ORIGINAL RESEARCH

A NOVEL SIROLIMUS-ELUTING BIODEGRADABLE MAGNESIUM-BASED ALLOY SCAFFOLD: SIX-MONTH RESULTS IN PORCINE PERIPHERAL ARTERIES

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ABSTRACT

Purpose: Magnesium-based alloy scaffold is a promising biodegradable stent due to its intrinsic mechanical performance and biocompatibility. Based on our preliminary experiments, we designed a novel sirolimus-eluting magnesium-based alloy scaffold. This work aimed to assess its safety and degradation performance *in vivo*.

Methods: The scaffolds were implanted in the lower limb arteries of Bama mini-pigs. Safety was defined as no immediate thrombosis or >30% residual stenosis, which was assessed with optical coherence tomography and digital subtraction angiography. Blood biochemical analyses were performed to evaluate hepatorenal toxicity. The degradation process of the scaffolds, the endothelialization, and lumen loss of the stented-vessels were detected with scanning electron microscopy, immunohistochemical, hematoxylin-eosin staining and optical coherence tomography.

Results: Twenty-four scaffolds were successfully implanted in six pigs with no signs of immediate thrombosis or >30% residual stenosis. The scaffolds were covered by endothelium at one month and absolutely resorbed at six months post implantation. Blood analysis showed that the hepatorenal function except for alanine aminotransferase and γ -glutamyl transpeptidase was normal. Obvious intimal hyperplasia and lumen loss were found in the stented vessels at three months, while the diameters and inner lumen areas of stented segments had increased significantly at six months ($p < 0.001$).

Conclusion: The scaffolds can be safely implanted in the porcine peripheral arteries and fully resorbed at six months after implantation. The stented vessels present positive vascular reconstruction when the scaffolds were fully resorbed. Future improvement for this scaffold should focus on the inhibition of intimal hyperplasia.

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INTRODUCTION

Peripheral arterial disease (PAD) affects 10%–20% of people worldwide and the incidence of PAD increases with age [1–3]. Endovascular interventions with stents are the main treatment for patients with PAD [4,5]. In-stent restenosis (ISR) has become a major challenge for patients received stents: ISR has been reported to occur in 30%–40% of patients with PAD within two to three years of treatment [6]. In recent years, the introduction

of drug-eluting stent (DES) has reduced the incidence of ISR; however, up to 3%–20% of these patients who received DES still experienced restenosis [7]. In addition, the existence of an implanted stent limits the recovery of normal vasomotor function and the choice of therapeutic strategies.

Biodegradable vascular scaffold is considered a promising stent for treating the cardiovascular diseases due to the degradable performance. Current biodegradable

vascular scaffold is mainly composed of either a polymeric scaffold or a biodegradable metallic stent [8]. Due to the inferior mechanical properties and late-phase thrombosis, the poly-L-lactic acid polymer stents have limited its benefits for vessel long-term patency [9,10]. Compared with polymeric scaffolds, magnesium-based alloy scaffolds exhibit better mechanical performance and biocompatibility [11]. As a catabolic product, Mg^{2+} is essential for many metabolic reactions and biological mechanisms [12]. Recently, magnesium-based alloy scaffolds have been reported in animal research or clinical trials [13,14]. However, results showed that magnesium-based alloy scaffolds degraded rapidly after implantation [15]. Thus, researchers have modified scaffold surfaces, improved coating techniques, or changed alloy components to improve the degradation rate [16-18].

To date, magnesium-based alloy scaffolds suitable for the peripheral arteries have not been reported [19]. Based on our preliminary experiments, we designed a novel magnesium-based alloy scaffold consisted of magnesium (97%), zinc (2%) and calcium (1%) and they were coated with the mixture of sirolimus and poly (lactic-co-glycolic acid). These scaffolds were implanted into the lower limb arteries of Bama mini-pigs to observe their safety and the degradable process.

MATERIALS AND METHODS

Animals

Healthy male Bama mini-pigs (10 months old, weight 25–35 kg) were purchased from Beijing Farm Animal

Research Center and housed in Center for Cardiovascular Experimental Study and Evaluation of Fuwai Hospital. All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications NO.85-23, revised 1996), and the study was reviewed and approved by the animal ethics committee of Fuwai Hospital (No.0099-1-8-HX(X)).

Characteristics of the biodegradable magnesium-based alloy scaffolds

The scaffolds are composed of magnesium (97%), zinc (2%) and calcium (1%) and coated with the mixture of sirolimus (drug loading 1.2 $\mu\text{g}/\text{mm}^2$) and poly (lactic-co-glycolic acid). These scaffolds were manufactured by Lepu Medical Technology (Beijing). The scaffold model used in the present study was 3.0 mm x 21.0 mm (Figure 1). The scaffolds were delivered using a balloon-expandable system, which was compatible with a 0.014-inch guide wire and a 6-French (Fr) vascular sheath.

Stenting procedures

After general anesthesia was administered, the right carotid artery was surgically exposed. Systemic heparinization was achieved with an intra-arterial injection of heparin (5,000 U; 200 U/kg). A 6-Fr guiding sheath was delivered into the artery of the lower limb under digital subtraction angiography (DSA). The scaffolds were implanted in the superficial femoral and popliteal artery (Figure 1A) with a stent-to-artery ratio of 1.1:1. After implantation, optical coherent tomography (OCT)

FIGURE 1.
MAGNESIUM-BASED ALLOY SCAFFOLDS.

(A) Unreleased stent installed in the delivery system. (B) Image of the released stent.

and DSA were performed to observe whether there was immediate thrombosis or residual stenosis. Animals received aspirin (100 mg/day) and clopidogrel (75 mg/day) from three days before the procedures till the end of follow-up.

Optical coherence tomography imaging

The OCT detection (Model C7 XR OCT Imaging System, Light Lab Imaging) was performed immediately after implantation, and at one, three and six months post operation. Briefly, an OCT catheter (2-Fr) was positioned in the distal vessels of the stented segment with 0.014" guide wire through 6-Fr guiding catheter. The stented segment was scanned with automatic pull-back at 20 mm/s. The scaffold status, vessel diameters and luminal areas were obtained from the OCT pictures.

Scanning electron microscopy analysis

All tissue slides were fixed with 2.5% glutaraldehyde solution for 30 min, followed by three phosphate-buffered saline washes. An ethanol series dehydration was performed in which samples were washed in 30%, 50%, 70%, 90% and 100% ethanol for three minutes each time. The samples are left to air dry overnight. Scanning electron microscopy was performed on a field emission scanning microscope (Zeiss MERLIN).

Blood analysis

To observe the impact of the scaffold on the hepatorenal function, both the blood routine and hepatorenal function were analyzed prior to implantation and at one, two, four, eight and 12 weeks after operation. The blood samples were analyzed at the Centers of the Experimental Animals of Fuwai Hospital.

Histopathologic analysis

The stented-vessels were fixed with 4% paraformaldehyde in phosphate buffered saline for 24 h followed by standard procedures for graded alcohols and xylene and paraffin-embedding. Paraffin-embedded slices were serially sectioned at 4- μ m intervals. The slides were stained using hematoxylin-eosin (HE) staining and immunohistochemical staining (IHC). The primary antibody to alpha-smooth muscle actin (α -SMA, 1:500, ab32575), to CD31 (1:200, ab32457), to CD8 (1:200, ab4055), and to CD68 (1:200, ab125212) were purchased from Abcam. The stained slides were analyzed by Image J 1.45.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8. Results are expressed as mean $\pm$ standard

deviation (SD). Data are analyzed using Student's t-test between two groups. Differences are considered significant at $p < 0.05$; *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

RESULTS

Animals and interventions

Twenty-four scaffolds (100%) were successfully implanted in six pigs and all the animals survived the scheduled follow-up periods. For each animal, four scaffolds were implanted in the lower limb arteries (Figure 2F). The patency of stented vessels was confirmed by DSA immediately after implantation as being without $>30\%$ residual stenosis (Figure 2A, B and E). The OCT results showed that the scaffolds were of suitable diameter for the vessels and attached well with no evidence of immediate thrombosis (Figure 2C and D).

Stented vessels one month post implantation

Endothelialization of artificial vascular graft contributes to improve vascular antithrombotic ability and vascular long-term patency. To evaluate the endothelialization of stented vessels, eight stented vessels from two animals were obtained one month after implantation. The HE staining results showed all eight stented vessels (100%) were successfully covered by endothelial cells (Figure 3A and B). The scanning electron microscopy results also confirmed the presence of endothelial cell in the stented vessels (Figure 3C and D). Furthermore, the IHC staining using anti-CD31 antibody confirmed the presence of endothelial cells (Figure 3E and F).

Scaffolds at three months post implantation

To observe the status of the scaffolds, the stented vessels of two animals was obtained at 3-month after operation. The HE results showed that the scaffolds turned into some amorphous particles without primary shapes (Figure 4A and B). Similarly, the OCT results also showed that the stented vessels were characterized by the amorphous particles and hyperplastic intima (Figure 4E). However, the stented vessels were characterized by obvious intimal hyperplasia. To explore the cell type in the hyperplastic intima, IHC was performed. Results showed that the hyperplastic intima was mainly composed of smooth muscle cells (Figure 4C and D). The alanine aminotransferase (ALT) and γ -glutamyl transpeptidase (GGT) slightly elevated at 1-2 weeks after operation, while return to normal levels at three months post operation (Figure 4F). Other blood analyses indicated that hepatorenal function was normal (data not shown).

FIGURE 2.
REPRESENTATIVE RESULTS DURING THE PROCESS OF SCAFFOLD IMPLANTATION.

(A) Release process of the distal scaffold shown by digital subtraction angiography (DSA). (B) Release process of the proximal scaffold shown by DSA. (C) Distal scaffold was confirmed by optical coherence tomography to be suitable for the vessels (without thrombosis). (D) Optical coherence tomography results showed that the scaffold matched well with the vessels without immediate thrombosis. (E) The DSA confirmed that the stented vessels were patent after implantation. (F) Schematic diagram of the operation (supine position).

FIGURE 3.
STENTED VESSELS WERE EFFECTIVELY COVERED BY ENDOTHELIAL CELLS.

Eight stented-vessels samples were obtained from two pigs one month after implantation. The femoral arteries proximal to the scaffold segments were considered as the normal control. (A) HE staining showed healthy endothelium in non-stented vessels (control). (B) HE staining showed that the stented vessels were covered by endothelial cells. (C) Scanning electron microscopy confirmed normal control of endothelial cells in non-stented vessels. (D) Scanning electron microscopy results confirmed effective endothelialization in the stented vessels. (E) IHC staining confirmed the existence of the endothelium in normal vessels (positive control). (F) IHC staining (antibody to CD31) confirmed that the stented vessels were covered by endothelial cells.

Scaffolds at six months post implantation

According to the schedule of follow-ups, the stented vessels of two animals were harvested at six months post implantation. The HE staining showed that the scaffolds were completely resorbed (Figure 5A and B). Similarly, the OCT results indicated that the scaffolds were completely resorbed and the vascular lumen was patent (Figure 5C and D). The diameters of the stented vessels and the inner lumen areas at six months were increased significantly compared with three months ($p < 0.001$) (Figure 5E and F).

DISCUSSION

Based on the results of the current study, we conclude that these novel magnesium-based alloy stents can be safely implanted in the peripheral arteries of mini-pigs. In the literature, both immediate thrombosis and residual stenosis have been considered as concerns for biodegradable vascular scaffold [20]. With our present scaffolds, OCT and DSA results showed no immediate thrombosis and residual stenosis in stented segments after implantation. Compared with conventional polylactic acid polymer stents, the magnesium-base alloy

FIGURE 4.
SCAFFOLDS BIODEGRADED INTO AMORPHOUS PARTICLES WITH OBVIOUS INTIMAL HYPERPLASIA
AT THREE MONTHS POST OPERATION.

(A) HE staining showed that the scaffolds biodegraded into amorphous particles with obvious intimal hyperplasia. Bar=500 μm . (B) Increased magnification of the square area shown in A. Bar=20 μm . (C) IHC staining showed that the intima was mainly composed of smooth muscle cells. Bar=500 μm . (D) Increased magnification of the square area shown in C. Bar=20 μm . (E) Optical coherence tomography results showed some amorphous particles and obvious intimal hyperplasia in stented vessels. The arrows indicate areas with amorphous particles (low signal intensity) and the areas marked with asterisks represent the intima (high signal intensity). (F) Time-dependent changes in blood alanine aminotransferase (ALT) and γ -glutamyl transpeptidase (GGT) post operation.

FIGURE 5.

SCAFFOLDS WERE FULLY DISSOLVED AND THE STENTED VESSELS SHOWED POSITIVE VASCULAR RECONSTRUCTION 6 MONTH POST OPERATION.

(A) HE staining showed that the scaffolds were completely resorbed. Bar=500 μm . (B) Increased magnification of the square area in A. Bar= 20 μm . (C) Optical coherence tomography results showed that the scaffolds were resorbed. Bar=1 mm. (D) Digital subtraction angiography showed the patency of the stented vessels at six months after operation. (E) Graph showed that the diameters of the stented vessels had increased significantly at six months compared with three months using Student's t-test ($*p<0.001$, $n=8$ stents). (F) Graph showed the inner lumen areas of stented vessels had increased significantly compared within three months using Student's t-test ($*p<0.001$, $n=8$ stents).

stents have an excellent mechanical performance that provides a reliable radial force [21]. Consistent with our results, previous studies of other types of magnesium-alloy scaffolds showed no immediate thrombosis [22, 23], which may be due to 1) the ability of magnesium ion to reduce coagulation and 2) the better biocompati-

bility of the magnesium alloy stents [24].

Both ALT and GGT levels increased slightly and peaked at 1-2 weeks after implantation; however, the peak values were less than double the normal values, and returned to normal values within three months after implan-

tation. A previous study indicated that a magnesium neodymium alloy stent (MgNd₂) was implanted in an animal, which subsequently showed no abnormal biomarkers [25]. In our present study, the increase of these two biomarkers may be caused by overload of sirolimus in the blood: in our study, four scaffolds were implanted in one animal and the drug amount on each stent was 1.2 µg/mm². Furthermore, the time for ALT and GGT levels to return to normal was consistent with the duration of the sirolimus coating (approximately two months after stent implantation). Of course, the increase of those two biomarkers may be also associated with other drugs used during the operation, including anesthetics and antiplatelet drugs. This will need to be investigated in a follow-up study.

Except for the safety of scaffolds implantation, the degradable performance was one of the most critical parameters for biodegradable vascular scaffold. In the present study, the HE and OCT results indicated that the scaffolds began to be resorbed by one month and only amorphous particles were visible by three months, eventually degrading completely by six months after the implantation. As to the degradation time, no consensus had been obtained among various researchers, with the degradation time varying from one month to two years [26]. In the clinical setting, ISR after stenting with bare metal or drug-eluting stents usually occurred within six months after operation [27]. With the magnesium-alloy degradable stents, there has been no consensus about the ISR time point. Mao et al. reported a new magnesium-based alloy scaffold that could keep stent shape up to six months after operation [28]. Matching the stent degradation time and the ISR time point needs more research. The stent degradation time has been linked to many factors, including the components of the scaffolds, coating techniques and implantation techniques [29]. In our experience, the rate of degradation of the scaffolds may also depend on appropriate balloon-dilatation after implantation of the magnesium-alloy scaffolds.

Unfortunately, the stented vessels presented obvious intimal hyperplasia, especially at three months after implantation. We speculated that the reason for the intimal hyperplasia was associated with several factors. First, the scaffolds were implanted into the arteries near the joint. Evidence from clinical research has revealed that the stents located in the joint areas more easily underwent restenosis [30,31]. In our present study, the available stents were a model with a diameter of 3 mm and we had to implant them into the peripheral arteries near the joint to achieve an optimal match with the vessels. In the future, a customized stent could be implanted into the vessels farther away from the joints. Secondly, the sirolimus coat was resorbed at an early stage post operation and thus could not inhibit the proliferation

of smooth muscle cells. Third, the stents were implanted in normal arterial vessels, and intimal hyperplasia is a heal response to injury. Healthy vessels may have a stronger heal response than atherosclerotic vessels. Fourth, at three months after implantation, the presence of amorphous particles from the stents may be another reason for intimal hyperplasia. The chemical component of these amorphous particles and their impact on the vascular smooth muscle proliferation remains unknown. Finally, the length of stents may be associated with restenosis or intimal hyperplasia [32]. The stents used in the present study were long up to 21.0 mm, which could promote intimal hyperplasia.

To confirm the cell type in the hyperplastic intima, we performed IHC staining. The results showed that the cells in the intima consisted mainly of smooth muscle cells. We also tried to identify lymphocytes or macrophage using IHC staining, but those two inflammatory cells were not found in the hyperplastic intima (data not shown). A previous study showed that inflammatory cells gathered in the scaffolds within two weeks after implantation [33]. However, vessel samples used in present study were from animals 1–3 months post operation.

Interestingly, a positive vascular reconstruction was found after the scaffolds were resorbed. The inner lumen areas at six months were higher than that at three months. The diameters of stented vessels were also increased relative to that seen at three months. Similarly, a previous study by Heublein et al. indicated that the inner lumen areas of stented vessels had a 25% enlargement after the stents were resorbed [34]. If intimal hyperplasia was significant, especially at three months after implantation, due to early release of the drug coat, delaying the duration of anti-proliferation drug may reduce the intimal hyperplasia. However, the ideal duration of this anti-proliferation was still not consistent in the literature.

Although significant intimal hyperplasia was noted in the scaffolds, no obvious thrombosis was confirmed during the periods of follow-up. This indicated magnesium-based alloy scaffolds exhibited a better antithrombotic ability—not only immediately after implantation but also during the process of biodegradation. Thrombotic events have been frequently reported with polylactic acid polymer stents, even when they are used with double anti-platelet drugs [35]. It is well known that endothelial cells are important to the prevention of thrombosis. With these magnesium alloy stents, endothelialization of artificial vascular graft was observed at one-month post implantation, which may also contribute to improved vascular antithrombotic ability.

The present study had some limitations. First, the novel scaffolds were implanted in normal vessels of healthy

pigs and the reactions of atherosclerotic vessels may differ from that of healthy vessels after stent implantation. Second, this study included only a limited number of animals. Although only six pigs were used in this study, we implanted four stents per pig. Thus, a total of 24 stents samples were implanted, providing additional data for analysis. Third, the follow-up period was six months. The OCT results at six months showed the total biodegradation of the stents and therefore we ended up this study at six month post operation. However, determining whether the positive vascular reconstruction persists would require additional study with a longer follow-up period.

CONCLUSIONS

The results of this study suggest that the novel magnesium-alloy scaffolds fully biodegrade *in vivo* and that the stented vessels show an obvious positive vascular reconstruction after the scaffolds were resorbed. Due to the observation of significant intimal hyperplasia, planned improvements for these scaffolds should focus on the inhibition of intimal hyperplasia by accurately controlling and delaying the release of the drug layer. Furthermore, because healthy animals were used for this study, the efficacy of the stent needs to be elevated in animals with atherosclerotic lesions. Presently, this newly developed stent is currently limited to experimental use and—though far from clinical application—shows promise.

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