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# Effects of a Matrix Metalloproteinase Inhibitor-Eluting Stent on In-Stent Restenosis

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Data Interpretation D  
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**Background:** The aim of this study was to compare changes in the extracellular matrix after implantation of a stent that elutes a matrix metalloproteinase (MMP) inhibitor (GM6001); and to determine the effects of the GM6001-eluting stent upon prevention of in-stent restenosis (ISR).





**Material/Methods:** We included 48 Guangxi Bama mini-pigs in this study. A GM6001-eluting stent was placed in one iliac artery and a stent that did not elute GM6001 was placed in the contralateral iliac artery. The iliac arteries were removed at 6 hours as well as 1, 7, 14, 56, 84, and 336 days after stent placement. Arteries were analyzed for morphometry, gelatinase content, different phenotypes of vascular smooth muscle cells (VSMCs), collagen content, apoptotic rate, and cell density.

**Results:** The vascular lumen areas of the GM6001 group were significantly increased and the neointimal areas were significantly reduced compared with the control group from the 7 days to the 336 days. In the 2 groups, expression of MMP-2 and MMP-9 peaked simultaneously, but GM6001-eluting stents inhibited expression of MMP-2 and MMP-9 in the vascular media and neointima (especially around the struts) significantly. In the GM6001 group, expression of tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2, myosin heavy chain 10 (MYH-10, marker of the proliferative phenotype of VSMCs), collagen content, percentage of apoptotic cells, and cell density were also decreased significantly compared with those in the control group.

**Conclusion:** Use of GM6001-eluting stents resulted in persistent and potent inhibition of intimal hyperplasia, an increase in luminal area, and no obvious thrombosis in the arteries of the mini-pigs.

**MeSH Keywords:** **Matrix Metalloproteinase 2 • Matrix Metalloproteinase 9 • Muscle, Smooth, Vascular**

**Full-text PDF:** <https://www.medscimonit.com/abstract/index/idArt/922556>

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## Background

Atherosclerosis is the main cause of coronary heart disease, cerebral infarction, and peripheral vascular disease. Atherosclerosis causes thickening and hardening of the arterial wall and narrowing of the vascular lumen, and the tissue/organ supplied by the artery will become ischemic or necrotic [1].

Thanks to developments in endovascular methods of treatment, increasing numbers of patients are undergoing endovascular procedures. In most centers, stents are used in 80% of interventional procedures because they provide a more reliable immediate result and improved prevalence of restenosis compared with those obtained with balloon angioplasty [2]. However, in-stent restenosis (ISR) following angioplasty and stenting are the most serious problems in arterial therapy [3]. The prevalence of restenosis after bare-metal stent (BMS) placement is ~10% [4]. Use of drug-eluting stents (DESs) reduces the risk of restenosis and revascularization of the target lesion, but the absolute number of instances of ISR cannot be ignored [5]. ISR remains an important issue in arterial stenting.

Stents coated with a pharmacotherapeutic agent (e.g., heparin, hirudin, sirolimus, paclitaxel, and inhibitors of glycoprotein IIb/IIIa) can be used to reduce the risk of ISR [6,7]. Such coated stents reduce the degree of ISR gradually, but cannot achieve complete eradication of ISR. The main reason is that, although the DES inhibits the proliferation of smooth muscle cells (SMCs), it also prolongs the endothelialization time of the stent [8].

Studies have shown that matrix metalloproteinases (MMPs) can enhance the proliferation and migration of vascular smooth muscle cells (VSMCs). Among them, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have key roles in degradation of the extracellular matrix (ECM), which is required for cell migration into the intima after arterial injury [9]. Matrix metalloproteinase inhibitors (MMPiS) can inhibit MMP activity and reduce the proliferation and migration of VSMCs [10,11].

We created GM6001-eluting stents, implanted them, and then the stents were removed without damaging the artery. Then, we measured the expression of MMP-2, MMP-9, tissue inhibitor matrix metalloproteinase (TIMP)-1, TIMP-2, different phenotypes of VSMCs during ISR, and noted changes in collagen content, the percentage of apoptotic cells, and cell density in the neointima. In this way, we explored the effects of GM6001-eluting stents in the prevention of ISR.

## Material and Methods

### Ethical approval of the study protocol

The protocol for animal experiments was approved by the Animal Care Committee of China Medical University (Beijing, China).

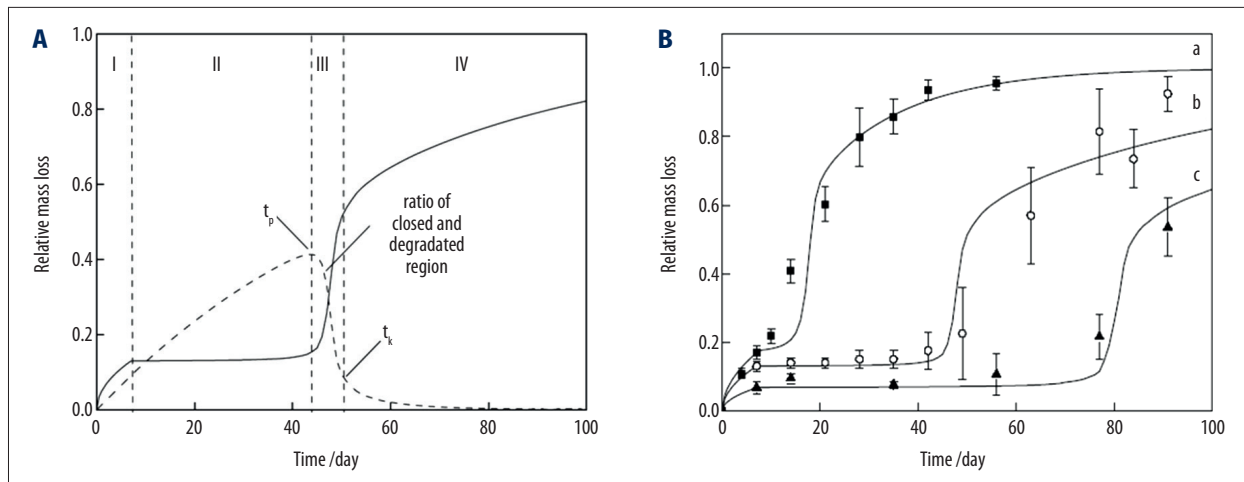
### Preparation and screening of GM6001-eluting stents

Lactic acid and glycolic acid were dissolved in dimethyl sulfoxide in certain proportions to prepare poly lactic-co-glycolic acid (PLGA) in 3 ratios (50/50, 70/30, and 75/25). GM6001 was added to these ratios of PLGA to make a GM6001-PLGA composite, which was applied to the surface of a Z-type BMS to form a DES (Figure 1). Different ratios of PLGA DESs were immersed completely in a certain amount of phosphate-buffered saline (PBS). The GM6001 concentration in PBS was measured by liquid chromatography every day for 0–100 days. A time-drug concentration curve was plotted according to the measurement results. The pharmacokinetics of drugs released by different ratios of the GM6001 DES were analyzed. The surface of the drug-coated stent was observed by scanning electron microscopy once a week for 0–180 days to determine PLGA degradation (Figures 2, 3). Finally, the coating with stable release of drug and duration of release >3 months was selected: 70/30. GM6001-eluting (each stent contained 0.5 mg of GM6001) and eluting stents not containing GM6001 were prepared for animal experiments. The eluting stents not containing GM6001 still contain a PLGA coating, in order to eliminate the interference of PLGA on the experimental results. Z-type stents made of a 316L stainless-steel wire were used in these experiments. The diameter of the strut was 0.15 mm, the diameter of the stent was 6 mm, and the length of the stent was 15 mm.

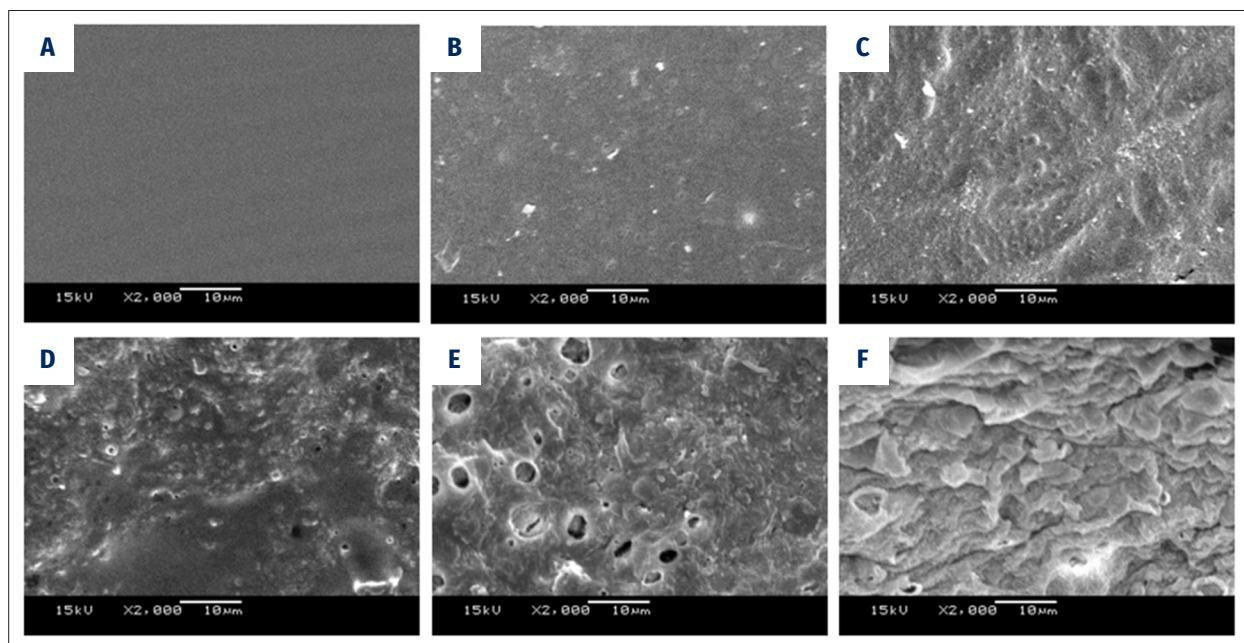
### Animal grouping, stent implantation and specimen preparation

Male and female Guangxi Bama mini-pigs (25–35 kg, 6 months old) were provided by the Sichuan Minipig Breeding Center (Beijing, China). All animals were fed a standard laboratory diet throughout the study.

Forty-eight Guangxi Bama mini-pigs were divided into 8 groups of 6 min-pigs in each group according to the time they were euthanized (6 hour; 1, 7, 14, 56, 84, and 336 days; and a sham group who did not receive stent implantation). The femoral artery was exposed surgically, and a 6-Fr sheath inserted by the Seldinger method. Forty-two GM6001-eluting stents were implanted randomly in the right or left iliac arteries. An eluting stent not containing GM6001 was inserted in the contralateral iliac artery, and this formed the



**Figure 1.** (A) The 4 stages of PLGA membrane degradation process: (I) initial rapid weight loss phase; (II) quasi-stable phase; (III) rapid weight loss phase; (IV) material disintegration phase. (B) Comparison of MC method simulation results of PLGA membrane degradation with experimental values: (a) PLGA50/50; (b) PLGA 70/30; (c) PLGA75/25, correlation coefficients were 0.9819; 0.9677; 0.9762.



**Figure 2.** Surface morphology of PLGA70/30 samples with different soaking times (A) before soaking; (B) after soaking for 1 week; (C) after soaking for 2 weeks; (D) after soaking for 4 weeks; (E) after soaking for 7 weeks; (F) after soaking for 10 weeks.

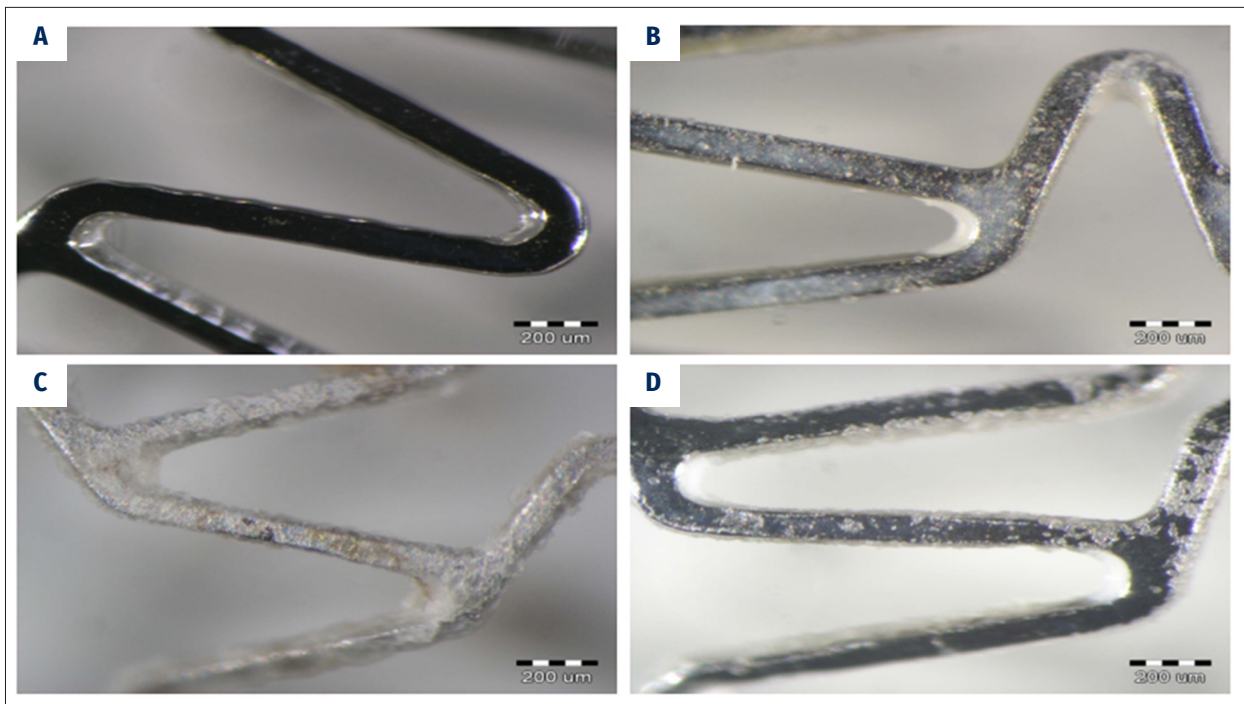
control group. After the stent was implanted, the 6.0 mm diameter balloons were over-inflated, initially at 6 atms and then at 8 atms. Heparin (3000 units every 12 hours) and penicillin (1.6 million units every day) were injected (intramuscularly) routinely 1 week after stents implanted to prevent thrombosis and infection. The pigs received dual antiplatelet therapy after surgery. Arterial angiography 6 hours as well as 1, 7, 14, 56, 84, and 336 days after surgery was done to observe the position and patency of the stents. After the animals had been euthanized, the iliac arteries were

harvested, pressure-fixed in formalin then embedded with optimal cutting temperature compound (OCT), and cryopreserved in a liquid nitrogen tank. Stents were removed without damaging the artery (Figure 4).

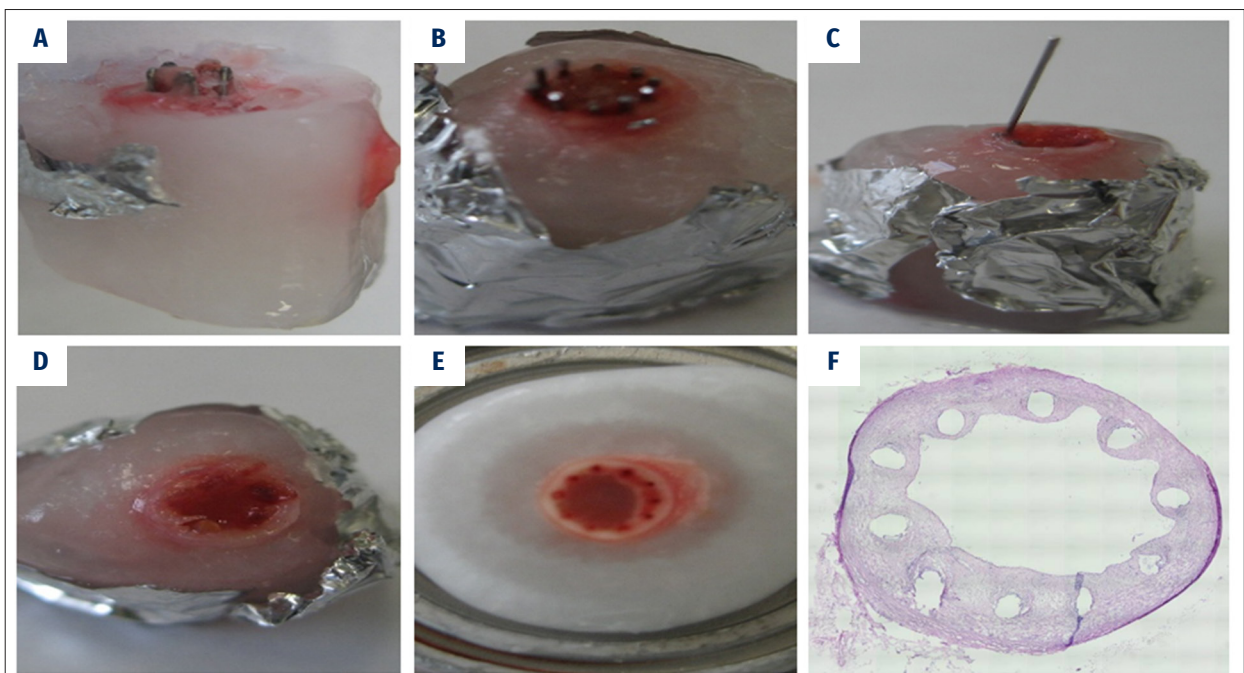
#### Morphologic and immunohistochemical staining

Artery sections (10 μm) were stained with hematoxylin and eosin (H&E). The vascular lumen and neointima were analyzed using ImageJ (National Institutes of Health,





**Figure 3.** Surface morphology of the PLGA coated stent after scouring: (A) initial; (B) 3 days; (C) 30 days; (D) 60 days.



**Figure 4.** (A-F) Stents were removed without damaging the artery. In the frozen state, the connection points at both ends of the stent were exposed, all the connection points were removed using forceps, so that all the struts of the stent were separated from each other and the strut was pulled out lengthways.

Bethesda, MD, USA) by an investigator blinded to the experimental procedure [12]. An ultrasensitive streptavidin-peroxidase kit (Maixin Biotech, Fujian, China) was used to measure the expression and distribution of MMP-2, MMP-9, TIMP-1,

TIMP-2, myosin heavy chain 10 (MYH-10), and smooth muscle protein 22-alpha (SM22 $\alpha$ , marker of the contractile phenotype of VSMCs) according to manufacturer instructions.

**Table 1.** Areas of vascular lumen.

Area of lumen (mm <sup>2</sup> )	Control	GM6001	P
6 h	8.11±0.46	7.98±0.41	>0.05
1 day	8.32±0.21	8.4±0.29	>0.05
7 days	5.07±0.22	8.26±0.20	<0.05
14 days	3.31±0.20	7.41±0.23	<0.05
56 days	1.24±0.13	3.59±0.20	<0.05
84 days	1.51±0.24	3.27±0.12	<0.05
336 days	7.09±0.17	7.72±0.32	<0.05

The primary antibodies we employed were MMP-2 (1:400 dilution; bsm-33327m; Bioss, Beijing, China), MMP-9 (1:400; bs-4593r; Bioss), TIMP-1 (1:400; bs-415r, Bioss), TIMP-2 (1:400; bs-416r; Bioss), MYH-10 (1:400; sc-376942; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SM22 $\alpha$  (1:600; ab14106; Abcam, Cambridge, UK). Antibodies were allowed to incubate with tissue sections overnight at 4°C. Secondary antibodies were added and allowed to incubate with tissue sections in an incubator at 37°C for 30 minutes. Tissue sections were incubated with 3,3'-diaminobenzidine and counterstained with hematoxylin. The distribution of target proteins was captured under a microscope (BX53; Olympus, Tokyo, Japan) at equal-exposure conditions. ImageJ was used to analyze the mean integrated optical densities (IODs) of MMP-2, MMP-9, TIMP-1, TIMP-2, MYH-10, and SM22 $\alpha$  in the vascular wall.

### Western blotting

Protein extraction was carried out. Briefly, a 1-mm segment was cut from ISR tissue. Loose connective tissue was removed, 200  $\mu$ L of ice-cold radio immunoprecipitation assay (RIPA) lysis buffer and 2  $\mu$ L of phenylmethylsulfonyl fluoride were added, and the tissue segment was homogenized. The homogenate was centrifuged at 12 000 g for 30 minutes at 4°C, and the supernatant was collected. The protein concentration was determined using a bicinchoninic acid (BCA) assay kit (p1511; Applygen, Beijing, China) following manufacturer instructions. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. The latter were washed with TBST (tris-buffered saline and Tween 20), blocked with 10% skimmed milk for 2 hours, and incubated with the same primary antibodies (1:1000 dilution) employed for immunohistochemical staining. PVDF membranes were washed in TBST buffer and probed with secondary antibodies: goat anti-rabbit immunoglobulin (Ig)G (1:4000; A21020; Abbkine Scientific, Beijing, China) and goat anti-mouse IgG (1:4000;

**Table 2.** Areas of neointima.

Area of neointima (mm <sup>2</sup> )	Control	GM6001	P
14 days	3.25±0.22		
56 days	6.26±0.36	3.12±0.26	<0.05
84 days	5.16±0.36	4.19±0.46	<0.05
336 days	4.32±0.24	2.97±0.26	<0.05

A21010; Abbkine Scientific) and visualized using an enhanced chemiluminescence (ECL) kit (Beyotime, Shanghai, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; A01020; Abbkine Scientific) was used as an endogenous control.

### Picrosirius Red staining

Tissue sections were stained with Picrosirius Red following manufacturer instructions (G1470, Solarbio, China). Images were captured using a microscope (BX60; Olympus) equipped with filters to provide circularly polarized illumination and which could be adjusted to allow differentiation of collagen of varying maturity [13]. The mean IOD values of mature collagen and new collagen were analyzed by split channels within ImageJ.

### Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

DNA fragmentation was detected by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (11684817910; Roche, Basel, Switzerland). Apoptosis in the neointima was investigated by staining tissue sections at days 56–336 days according to manufacturer instructions. ImageJ was used to calculate the percentage of apoptotic cells, and to measure the density of cells in the neointima.

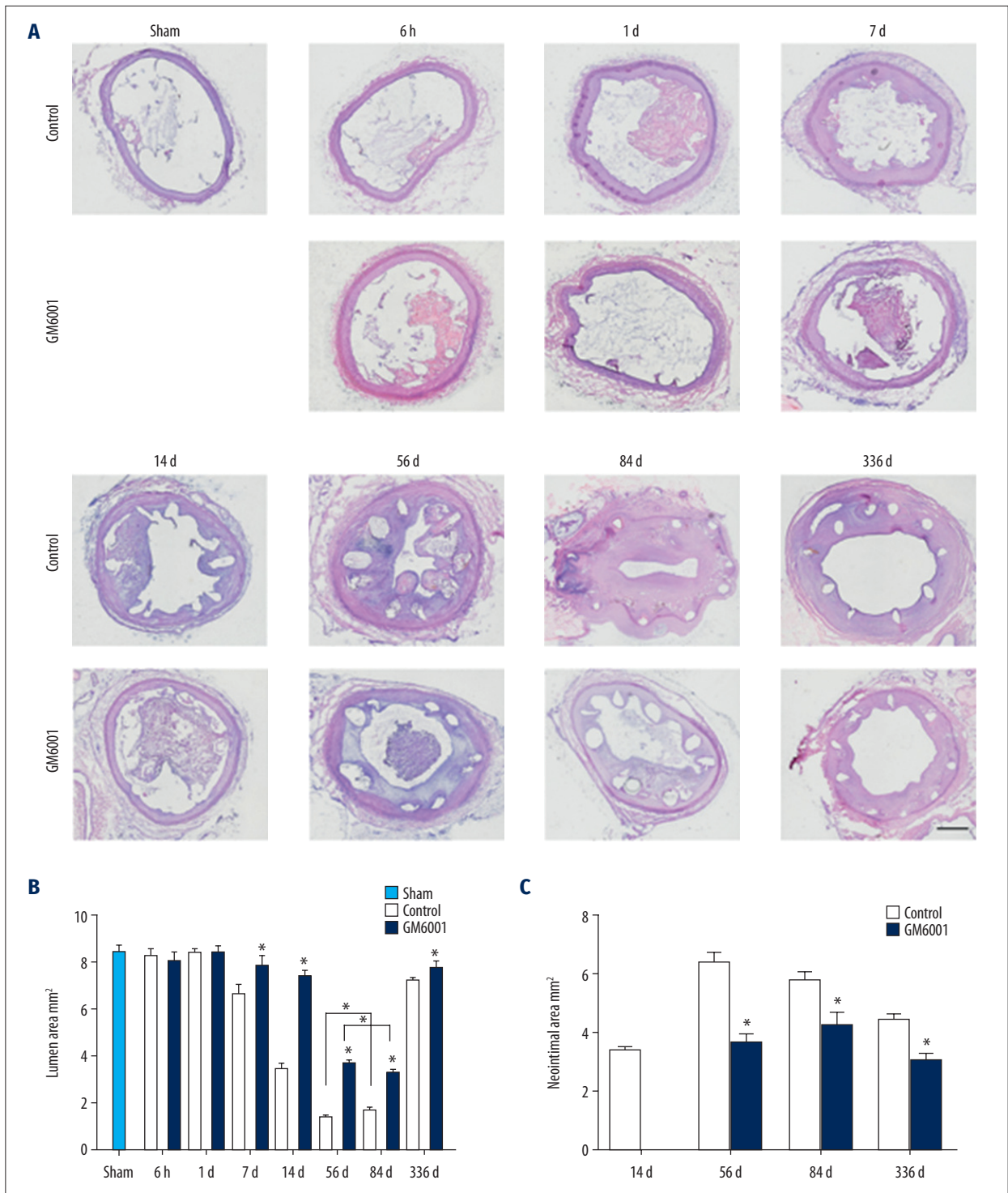
### Statistical analyses

Data are the mean  $\pm$  standard deviation (SD) as analyzed by SPSS v17.0 (IBM, Armonk, NY, USA). Significant differences were analyzed using the Student's *t*-test or ANOVA followed by a Dunnett multiple-comparison post hoc test. The results of western blotting and immunohistochemical staining were analyzed by linear regression. *P*<0.05 was considered significant.

## Results

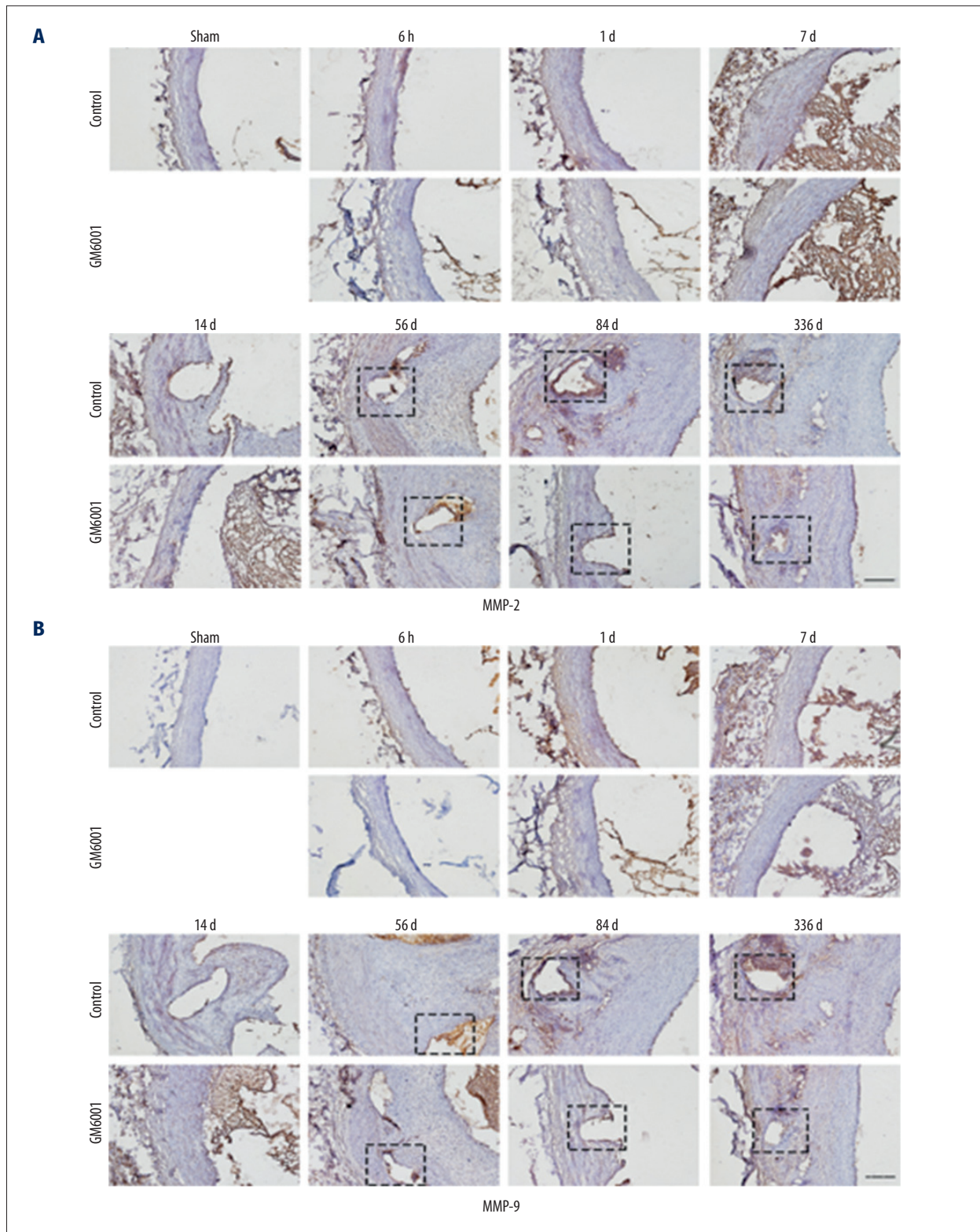
### Histopathological and histomorphometric data

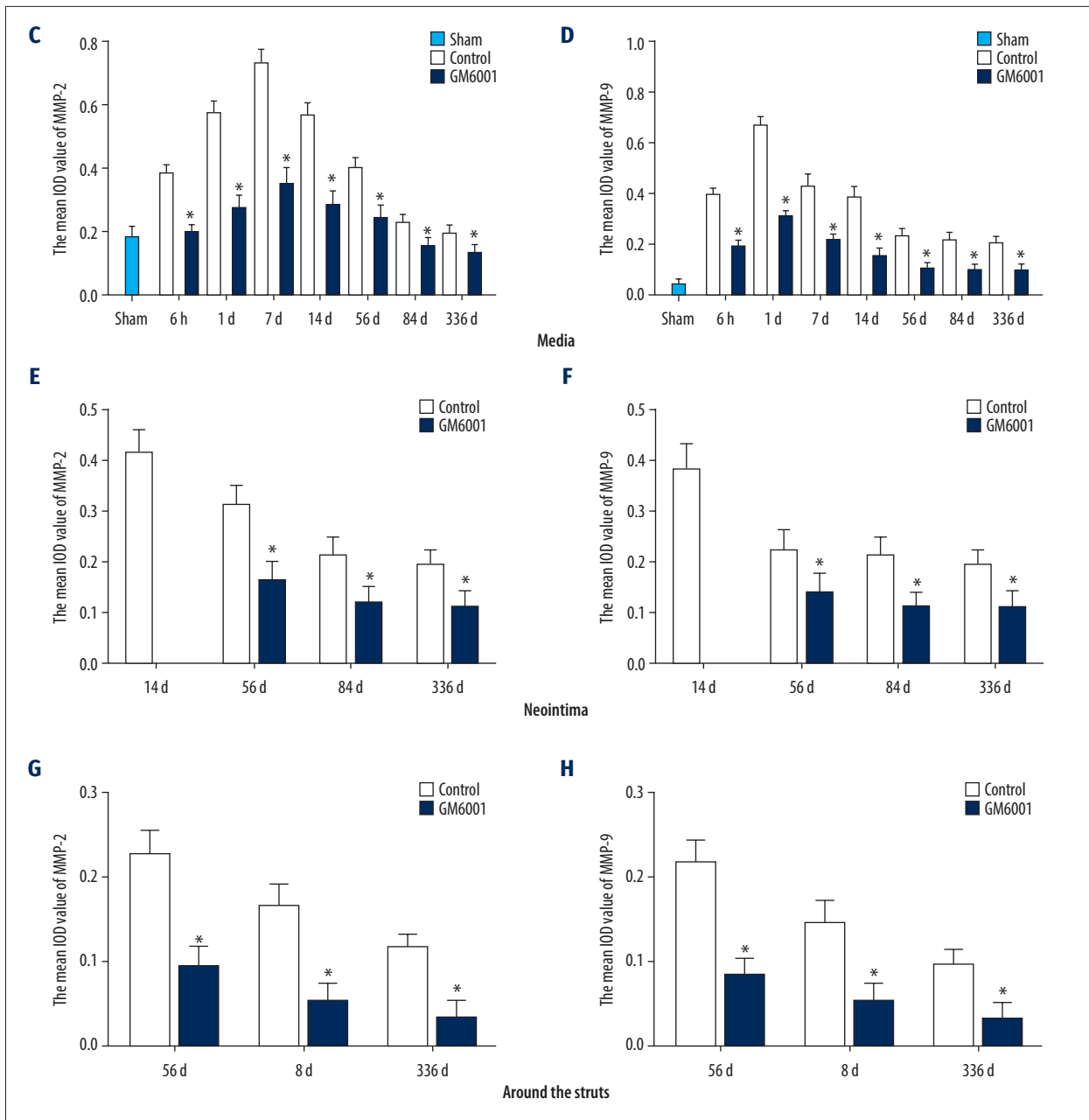
The histological data of different groups are presented in Tables 1 and 2. Histological hallmarks of ISR are neointimal



**Figure 5.** Hematoxylin and eosin (H&E) staining. **(A)** After the application of matrix metalloproteinase inhibitors (MMPI) GM6001, the degree of stent restenosis was significantly reduced compared to the control group from the 7 days to 336 days ( $P<0.05$ ). **(B, C)** The area of vascular lumen and neointima changes in the 2 groups; bar=1000  $\mu$ m.







**Figure 6.** (A, B) Immunohistochemistry to detect the distribution and time course of the expression of matrix metalloproteinase (MMP)-2 and MMP-9. (C-H) The expression of MMP-2 and MMP-9 in the media, neointima and around the struts. The expression of MMP-2 and MMP-9 in the GM6001 group was reduced compared with the control group ( $P < 0.05$ ); bar=200  $\mu\text{m}$ .

growth, proliferation of VSMCs, and formation of abundant ECM. Significant differences between the 2 groups were clearly observed from H&E staining. Provisional matrix was formed between struts at 1 day of the control group and at 7 and 14 days of the GM6001 group. The neointima was observed at 14 days in the control group. In the GM6001 group, neointima appearance occurred later than that in the control group: 56 days. The neointimal area of the GM6001 group was smaller than that of the control group at the

same time point (Figure 5A). A significant increase in the arterial luminal area of mini-pigs in the GM6001 group compared with that in the control group from 7 days to 336 days was noted ( $P < 0.05$ ). The arterial luminal area of mini-pigs in the control group was smallest at 56 days, in the GM6001 group was smallest at 84 days, and the minimum luminal area in the GM6001 group was larger than that in the control group ( $3.27 \pm 0.12$  versus  $1.24 \pm 0.13$   $\text{mm}^2$ ,  $P < 0.05$ ). At 336 days, the arterial luminal area of mini-pigs in the GM6001



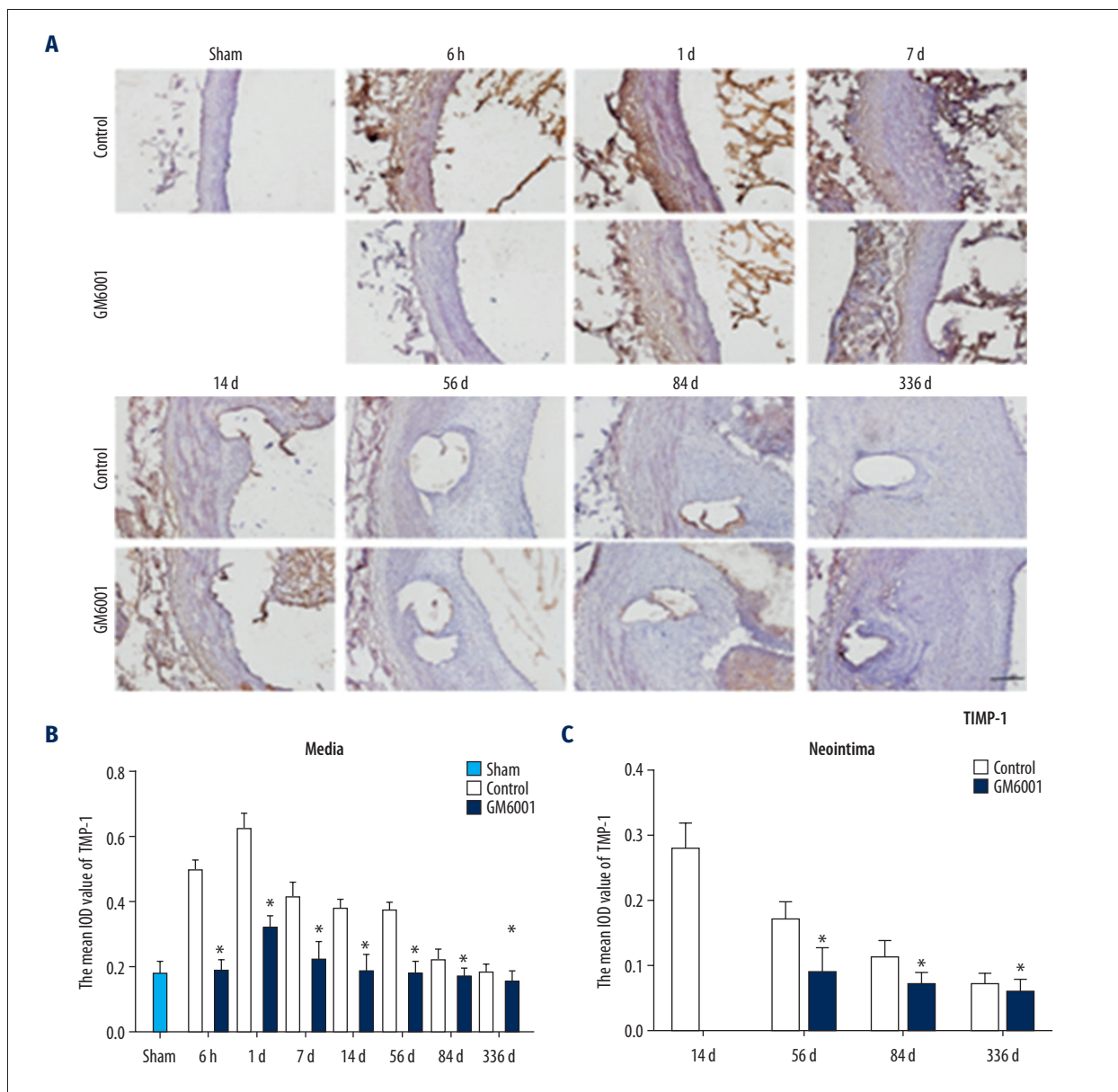
group continued to be larger than that in the control group ( $7.72 \pm 0.32$  versus  $7.09 \pm 0.16$  mm<sup>2</sup>,  $P < 0.05$ ). There was no obvious long-term thrombosis in the 2 groups (Figure 5B, 5C).

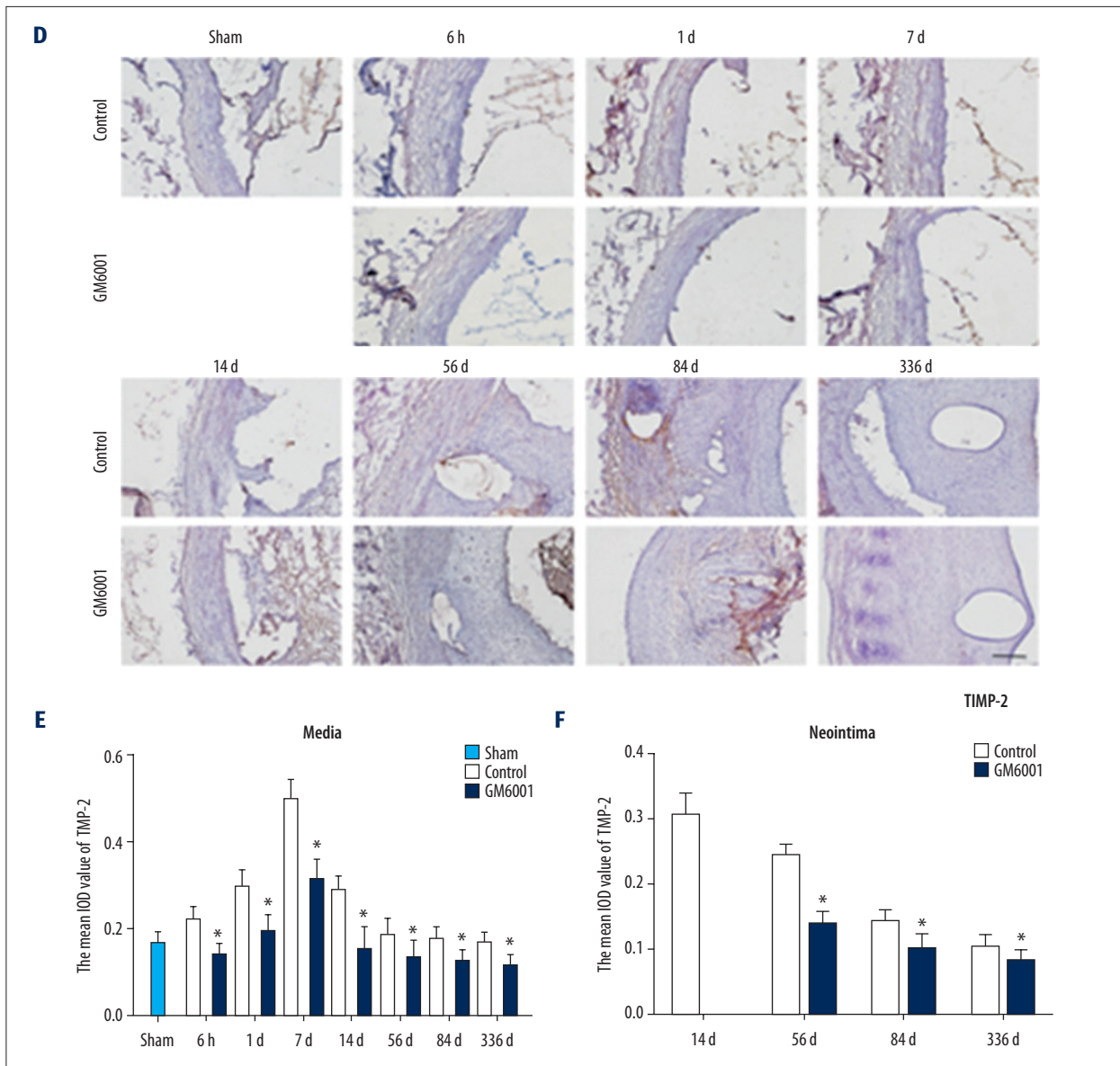
### Expression and distribution of MMP-2, MMP-9, TIMP-1, TIMP-2, MYH-10, and SM22 $\alpha$

Immunohistochemical staining showed that MMP-2, MMP-9, TIMP-1, TIMP-2, MYH-10, and SM22 $\alpha$  were distributed mostly near the internal elastic lamina or in parts of the intimal injury, around the struts, and neointimal lumen surface.

MMP-2 was expressed continuously in the vascular media of mini-pigs in the control group, and decreased gradually after

reaching a peak at 7 days. In the GM6001 group, MMP-2 expression also decreased gradually after reaching a peak at 7 days, but decreased significantly at all time points compared with that in the control group. Expression of MMP-2 in the control group was about 2-fold that in the GM6001 group at 7 days. In the 2 groups, MMP-2 expression in the neointima was high at 14 days and 56 days, and then decreased gradually, and MMP-2 expression also decreased significantly in the GM6001 group ( $P < 0.05$ ). Neointimal MMP-2 expression in the GM6001 group decreased 48.3%, 42.8%, and 42.1% compared with that in the control group at 56, 84, and 336 days, respectively. We selected the same area around the struts to measure MMP-2 expression. Expression of MMP-2 in the GM6001 group around the struts decreased 59.1%, 68.8%,



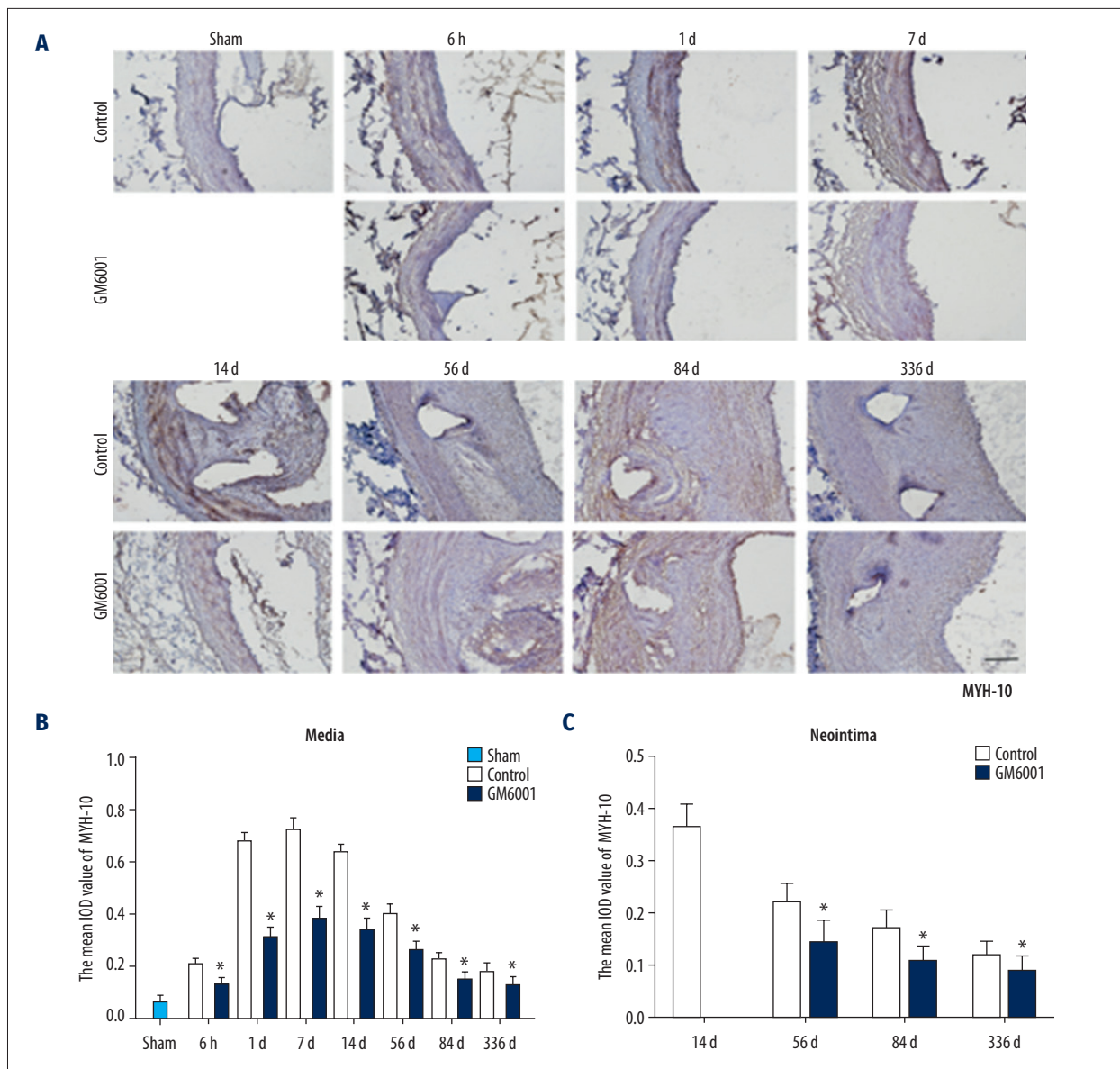


**Figure 7.** (A) Immunohistochemistry to detect the distribution and time course of the expression of tissue inhibitor of matrix metalloproteinase (TIMP)-1. (B, C) The expression of TIMP-1 in the media and neointima. The expression of TIMP-1 in the GM6001 group was reduced compared with the control group ( $P<0.05$ ); bar=200  $\mu\text{m}$ . (D) Immunohistochemistry to detect the distribution and time course of the expression of TIMP-2. (E, F) The expression of TIMP-2 in the media and neointima. The expression of TIMP-2 in the GM6001 group was reduced compared with the control group ( $P<0.05$ ); bar=200  $\mu\text{m}$ .

and 72.7% compared with that in the control group at 56, 84, and 336 days, respectively. The degree of reduction of MMP-2 around the struts was significantly higher than that in the neointima ( $P<0.05$ ) (Figure 6).

In the control group, peak expression of MMP-9 in vascular media appeared at 1 day and then decreased gradually. Similarly, in the GM6001 group, peak expression of MMP-9 appeared at 1 day and then decreased gradually but, compared with the control group, MMP-9 expression was also

decreased significantly ( $P<0.05$ ). Expression of MMP-9 in the control group was about 2-fold that of the GM6001 group at 1 day. In the neointima, MMP-9 expression in the GM6001 group was also decreased significantly compared with that in the control group ( $P<0.05$ ). MMP-9 expression in the neointima of arteries of mini-pigs in the GM6001 group decreased 36.3%, 47.6%, and 42.1% compared with that in the control group at 56, 84, and 336 days, respectively. The same area around the struts was used to measure MMP-9 expression. Expression of MMP-9 in the GM6001 group around the



**Figure 8.** (A) Immunohistochemistry to detect the distribution and time course of the expression of myosin heavy chain 10 (MYH-10). (B, C) The expression of MYH-10 in the media and neointima. The expression of MYH-10 in the GM6001 group was reduced compared with the control group ( $P<0.05$ ); bar=200  $\mu\text{m}$ .

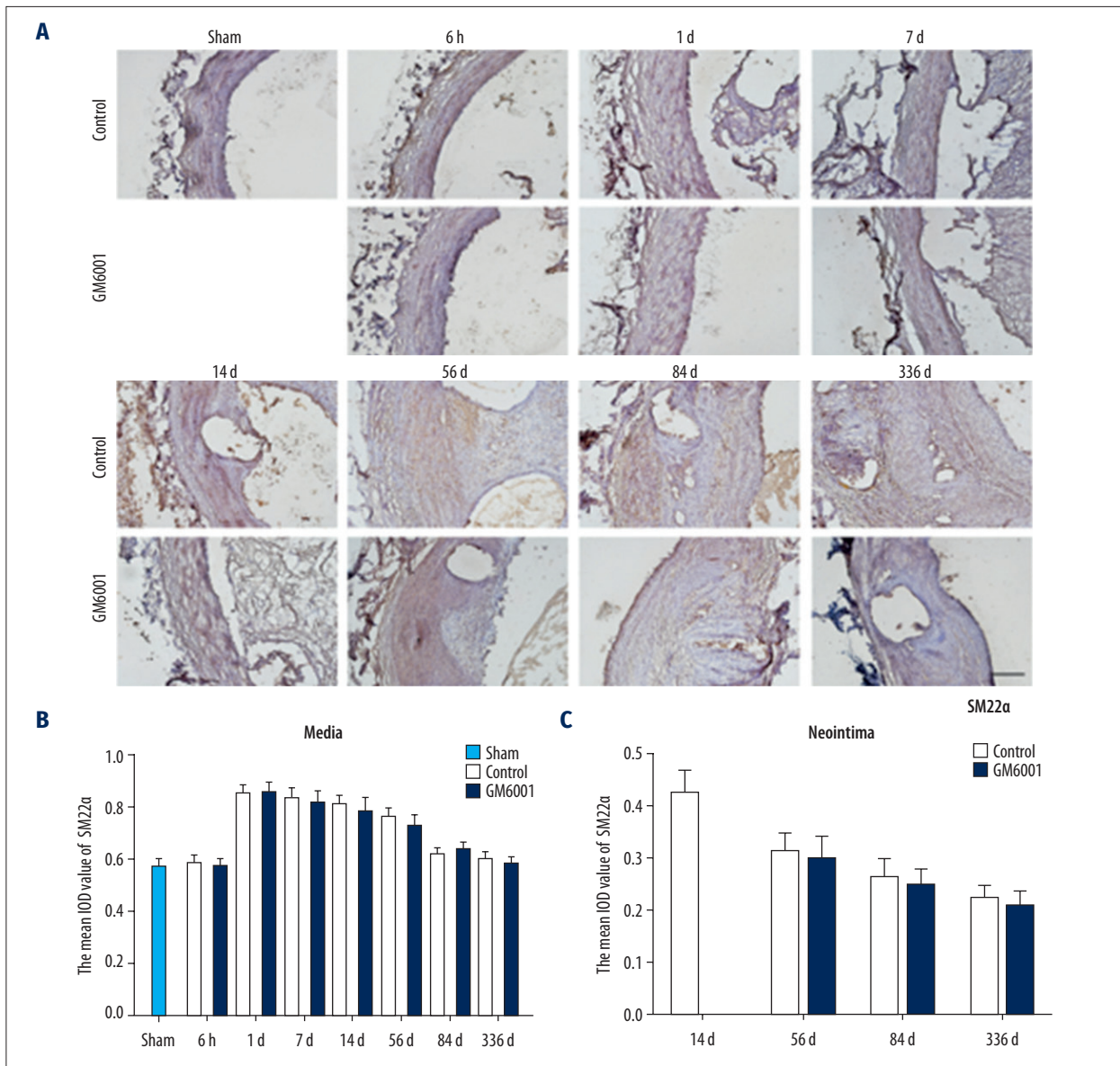
struts decreased 61.9%, 64.3%, and 67.8% compared with that in the control group at 56, 84, and 336 days, respectively. The degree of reduction of MMP-9 expression around the struts was significantly higher than that in the neointima ( $P<0.05$ ) (Figure 6).

In the media of arteries of mini-pigs in the control group, TIMP-1 expression peaked at 1 day, TIMP-2 expression peaked at 7 days, and then decreased gradually. The trend of expression of TIMP-1 and TIMP-2 in the vascular neointima was approximately identical and decreased gradually after reaching a peak at 14 days and 56 days. Expression of

TIMP-1 and TIMP-2 in the GM6001 group was also decreased significantly compared with that in the control group at the same time point ( $P<0.05$ ) (Figure 7).

In the control group, peak expression of MYH-10 in the vascular media of mini-pigs appeared at 7 days and then decreased gradually. Similarly, in the GM6001 group, peak expression of MYH-10 appeared the 7 days and then decreased gradually but, compared with the control group, MYH-10 expression was decreased significantly ( $P<0.05$ ). In the 2 groups, MYH-10 expression in the neointima was high at 14 days and 56 days and then decreased gradually, and MYH-10





**Figure 9.** (A) Immunohistochemistry to detect the distribution and time course of the expression of smooth muscle protein 22-alpha (SM22 $\alpha$ ). (B, C) The expression of SM22 $\alpha$  in the media and neointima, the expression of SM22 $\alpha$  was not significantly different between the two groups ( $P>0.05$ ); bar=200  $\mu$ m.

expression was also decreased significantly in the GM6001 group ( $P<0.05$ ) (Figure 8). SM22 $\alpha$  expression between the 2 groups was not significantly different ( $P<0.05$ ) (Figure 9).

Western blotting confirmed the time course of expression of target proteins. Expression of MMP-2, MMP-9, TIMP-1, TIMP-2, and MYH-10 in the GM6001 group was significantly lower than that in the control group ( $P<0.05$ ). SM22 $\alpha$  expression was not significantly different between the 2 groups. Linear regression analyses of western blots and immunohistochemical staining of each target protein revealed a linear relationship ( $P<0.05$ ) (Figure 10).

### Apoptosis and collagen content

The apoptosis percentage and cell density decreased gradually with time in the neointima. The apoptosis percentage and cell density in the GM6001 group were significantly lower than that in the control group ( $P<0.05$ ) (Figure 11A).

The content of mature collagen in the neointima increased with increasing time. In the control group, the content of new collagen decreased gradually from 56 days but, in the GM6001 group, the content of new collagen decreased after reaching a maximum at 84 days. The content of new collagen

and mature collagen in the GM6001 group were significantly lower than that in the control group ( $P < 0.05$ ) (Figure 11B).

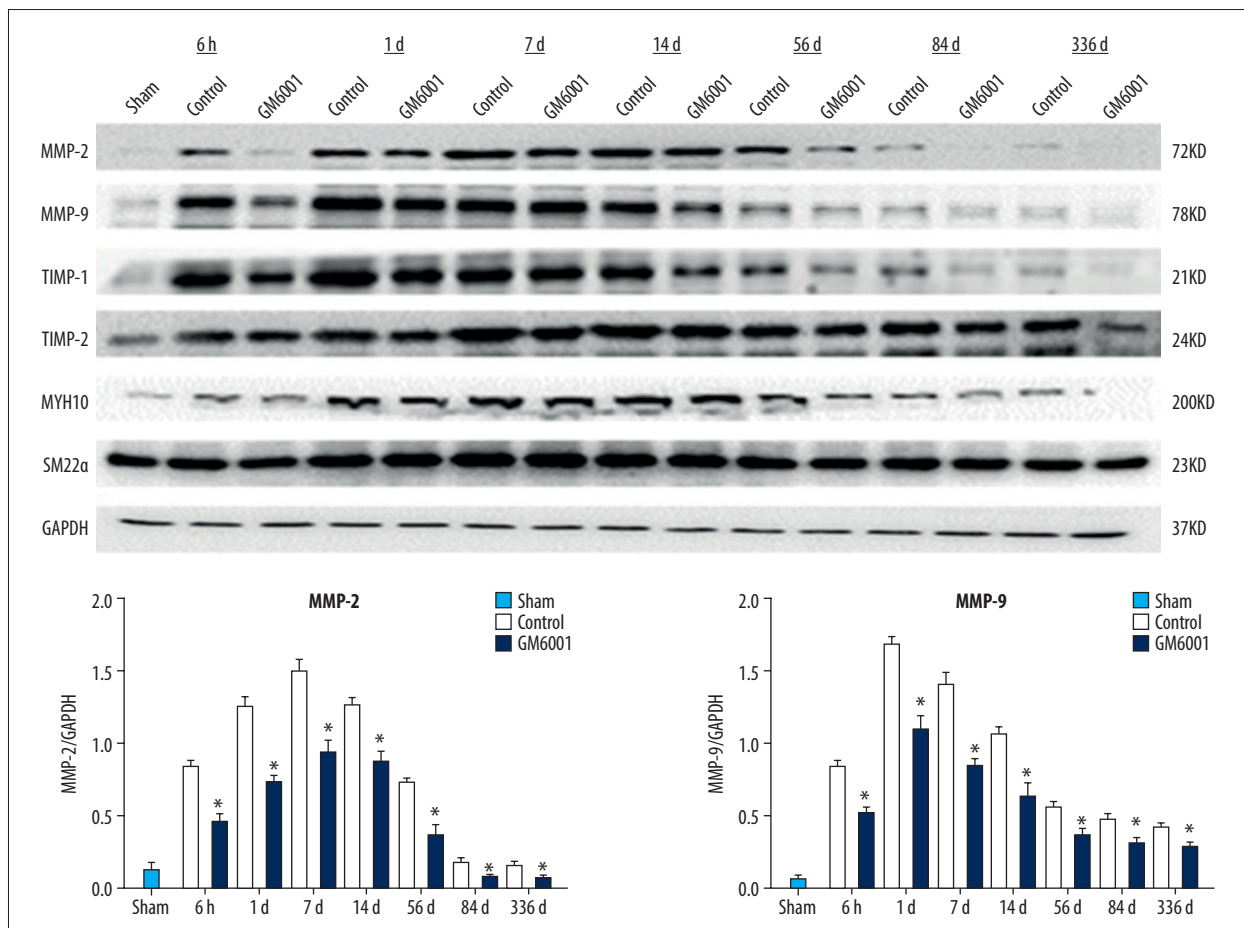
## Discussion

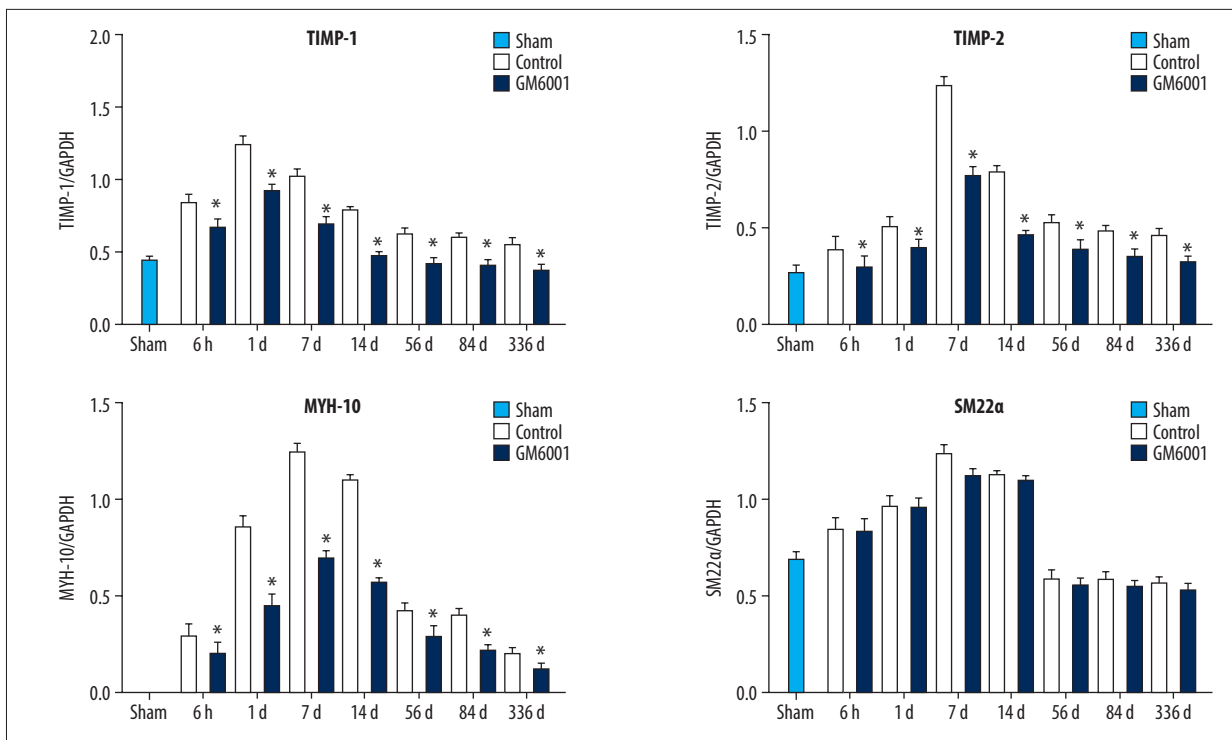
The common mechanisms of ISR include injury to vascular endothelial cells, proliferation and migration of VSMCs, ECM deposition, and vascular remodeling [10]. The proliferation and migration of VSMCs are necessary for stent restenosis. The migration of VSMCs from the vascular media to the intima must break through the "migration barrier" around the cells [14,15]. This barrier is complex (it comprises elastic fibers, internal elastic lamina, collagen I, III, and IV) [8]. The ECM has important roles not only as a cytoskeleton but also in phenotypic transformation of VSMCs and subsequent vascular remodeling. Therefore, ECM degradation plays an important part in promoting VSMC migration [16].

Studies have shown that MMPs can promote the transformation of VSMCs into proliferative phenotypes and enhance the proliferation and migration of VSMCs by degrading the matrix components around VSMCs [17]. Among them, MMP-2

and MMP-9 have key roles in ECM degradation [18]. Several studies have identified increased expression of MMP-2 and MMP-9 to coincide with VSMC migration from the media to the intima. MMP-2 and MMP-9 mainly degrade gelatin, collagen (types IV, V, and VII), fibronectin, laminin and elastin [19]. MMP-2 and MMP-9 contribute to intimal hyperplasia mainly through VSMC migration from the media into the intima by degrading and breaching the ECM proteins surrounding each cell and the internal elastic lamina [20,21].

We compared the impact of GM6001-eluting stents and eluting stents not containing GM6001 on expression of MMP-9, MMP-2, TIMP-1, TIMP-2, MYH-10, SM22 $\alpha$ , collagen content, percentage of apoptotic cells, and cell density in the iliac arteries of mini-pigs (Table 3). Expression of MMP-2 and MMP-9 increased significantly in the early stage then decreased gradually after implantation of stents that did not contain GM6001. Expression of MMP-2 and MMP-9 decreased significantly after implantation of GM6001-eluting stents. From 56 days, especially around the struts, the degree of reduction of MMP-2 and MMP-9 was significantly higher than that in the neointima. The closer the distance to the struts, the more pronounced was inhibition of expression of MMP-2





**Figure 10.** Western blot to detect the time course of the expression of target proteins. The expression and change trend of MMP-2, MMP-9, TIMP-1, TIMP-2, MYH-10, and SM22α in the 2 groups. The linear regression analysis of western blot and immunohistochemical results of each target protein revealed a linear relationship ( $P < 0.05$ ). MMP – matrix metalloproteinase; TIMP – tissue inhibitor of matrix metalloproteinase; MYH-10 – myosin heavy chain 10; SM22α – smooth muscle protein 22-alpha.

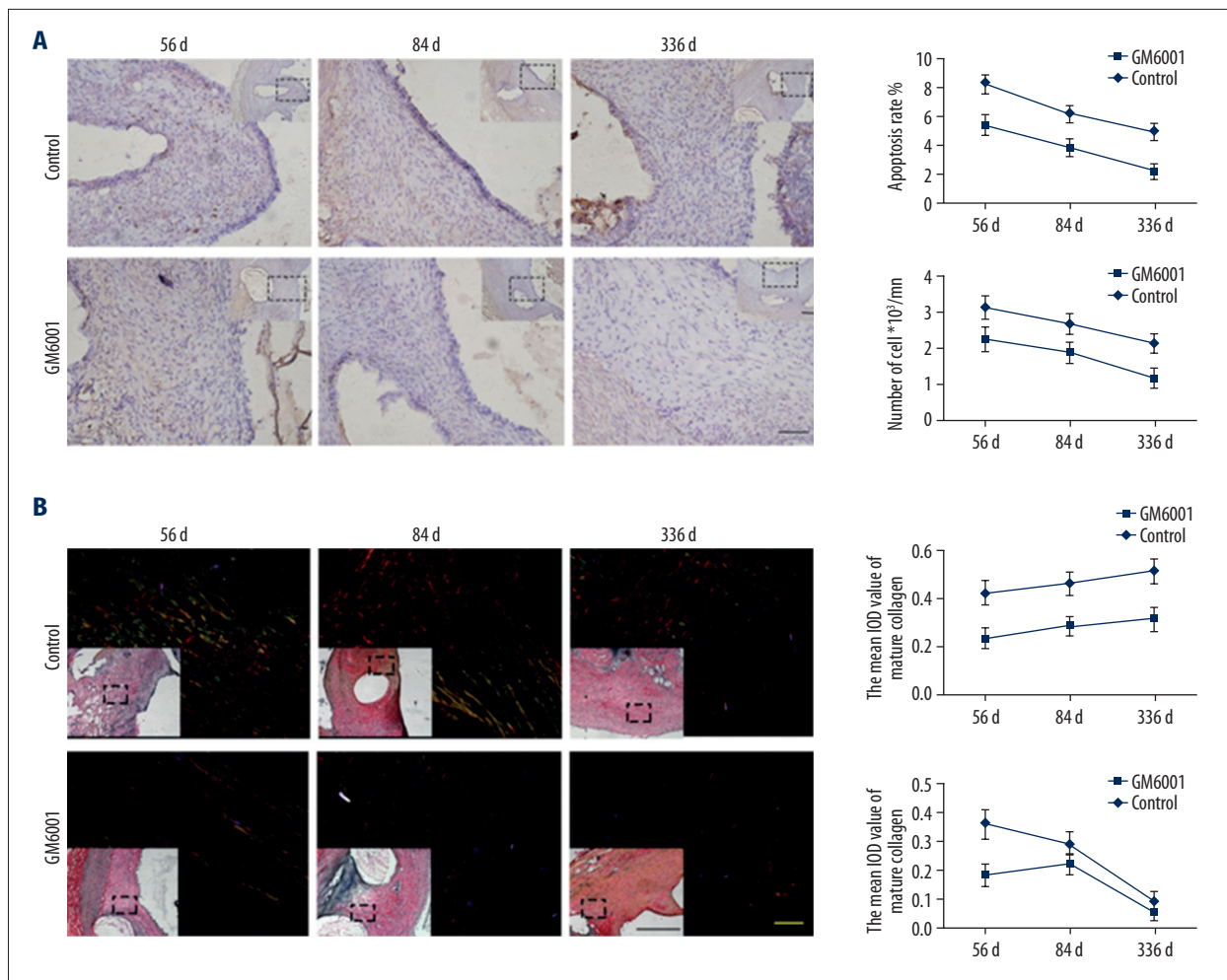
**Table 3.** Summary table of all results. The changes of MMP-2, MMP-9, TIMP-1, TIMP-2, MYH-10 and SM22α in the media and neointima after GM6001 eluting stent implantation. The changes of apoptosis percentage, cell density, new collagen and mature collagen in the neointima after GM6001 eluting stent implantation.

	Change after GM6001-eluting stent implantation	P
MMP-2	Decrease	<0.05
MMP-9	Decrease	<0.05
TIMP-1	Decrease	<0.05
TIMP-2	Decrease	<0.05
MYH-10	Decrease	<0.05
SM22α	No significant difference	>0.05
Apoptosis percentage	Decrease	<0.05
Cell density	Decrease	<0.05
New collagen	Decrease	<0.05
Mature collagen	Decrease	<0.05

and MMP-9. This finding also indicated that the decrease in expression of MMP-2 and MMP-9 was due to the effect of GM6001-eluting stents.

The reduced response of TIMP-1 and TIMP-2 after implantation of GM6001-eluting stents could have been due to several factors. TIMP expression changes with reductions in expression of MMPs. Hence, expression of MMP-2 and MMP-9 decreased so expression of TIMP-1 and TIMP-2 decreased. The exogenous MMPi GM6001 inhibited expression of TIMP-1 and TIMP-2 directly. The TIMP and exogenous inhibitor all retarded intima formation. In one study, TIMP-1 and TIMP-2 were transfected into cultures of blood vessels from rats and humans *in vitro*, and it was found that overexpression of TIMP-1 and TIMP-2 inhibited VSMC proliferation in rat arteries and human veins [22,23]. Li et al. implanted stents into the iliac arteries of rabbits and treated them with GM6001 via a peripheral vein [24]. They found that GM6001-treated animals had significant reductions in intimal cross-sectional area as well as an increase in luminal area compared with placebo-treated animals at 10 weeks. However, studies have shown that synthetic GM6001 has a low molecular weight and high activity *in vitro*. However, its water solubility is extremely low, and bioavailability in the whole body is low. Scholars have not observed long-term continuous changes





**Figure 11.** (A) The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, apoptotic rate, and cell density of the neointima. Bar=100  $\mu$ m for large images and 500  $\mu$ m for small images. (B) Picosirius Red staining: yellow staining for immature collagen and red staining for mature collagen. Bar=40  $\mu$ m for large images and 500  $\mu$ m for small images.

in expression of MMP-2 or MMP-9 with implantation of MMPI-eluting stents. We demonstrated that expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 was reduced significantly after implantation of GM6001-eluting stents.

During ISR, VSMCs undergo complex changes, including migration and proliferation from the media towards the intima [14,25]. Proliferation of VSMCs in the media and subsequent migration into the intima requires the transition of VSMCs from a contractile phenotype to a proliferative phenotype, with eventual ECM deposition in the intima [26]. We demonstrated that MYH-10 expression was reduced significantly in the media and intima after implantation of GM6001-eluting stents. There was no significant change in SM22 $\alpha$  expression in the 2 groups. The cell density and percentage of apoptotic cells in the GM6001 group in the neointima were also reduced significantly compared with those in the control group. This finding indicated that the number of VSMCs

with a proliferative phenotype in the media and neointima was reduced compared with that in the control group after implantation of GM6001-eluting stents.

Pathology studies using animals have shown that the cell components in the neointimal tissue of arteries after stent implantation account for a small part of the total volume, and that the ECM is the main component of the neointima, accounting for >50% of the total volume [27]. This proportion far exceeds the composition of the ECM and cellular components of the normal vascular wall. If the ECM is reduced, the risk of ISR will be inhibited significantly [28]. Collagens secreted by VSMCs are important components of neointimal ECM [29]. We revealed a significant decrease in the content of new collagen and mature collagen in the neointima of arteries of mini-pigs in the GM6001 group. Collagen is the main component of the neointima, so the neointimal

area in the GM6001 group was reduced, the vascular luminal area was increased, and the degree of ISR was reduced.

In our study, the neointima regressed at 336 days after hyperplasia. The Kim et al. study suggests that the mechanism of in-stent neointimal regression may be maturation of the neointima [30]. The early neointima contains large quantity of proteoglycans, which provide both the volume of the neointima, because of their high water content, and facilitation of cell migration and proliferation [31]. However, as the neointima ages, it loses the bulky, hydrated proteoglycans, which results in reduction in the neointimal volume and the stimulus for smooth muscle cell proliferation. Before the intimal hyperplasia, some suspected thrombus substances appeared in the vascular cavity. According to previous research, in the first phase of matrix formation is the formation of "provisional matrix" which is formed by the plasma proteins such as fibrin, fibrinogen, and fibronectin [32]. This matrix allows more SMCs to bind and proliferate while also trapping inflammatory cells that release MMPs that digest the hyaluronan so that a more permanent matrix collagen type I and III production can occur allowing the wound to completely heal with eventual fibrosis [33].

In previous studies, the anti-cell-proliferative agents used in DESs have prevented ISR by inhibiting VSMC proliferation,

but the proliferation of endothelial cells is also inhibited, leading to delayed endothelialization, which cannot inhibit ISR in the mid- and long-term [8,34]. Stent removal enabled clear detection of specimens containing metal stents. The most important finding of our study was that, by inhibiting expression of MMP-2 and MMP-9, a GM6001-eluting stent could reduce the number of VSMCs that in the neointima, and the collagen content of the main component of the ECM in the neointima decreased. These actions resulted in persistent and potent inhibition of intimal hyperplasia, an increase in luminal area, and no obvious thrombosis.

## Conclusions

Use of a GM6001-eluting stent resulted in persistent and potent inhibition of intimal hyperplasia, an increase in luminal area, and no obvious thrombosis in mini-pigs. Our study provides a theoretical basis for local release of exogenous MMPis to regulate MMP activity, SMCs, and ECM metabolism during ISR.

## Conflict of interests

None.

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