



## Biocompatibility and Biodegradation of Poly(*D,L*-lactic-*co*-glycolic acid) 90/10: In Vitro and In Vivo Assays

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### ABSTRACT

There has been concern regarding the long-term safety of the current drug-eluting stents (DESs) using "durable-polymer" technology. On this background, a number of second generation DESs using bioabsorbable-polymer are put under tests. In our study, we assessed the in vitro degradation and biocompatibility of poly (*D,L*-lactic-*co*-glycolic acid) (PLGA) 90L/10G, as a biodegradable material. In vitro degradation characteristics were evaluated by measuring decrease in the mass loss of PLGA films. In order to evaluate the cytotoxicity of PLGA, calf vascular smooth muscle cells (VSMCs) were incubated with PLGA films. Cells that were incubated in culture medium alone were used as controls. We determined the cell viability by a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and the distribution of cell cycle phases by flow cytometry. The morphology of VSMCs seeded on PLGA films was observed by scanning electron microscopy technique. PLGA films were inserted into the anterior wall of the left ventriculotomies and circumflex arteries of 6 dogs that served as test animals. The animals were sacrificed after 2 weeks, 1 month, and 2 months. Pathohistological and ultrastructural changes in the myocytes and vasculature were examined by light and electron microscopy. High-molecular-weight PLGA degraded slowly in the first 4 months. There was 10% mass loss at the 120th day and rapid mass loss thereafter. There were no differences in the cell viability and distribution of cell cycle phases between the control and PLGA groups. The cells attached favourably and grew well on the films. A slight inflammatory change occurred with regard to the pathohistology and ultrastructure of the myocardium and vessels. Thus, the low cytotoxicity, good histocompatibility, and biodegradable nature of PLGA 90/10 make it a promising material as a useful vehicle for locally administered drugs.

### Key Words:

biodegradable;  
biocompatible;  
PLGA;  
polymer;  
cytotoxicity.

### INTRODUCTION

The effectiveness of drug-eluting stents (DESs) in reducing the occurrence of in-stent restenosis and target lesion revascularization has been proven beyond doubt [1]. However, there has been concern regarding the long-term safety of the current DESs using "durable-polymer" technology [1–5]. Among the various safety issues,

the observations of delayed vascular healing and hypersensitivity reaction to the durable polymer, as well as the subsequent development of stent thrombosis is particularly prevailing. Although definite proof on causal relationship is lacking, it is conceivable that the presence of residual polymer after drug elution is the most likely culprit for

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the delayed healing as well as development of adverse events [6]. It is on this background that a number of second generation DESs using bioabsorbable-polymer and biodegradable drug-eluting stents are either in development or under clinical testing [7-12].

The recent study showed that slowing the release rate and lowering the dose of anti-proliferative drugs may favourably influences the vascular biological response to DESs implant, decreasing early toxicity and promoting stable healing while still suppressing neointima formation [13]. The property of the components or monomer units, especially their impact on biosystems, in biodegradable PLA copolymer or its blends is crucial in their performance [14,15]. In this research, we selected poly(*D,L*-lactic-*co*-glycolic acid) (PLGA) which was used in many medical fields and was proved to be biocompatible. The rate of biodegradation of PLGA increased rapidly with increases in the GA ratio. Thus, we used PLGA 90/10 which may show a longer period of degradation time than previous studies.

In our study, we have assessed for the first time the *in vitro* degradation kinetics and biocompatibility, and described the morphological changes of the biodegradable material PLGA 90/10.

## EXPERIMENTAL

### Materials and Methods

PLGA (90/10, molecular weight [MW] 160 kDa, and intrinsic viscosity, 1.4 dL/g) films were supplied by Shandong Medical Equipment Research Institute, shandong, china. Films were prepared by the solvent evaporation method as well.

### In Vitro Degradation

*In vitro* degradation characteristics were evaluated by measuring mass loss of PLGA films. For this purpose, preweighed 150- $\mu$ m-thick polymer films were placed in 2 mL of phosphate-buffered saline (PBS; pH 7.4) and incubated at 37°C. Samples were recovered periodically, dried in vacuum desiccators, and then weighed to determine the mass loss. The morphological changes of films surface during degradation were observed on a scanning electron microscope (SEM, Hitachi S-3400N, Japan).

### Cell Culture

VSMCs were obtained from the segments of aortas explanted from a newborn calf. The aortas were immersed in a solution of calcium- and magnesium-free PBS supplemented with gentamicin (400 U/mL). They were cut open longitudinally after removal of the adventitia. The endothelium was removed by gently rubbing the luminal side of the aortas over the surface of a tissue culture dish. Subsequently, the aortas were placed, lumen side down, at the bottom of a tissue culture flask and allowed to adhere for approximately 30 min. The tissues were then immersed in a growth medium comprising Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL) (Gibco, Grand Island, NY, USA). After 14 days, the aortic tissues were removed, and the VSMCs that had grown were detached using 0.25% trypsin (Hyclone, Logan, UT, USA). The detached cells were resuspended in the growth medium and seeded in tissue culture flasks.

### Immunocytochemistry

Subconfluent cultures at early passage were examined for the presence of  $\alpha$ -SM actin, (anti-smooth muscle actin, clone 1A4 [1:400], Sigma) After fixing in 1% phosphate-buffered formalin on ice for 30 min, the cells were permeabilized with 0.1% Triton X-100 for 30 min and subsequently incubated with the primary antibody for 12 h at 4°C. Secondary antibodies were conjugated with fluorescein isothiocyanate (FITC) (Sigma, Sigma Chemicals, St. Louis, MO). Immunofluorescent images were obtained using a fluorescence microscope (Nikon Eclipse, Japan) equipped with 20 $\times$ , 40 $\times$ , and 100 $\times$  objectives and a digital camera (Nikon DXM1200, Japan).

### Cell Viability Assay

Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which assesses the mitochondrial activity in living cells. VSMCs were plated on a 96-well plate ( $1.0 \times 10^5$  cells/mL) and cultured with PLGA films. Cells that were incubated in culture medium alone were used as controls. Cell viability

was detected on the 1st, 3rd, 5th, and 7th days of culturing. After refreshing the medium on the above mentioned days, 20  $\mu\text{L}$  of MTT (5 mg/mL in PBS) was added to each well, and the cells were incubated for another 4 h. After incubation, 150  $\mu\text{L}$  of dimethyl sulphoxide (DMSO, Sigma, St. Louis, MO) was added for 12 min to dissolve the formazan precipitate. Absorbance was measured at 490 nm using a microplate autoreader.

### Cell Cycle Phase

VSMCs were plated on a 6-well plate ( $1.0 \times 10^6$  cells/mL) and cultured with PLGA films. Cells that were incubated in culture medium alone were used as controls. After refreshing the medium on days 1st, 3rd, 5th, and 7th, the cell cycle was determined on the 7th day. The cells were harvested by trypsinization, washed with PBS, fixed in ice-cold 70% methanol for 24 h, washed again with PBS, and treated with 1 mg/mL RNase A in a buffer containing 1mM Tris-HCl (pH 7.4) and 1.5 mM NaCl for 30 min at 37°C.

The cells were then collected by centrifuging at 400 g for 5 min and stained with 250 mL of nuclear staining solution (10 mg propidium iodide, 0.1 mg trisodium citrate, and 0.03 mL Triton X-100 dissolved in 100 mL water) for 30 min at room temperature in the dark. After adding 750 mL of PBS, a flow cytometer (EPICS XL, Beckman Coulter, FL, USA) was used to determine the DNA content which was determined on the basis of the propidium iodide signal and served as an index of the cell cycle phase.

### Cell Morphology

For morphology observation, the VSMCs were seeded onto the samples by dipping the samples into a cell suspension of  $3 \times 10^5$  cells/100  $\mu\text{L}$ , and after 4 h of incubation, the culture wells were filled with 1 mL of the culture medium. After 7 days of culturing, cellular constructs were harvested and rinsed twice with PBS to remove nonadherent cells. For scanning electron microscopy (SEM) observations, the cellular constructs were fixed with 2.5% glutaraldehyde at 4°C for 4 h and subsequently dehydrated through a series of graded alcohol solutions and then air dried overnight. After drying, the samples were sputtered with gold and observed by SEM.

### Animal Preparation

For this study, we used 6 mongrel dogs of either sex that weighed 15-25 kg. They were randomly divided into the following 3 groups: 2-week group ( $n=2$ ), 1-month group ( $n=2$ ), and 2-month group ( $n=2$ ). The dogs were anesthetized with pentobarbital sodium (25 mg.kg<sup>-1</sup>). After intubation and mechanical ventilation, left thoracotomy was performed. The PLGA films were placed in the anterior wall of the left ventriculotomies and circumflex arteries.

### Morphological Evaluation

The test specimens with surrounding muscular tissue were excised. After fixing in 4% phosphate-buffered formalin, the samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Histological evaluation of the specimens was performed by blind analysis by a pathologist. The assessed biological response parameters included acute inflammatory changes, necrosis, chronic inflammatory changes, foreign body reaction, and fibrosis. Analysis was performed according to the ISO standards which do not include statistical analysis. Tissue reactions were scored semi-quantitatively according to the following criteria: 0 = no reaction, 1 = mild reaction, 2 = moderate reaction, and 3 = severe reaction.

For electron microscopy, ultrathin sections (50-100 nm) were cut from each sample, counterstained with uranium acetate and lead citrate, and examined under a transmission electron microscope (Philips 201, USA) by 2 professionals.

### Statistical Analysis

All results were presented as mean  $\pm$  standard deviation (SD) unless otherwise specified. Comparisons between quantitative data were made using a t test.  $P < 0.05$  was considered statistically significant. SPSS 10.0 software (SPSS, Chicago, Ill, USA) was used for statistical analysis.

## RESULTS AND DISCUSSION

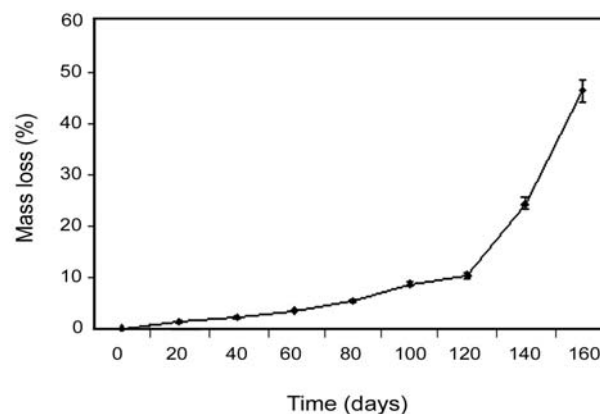
PLGA has shown good histological findings and durability when it was used to patch the canine pulmonary artery trunk [16]. PLGA has been used for ophthalmic

and orthopedic applications and has been found to be biocompatible [17,18]. Therefore, we selected PLGA from among several biodegradable polymers.

### In Vitro Degradation Kinetics and Morphological Changes

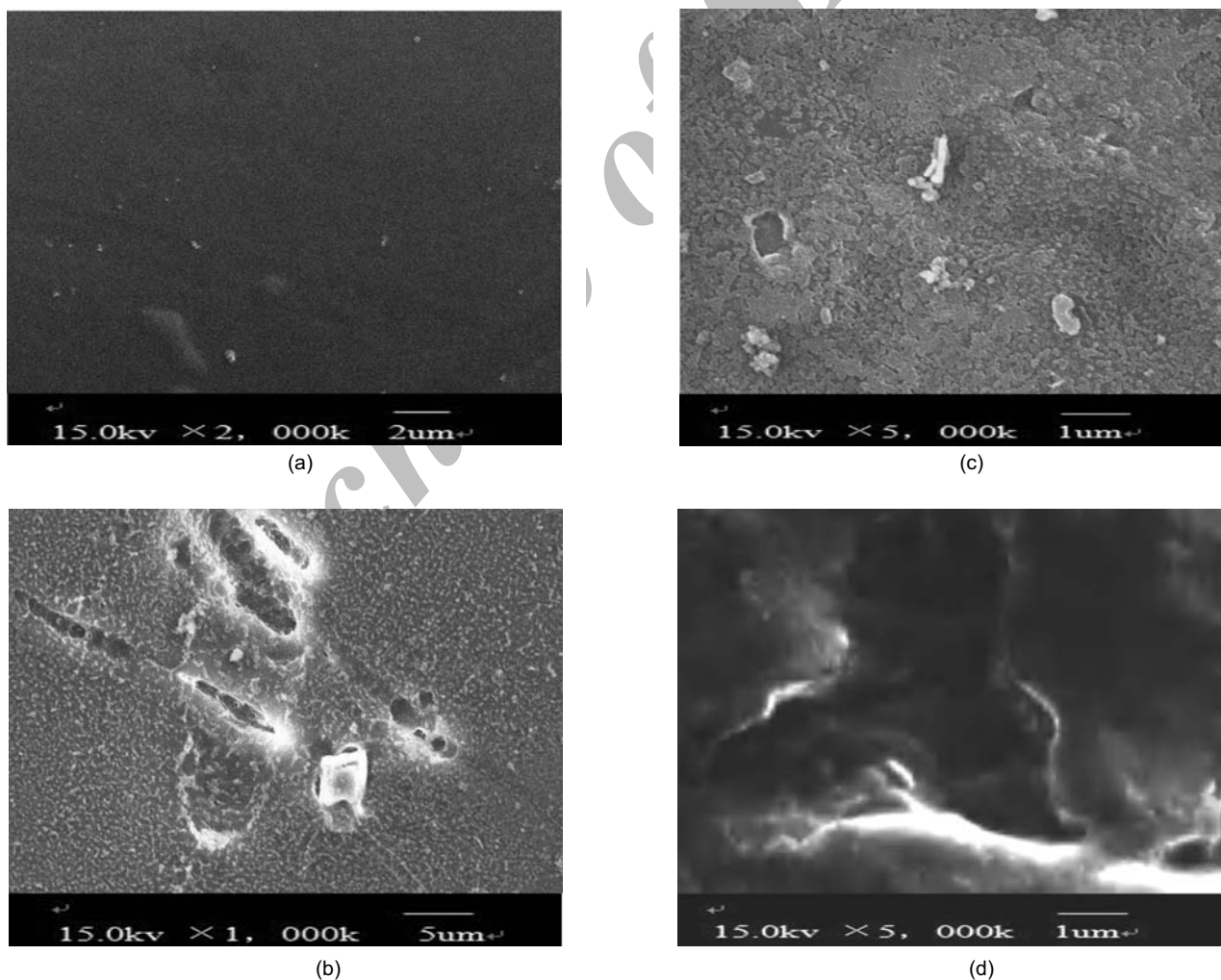
The rate of mass loss plays an important role in investigating the biodegradation process of polymers. Figure 1 shows the mass loss curve for a degrading PLGA polymer.

High-molecular-weight PLGA 90/10 degraded slowly in the first 4 months; there was 10% mass loss on the 120th day and rapid mass loss thereafter (Figure 1). The PLGA films which were prepared by the solvent evaporation method had a compact structure without any holes. PLGA 90/10 as a hydrophobic polymer tended to show surface erosion

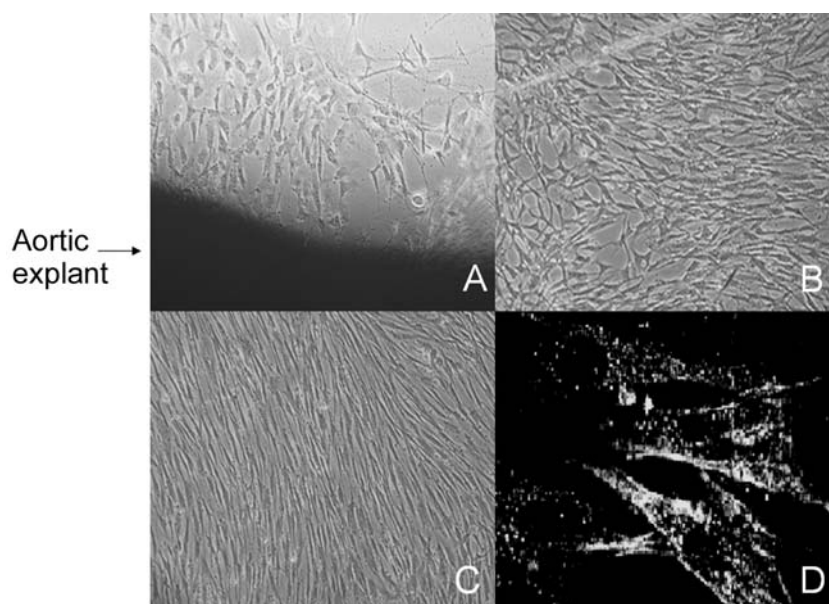


**Figure 1.** Changes in the mass of PLGA 90/10 during in vitro degradation.

characteristics [19]. In degradation process, the transparent films turned milky and slightly expanded and



**Figure 2.** SEM micrographs of PLGA 90/10 specimens after erosion in PBS: (a) as-prepared PLGA film; (b) degradation after 17 weeks; (c) degradation after 23 weeks; (d) degradation after 24 weeks.



**Figure 3.** Microscopic images of vascular smooth muscle cells: (A) growth of fusiform smooth muscle cells in tissue; (B) fusion of smooth muscle cells; (C) high-density smooth muscle cells (original magnification,  $\times 100$ ); (D)  $\alpha$ -smooth muscle actin was positive for immunofluorescence staining.

because the films became hard and brittle, the mechanical properties decreased as well. At 17th week, the surface of films became rough (Figure 2b). At 23th week, though the films maintained the former profile, it became brittle and apt to become fragile (Figure 2c), and finally at 24th week the surface of films collapsed (Figure 2d). Thereafter, the polymer films shrank all together.

Jabara et al [13] studied the PLGA coating, paclitaxel-eluting stent. The *in vitro* studies demonstrated a gradual elution over the course of 12 to 16 weeks. The animal experiment study results support the notion that slowing the release rate and lowering the dose of paclitaxel favourably it influences the vascular biological response to DES implant, decreasing early toxicity and promoting stable healing while still suppressing neointima formation. The rate of biodegradation of PLGA increased rapidly with increases in the GA ratio [20]. The molecular weight,

crystallinity, thermal history, and geometry of the implant considerably influence its degradation. The previous study showed that the PLGA 80/20 knitted stents lost about 35% of their initial weight at 11th week [21]. Our study showed that due to the large molecular weight and a larger proportion of the PLA, PLGA 90/10 had a longer degradation time [22] and it degraded slowly in the first 4 months followed by rapid mass loss thereafter.

### Cell Identification and Morphology

The primary cells in the culture were observed from the edge of the cultured tissues after 10 days. VSMCs of the calf aorta on first passage showed the “hill and valley” morphological characteristic after 14 days of culturing. VSMCs that had been cultured from the calf aortic explants for approximately 14 days stained positive for  $\alpha$ -SM-actin, thus confirming the smooth muscle nature of these cells (Figure 3).

**Table 1.** MTT test showed viability of VSMCs in control group and PLGA group cultured at 1, 3, 5, and 7 days *in vitro*. ( $P > 0.05$ )

Group	Cell viability			
	1 (day)	3 (days)	5 (days)	7 (days)
Control group	0.375 $\pm$ 0.154	0.381 $\pm$ 0.158	0.425 $\pm$ 0.110	0.494 $\pm$ 0.123
PLGA group	0.310 $\pm$ 0.110	0.334 $\pm$ 0.144	0.410 $\pm$ 0.119	0.466 $\pm$ 0.104

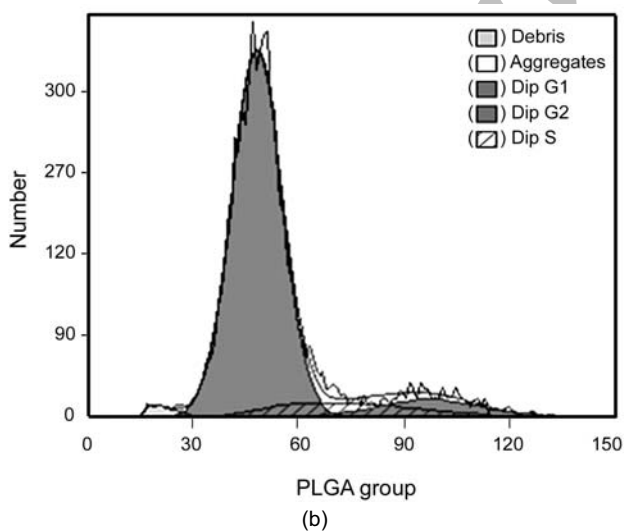
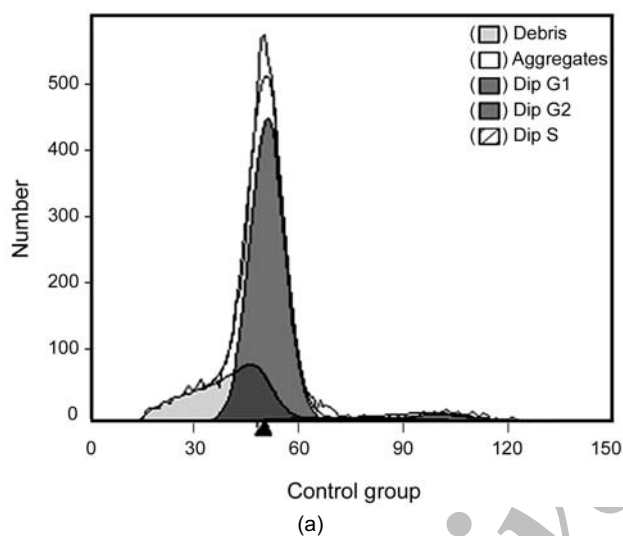
**Table 2.** The effect of PLGA on cell cycle in vascular smooth muscle cells. Cell-cycle analysis by flow cytometry. ( $P > 0.05$ )

Group	Cell viability		
	G0/G1	S	G2/M
Control group	91.59±1.086	3.275±0.11	3.89±0.111
PLGA group	87.84±2.901	5.8±1.824	5.113±0.745

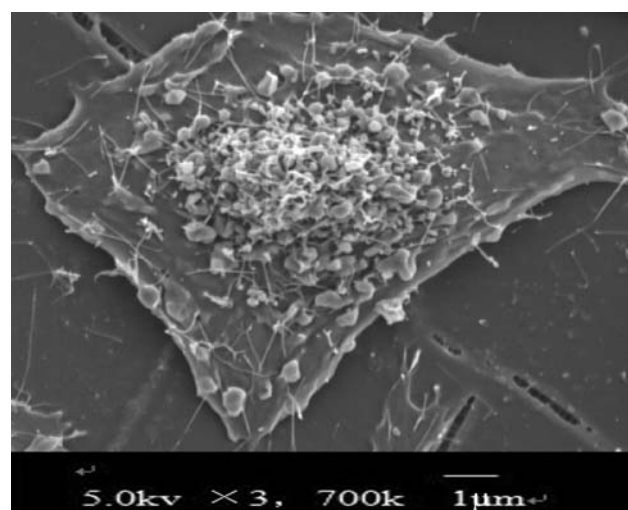
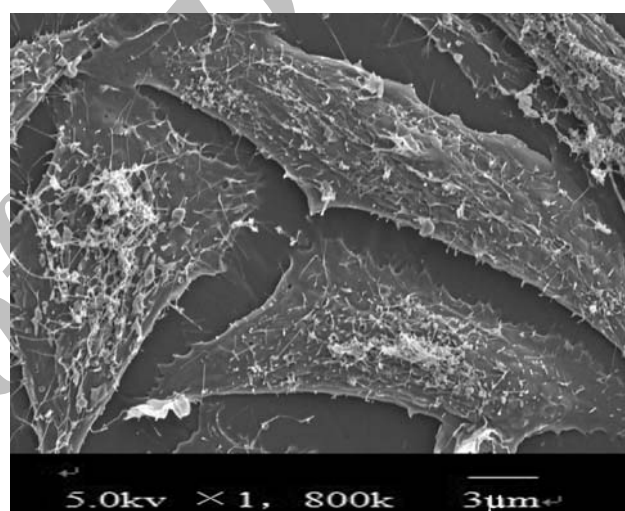
**Cell Viability and Cell Cycle Phase**

MTT test showed that there were no significant differences in the cell viability of VSMCs cultured at 1st,3rd, 5th, and 7th days in vitro between the PLGA

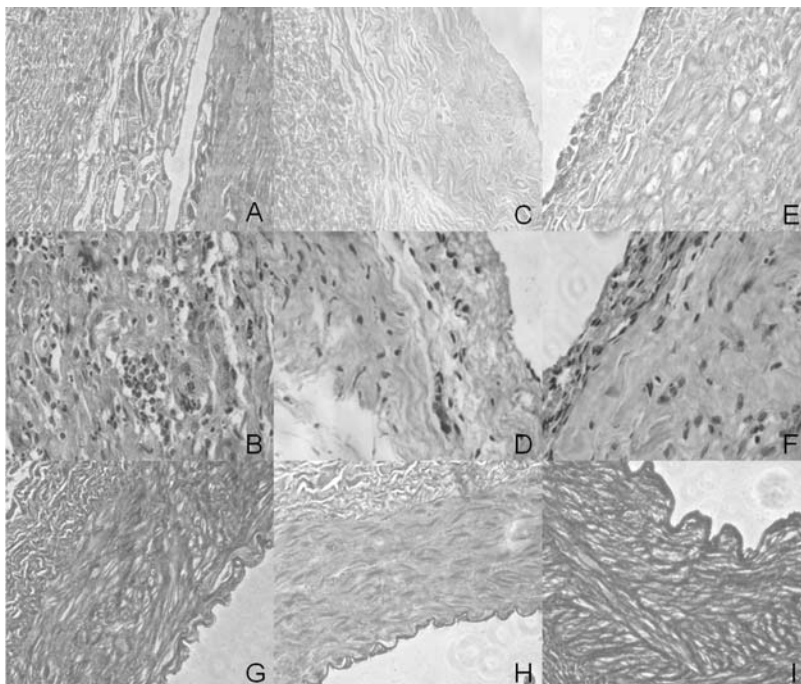
and control groups ( $P > 0.05$ ) (Table 1). There were no significant differences in the percentage of cells in the G0/G1, S, G2/M phase between the PLGA and control groups ( $P > 0.05$ ) (Table 2 and Figure 4).



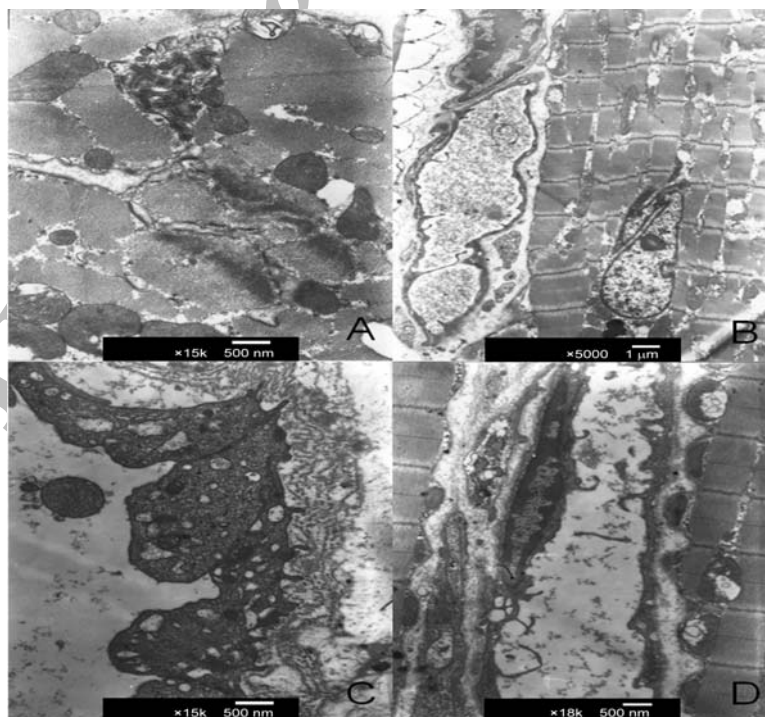
**Figure 4.** Flow cytometric analysis for the effect of PLGA on cell cycle in vascular smooth muscle cells. Cells that were incubated in culture medium alone were used as controls. Shown here is the cell cycle distribution of: (a) control group and (b) PLGA group.



**Figure 5.** Morphology of vascular smooth muscle cells cultured for: (a) 3 and (b) 7 days with PLGA films. (a) 1,800×; (b) 3,700×.



**Figure 6.** Histology of a PLGA film after 15 d, 1 month, and 2 months of intramuscular implantation in the dogs: (A) after 15 d, infiltration of neutrophilic granulocytes and lymphocytes were observed to some extent; (B) vascular proliferation and fibroblastic capsule formation began, (C) after 30 d, infiltration of lymphocytes reduced, (D) fibroblastic capsule was formed completely, (E) after 60 d, infiltration of lymphocytes, and (F) a compact fibroblastic capsule were observed; (G) after 15 d; (H) after 30 d; (I) after 60 d. (There was no obvious infiltration of inflammatory cells in vasculature.) (A, C, E, G, H, and I:  $\times 200$ ; B, D, and F:  $\times 400$ ).



**Figure 7.** Representative transmission electron micrographs of the ventricular myocardium and vasculature. A mild inflammatory reaction was observed in the myocytes and vasculature. The sarcoplasm network expanded mildly. Partial elastic membrane thickening was observed. There was no significant myocyte apoptosis or vascular necrosis. (A, B, C, and D: magnification  $\times 10,000$ ).

### Cell Morphology

Cell morphology and interaction between the cells and PLGA films were studied in vitro for 7 days. Figure 5a presents the SEM micrograph showing the morphology of VSMCs on the film cultured for 3 days. It was observed that VSMCs which are attached and spread on the films show normal morphology and phenotype. Figure 5b shows the confluence of VSMCs with polygonal shape on the films.

### Morphological Changes

The results from light microscopic analysis are shown in Figure 6. After 15 days of implantation, infiltration of neutrophilic granulocytes and lymphocytes were observed to some extent and vascular proliferation and fibroblastic capsule formation began. On 30th day after implantation there was a drop in infiltration of the lymphocytes, and the fibroblastic capsule was formed completely. After 60 days of implantation, the infiltration of the lymphocytes and a compact fibroblastic capsule were observed. There was no obvious infiltration of inflammatory cells in vasculature. Atrial myocardial ultrastructure was examined by electron microscopy (Figure 7).

Mild inflammatory reaction was observed in the myocytes and vasculature. The sarcoplasm network expanded mildly. Partial elastic membrane thickening was observed. There was no significant myocyte apoptosis or vascular necrosis.

### CONCLUSION

Our present study indicated that the proliferation and viability of VSMCs were not affected by the presence of PLGA. The VSMCs adhered on the films intimately and spread well. During the in vivo assessment, there were no behavioural changes or visible signs of physical impairment indicative of systemic or neurological toxicity. In general, PLGA induced the early inflammatory response, as indicated by the recruitment of lymphocytes and granulocytes and by fibrotic band thickness.

A significant acute inflammatory component of neutrophils was not detected. The ultrastructure of the myocytes and vasculature revealed a mild inflammatory reaction. There was no significant myocyte

apoptosis or vascular necrosis. Due to the large molecular weight and a larger proportion of the PLA, PLGA 90/10 has a longer degradation time. Our study demonstrated that PLGA 90/10 degraded slowly in the first 4 months; there was 10% mass loss on the 120th day and then rapid mass loss thereafter.

The low cytotoxicity, good histocompatibility, and biodegradable nature of PLGA 90/10 make it a promising material for a useful vehicle for locally administered drugs.

### Future Requirements

Important issues that need to be addressed in the future during further stent development are fatigue testing under dynamic conditions, identification of appropriate sterilization protocols, and options for drug incorporation. The in vivo performance and degradation of the stent should also be evaluated.

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