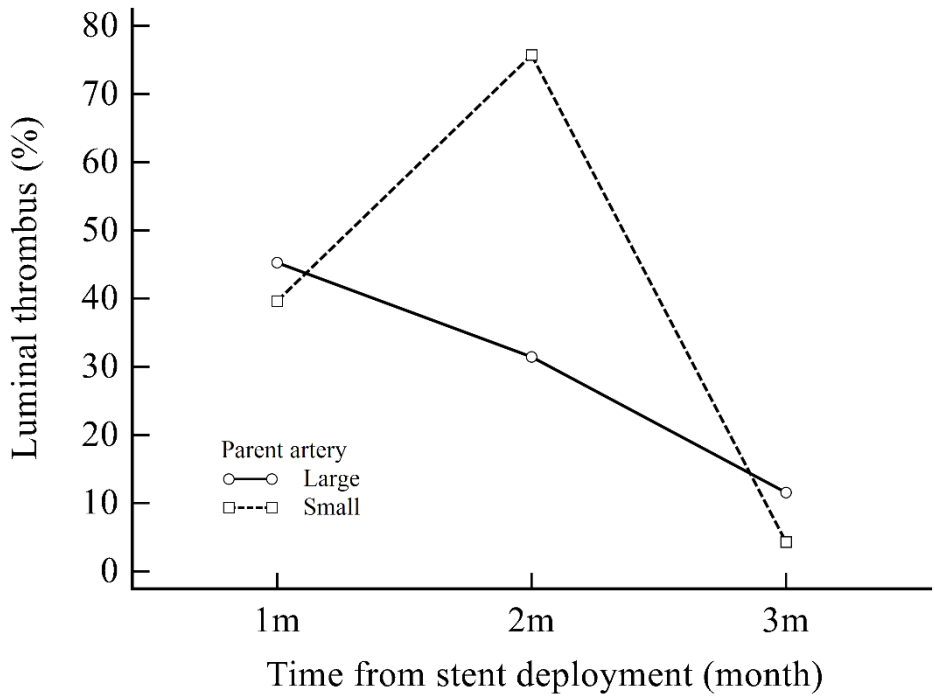


Figure 21. Neointimal hyperplasia and the parent artery diameter. The small group shows relatively severe neointimal hyperplasia upon a one-month follow-up (p=0.020). There is no significant difference at two and three-month follow-up (p=0.316, p=0.106, respectively).

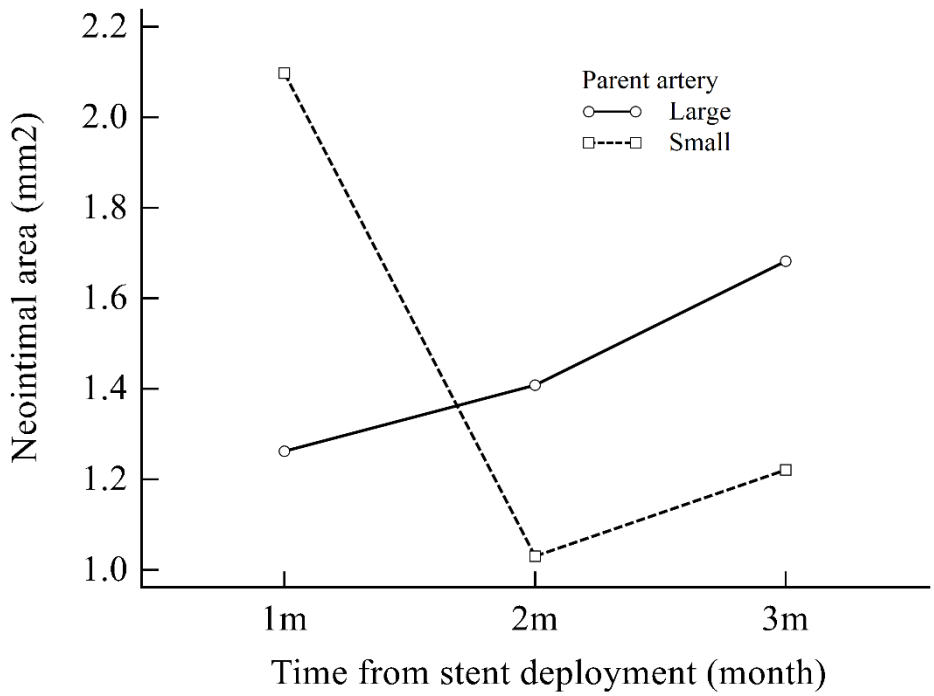


Figure 22. Inflammation score and the parent artery diameter.

The inflammation score is higher in the small group; however, there is no statistical significance (p=0.203 at one month and p=0.408 at two months).

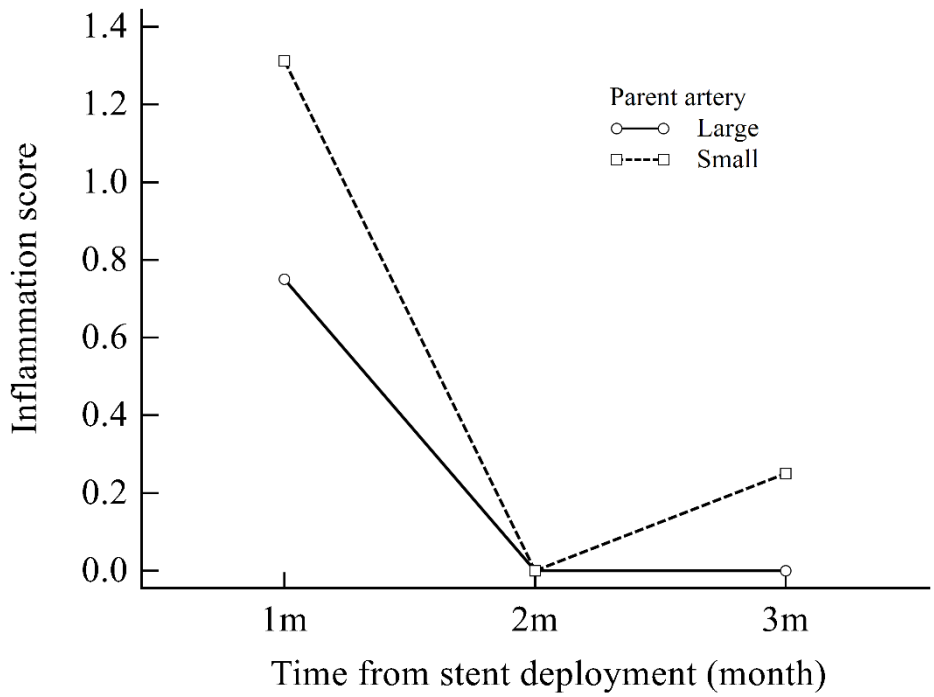


Figure 23. CD 68(+) cells and the parent artery diameter.

The numbers of CD 68(+) cells / 400 HPF are 4.8±7.5 in the large group and 9.3±16.0 in the small group at one-month follow-up. However, there is no statistical significance (p=0.546).