ABBS

Original Article

Electrical stimulation inhibits neointimal hyperplasia after abdominal aorta balloon injury through the PTEN/p27Kip1 pathway

Ping Zhang[†], Zhitao Liu[1†], Guoxiang He[*], Jianping Liu, and Jian Feng

Department of Cardiology, Southwest Hospital, Third Military Medical University and Chongqing Institute of Interventional Cardiology, Chongqing 400038, China
[†]These authors contribute equally to this work.
[*]Correspondence address. Tel: +86-23-68754268; Fax: +86-023-68754007; E-mail: heguoxiangcq@yahoo.com.cn
[1]Present address: No. 401, Hospital of the Chinese People's Liberation Army, Qingdao 266000, China

Electric fields (EFs) exert biological effects on promoting wound healing by facilitating cell division, cell proliferation, and cell directional migration toward the wound. In this study, we examined the inhibitory effect of direct-current (DC) EFs on the formation of neointimal hyperplasia and the possible mechanism in an abdominal aorta balloon injury rabbit model. Sixty rabbits were divided into normal, control, and experimental groups. After establishment of the abdominal aorta balloon injury model, electrodes were implanted into the bilateral psoas major muscle in control and experimental groups. Only the experimental group received electric stimulation (EFs applied at 3 or 4 V/cm for 30 min/day) for 1, 2, and 4 weeks, respectively. Neointimal hyperplasia of the abdominal aorta and proliferation of vascular smooth muscle cells (VSMCs) were measured. Expressions of collagen, p27^{Kip1}, and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) were detected. Results showed that the ratio of the tunica intima area to the tunica media area, the expression of type-I collagen in the neointimal, and the proliferating cell nuclear antigen index in experimental groups were significantly less than those in control groups 2 weeks post-operation ($P < 0.01$). Expressions of p27^{Kip1} and PTEN were increased in experimental groups compared with control groups ($P < 0.01$). In conclusion, our results suggested that the application of DC EFs could inhibit neointimal hyperplasia and reduce collagen expression after abdominal aorta balloon injury. This was probably induced by upregulation of PTEN/p27^{Kip1} expression, thereby inhibiting VSMC proliferation.

Keywords   electric stimulation; neointimal hyperplasia; balloon injury; PTEN; p27^{Kip1}

Introduction

Restenosis after successful percutaneous transluminal coronary intervention remains a major problem limiting the long-term efficacy of this procedure. Direct and indirect evidence strongly indicated that intimal hyperplasia or proliferation of medial smooth muscle cells may be one of the main reasons [1]. Medial vascular smooth muscle cells (VSMCs) constitute the cardinal component of the arterial wall. It is believed that restenosis involves the reactivation of quiescent VSMCs in the arterial wall that induced by mechanical or inflammatory means [2]. The proliferation of VSMCs is controlled by a complex interaction between stimulatory and inhibitory growth factors [3]. When the stimulating growth factors overcome the action of inhibitors, the proliferative activity of VSMCs is greatly increased and the healing process occurs [4,5]. The intrinsic proliferative potential of VSMCs may also play an important role in the growth regulation.

Currently, treatments for restenosis have focused on the conventional medicine, gene therapy, interventional therapy, and surgical procedures. These treatments can reduce the risk of restenosis to a certain extent, but their effects on the repair of vascular endothelium and the inhibition of neointimal hyperplasia in the injured vascular are incompletely understood.

Endogenous electric fields (EFs) are present in physiological and pathophysiological processes such as the wound healing, tissue regeneration, embryonic development, and tumor formation [6]. They also exist around blood vessels. Researchers have discovered that there are several types of electric potential difference around the vascular endothelium and that these fields are critical in regulation of the generation and development of thrombosis [7]. Electric potential difference can be generated when the vascular endothelium is injured [7]. The electric current

generated at the wound area and the surrounding epithelial area is a steady direct current (DC) [8]. These endogenous EFs exert biological effects on promoting the wound healing by facilitating cell division, cell proliferation, cell directional migration toward the wound, and by remodeling and stimulating the release of various cytokines and growth factors such as vascular endothelial growth factor [9]. If endogenous EFs cannot stimulate the wound healing, the application of DC to the injured and the non-injured regions could greatly benefit this process. It has been confirmed that the healing of bone fractures, chronic injuries to the soft tissue, intractable skin ulcers, as well as many other traumatic disorders can be improved (or even treated) by application of steady-DC EFs [10]. For example, the degree of local atherosclerosis is alleviated after EF stimulation by local implantation of electrodes in the psoas major muscle in an atherosclerosis model in rabbits [11].

Here, we examined the inhibitory effects of DC EFs on neointimal hyperplasia and the possible mechanism in an abdominal aorta balloon injury rabbit model.

Materials and Methods

Animal experiment
Healthy Japanese white rabbits were provided by the Laboratory Animal Center of the Third Military Medical University (Chongqing, China). They were kept in individual feeding cages at 20–22°C and 50–60% relative humidity on a 12-h light/12-h dark cycle. They were provided with 100 g of food (Nutrition Department of Da-Ping Hospital, Third Military Medical University) per day and water *ad libitum*.

The animal experiment protocol was approved by the Ethics Committee of Animal Research of China (Beijing, China). Sixty rabbits (30 males and 30 females; 3 months old; 1.98 ± 0.23 kg) were used in this study. Rabbits were randomly divided into normal group ($n = 6$), control (Con) group ($n = 18$), and experimental (Exp) group ($n = 36$). The Exp group was then divided into 3.0 V/cm Exp group (3Exp) and 4.0 V/cm Exp group (4Exp) according to the different EF intensity. The abdominal aorta balloon injury model was established in the Con and the Exp groups. The electrodes were implanted in all rabbits, but only the Exp group received electrical stimulation. Samples were collected at 1, 2, and 4 weeks post-balloon injury in each group.

Materials
Evans blue was purchased from Sigma-Aldrich (St. Louis, USA). Sirius red dye was purchased from Polysciences (Warrington, USA). The apoptosis kit was from Roche Pharmaceuticals (Nutley, USA). Mouse anti-collagen I and III antibodies were obtained from Abcam (Cambridge,

USA) and EMD Chemicals (Darmstadt, Germany), respectively. Anti-rabbit proliferating cell nuclear antigen (PCNA) polyclonal antibody was purchased from LifeSpan BioSciences (Seattle, USA). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) mouse polyclonal antibody and rabbit anti-Kip1 (p27[Kip1]) polyclonal antibody were purchased from Cell Signaling (Danvers, USA) and Upstate Incorporated (Charlottesville, USA), respectively. The internal standard, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, was purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

Anesthesia
Rabbits were sedated with xylazine hydrochloride (5 mg/kg, i.m.; Sigma-Aldrich) and atropine sulfate (0.04 mg/kg, i.m.; Zhongdan Pharmaceutical Company, Taizhou, China). Twenty minutes later, general anesthesia was induced with ketamine hydrochloride (50 mg/kg, i.m.; Xianju Pharmaceutical Company, Xianju, China) and maintained with isoflurane (0.5–1% in 100% oxygen; Abbott Laboratories Limited, Maidenhead, UK).

During the procedure, intermittent bolus doses of fentanyl (1 mg/kg/30 min, i.v.; Yichang Pharmaceutical Company, Yichang, China) were given to enhance analgesia. The pedal reflex was checked every 5 min throughout the surgical procedure.

Establishment of the rabbit model
After disappearance of the pedal reflex in the hind limbs, rabbits were placed in the supine position on a heated operating table. The right femoral artery, the inferior segment of the abdominal aorta, and common iliac artery were exposed. A percutaneous transluminal coronary angioplasty balloon of 2.5 mm × 20 mm was inserted into the inferior segment abdominal aorta through the right femoral artery. The distance between the balloon tip and the abdominal aorta crotch was 3 cm. A pressure of 12 atm was used in the balloon and released when the balloon was withdrawn at the crotch. These procedures were repeated three times. The balloon catheter was then withdrawn and the right femoral artery was ligated.

A pair of platinum electrodes was inserted into the bilateral psoas major muscle of the abdominal aorta. The electrodes were parallel to the abdominal aorta. The electrode tips were 2-mm above the abdominal aorta crotch, and the distance from the electrodes to the abdominal aorta was 1 cm. All local incisions and skin were sutured in a non-continuous style. Electrodes were fixed on the back of the neck through a subcutaneous tunnel from the abdominal cavity. Low-voltage DC stimulation (EFs 3 or 4 V/cm) was applied using a DC power supply (Shan-Jie Technological Limited Company, Shanghai, China) for 30 min/day from

the second postoperative day until the end of the experiment.

Prophylactic antibiotic treatment (ceftazidime 10 mg/kg/8 h, i.m., Xianju Pharmaceutical Company) was given for 3 days. The analgesic treatment with tramadol hydrochloride (1.5 mg/kg, s.c.; Shanghai Pharmaceutical Limited Company, Shanghai, China) was given every 4 h on the first day, every 6 h on the second day, and every 8 h on the third day.

Observation of the neointimal hyperplasia

After the rabbits were sacrificed, the abdominal aorta was perfused and fixed with 4% paraformaldehyde solution. Routine dehydration, paraffin embedding, and sectioning were carried out after the connective tissue of the tunica externa was sheared. The ratio of the tunica intima area to the tunica media area was calculated using hematoxylin and eosin (H&E) staining. Collagens I and III were stained by Sirius red dye. To minimize the variation across different batches of staining, the experiment procedure was performed strictly according to the manufacturer's instructions. The concentration of the dyes and the staining time and exposure time of the photograph should be coincided all the time. Images were observed using an Olympus DX71 Micro-image Acquisition System (Olympus Optical Company, London, UK). The integrated optical density (IOD) was calculated using Image Pro-plus 6.0 software (Media Cybernetics, Bethesda, USA).

Detection of the apoptosis and proliferation of VSMCs and reendothelialization

The apoptosis and the proliferation of VSMCs were detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method and PCNA immunohistochemistry, respectively. Values of the apoptosis index (AI) and proliferation index (PI) were calculated as the percentage of positive apoptosis and positive PCNA cell nuclei vs. the total amount of VSMCs, respectively. Reendothelialization of the injured intima was observed using Evans blue staining (1%, 1 ml/kg, i.v., 30 min before rabbit was sacrificed). Images were obtained using a Finepix S6500 digital camera (Fuji Company, Sendai, Japan), and IOD values were calculated using Image Pro-plus 6.0 software.

Immunohistochemistry

Tissue sections (4 μm) were deparaffinized, dehydrated, and subjected to antigen retrieval using microwave oven (2 min at 1000 W and 5 min at 200 W) followed by incubation with 0.05% trypsin in phosphate-buffered solution (PBS) for 15 min at room temperature. The endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide, and then the slides were treated with 5% bovine serum albumin for 15 min. PTEN or $p27^{Kip1}$ antibody was added, respectively. The incubation with primary antibodies was performed at 4°C overnight. Following washes with PBS, the slides were incubated with secondary biotinylated antibodies and incubated with avidin–horseradish peroxidase complex (Vector Laboratories, Burlingame, USA). After counter-staining with hematoxylin (DakoCytomation, Carpinteria, USA), the slides were examined.

Western blotting

Total protein of the abdominal aorta segment (~3 cm above the abdominal aorta crotch) was extracted, and the protein concentration was determined. Protein samples were denatured by heating at 95°C for 3–5 min. They were then loaded onto 10% sodium dodecyl sulfate polyacrylamide gels (30 μg protein/lane).

After electrophoresis, protein bands were transferred onto nitrocellulose membranes, incubated in the blocking buffer (5% defatted milk powder), and washed with $1 \times$ TBS-T (100 mM Tris–HCl, pH 7.5, 0.9% NaCl, and 0.05% Tween 20). The expression of each examined protein was identified using the corresponding antibody as described in the 'Materials' section. Images were formatted and recorded with a gelatin formatter (Bio-Rad, Hercules, USA). The absorbance of each target protein band and the internal control (GAPDH) was analyzed using Quantity One Software (Bio-Rad). The relative expression of each target protein was expressed as the ratio of the intensity of the target protein band vs. the one of the GAPDH.

Quantitative real-time reverse transcription–polymerase chain reaction

Total RNA was extracted from selected arteries by a standard protocol using Trizol reagent (Invitrogen, Carlsbad, USA). cDNA was synthesized by reverse transcription (RT) from 2 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative RT–polymerase chain reaction (qRT–PCR) was carried out on a RotorGene 2000 (Corbett Research, Sydney, Australia) using an SYBR green detection protocol. After an initial denaturation step of 5 min at 95°C, PCR amplification of the cDNA fragments corresponding to $p27^{Kip1}$, PTEN, and internal GAPDH genes was carried out through 45 cycles at 94°C for 30 s, 60°C for 5 s, and 72°C for 10 s. The following primers were used: $p27^{Kip1}$ forward 5′-GGG ACCTGGAGAAGCACTGC-3′, reverse 5′-TTTTGTTTTG AGGGGAAGAATC-3′; PTEN forward 5′-CGACGGG AAGACAAGACAAGTTCAT-3′, reverse 5′-GCTAGCCT CTGGATTTGACG-3′; GAPDH forward 5′-ACCCAT CACCATCTTCCAGGAG-3′, reverse 5′-GAAGGGGCGG AGATGATGAC-3′.

Statistical analysis

The SPSS11.5 software package for Windows (SPSS, Chicago, USA) was used for data analyses. Data were expressed as the mean $\pm$ SD. The significance of the means was tested by one-way ANOVA. The least-square difference (LSD) test, Student's t-test, and the Dunnett t-test were used for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

General health of rabbits

Three out of the 60 rabbits, one each from the Con 2 weeks (Con2w), 3Exp1w and 4Exp2w groups, died during the 4-week experiment. The overall mortality was 5%. The rabbit in the Con2w group died of the infection of the abdominal cavity, whereas the rabbit in the 3Exp1w group died of the intestinal obstruction. The cause of death for the rabbit in the 4Exp2w group was not known.

The ratio of the tunica intima area to the tunica media area

The neointimal began to form, and the thickness gradually increased 1 week after the tunica intima was injured. The ratios of the tunica intima area to the tunica media area in the Con1w, Con2w, and Con4w groups were 0.08 ± 0.01, 0.21 ± 0.02, and 0.47 ± 0.06, respectively. In the 3Exp group, these values were 0.08 ± 0.01, 0.16 ± 0.03, and 0.28 ± 0.04, at 1, 2, and 4 weeks after surgery, respectively, which were significantly reduced compared with those of the Con2w and Con4w groups ($P < 0.05$ and $P < 0.01$, respectively). Similar findings were observed in 4Exp1w, 4Exp2w, and 4Exp4w groups with ratios of 0.08 ± 0.02, 0.15 ± 0.02, and 0.28 ± 0.04, respectively ($P < 0.01$ compared with the Con2w and the Con4w groups). There was no significant difference between the 3Exp and the 4Exp groups at the corresponding time points [**Fig. 1(A,B)**].

### Apoptosis and proliferation of VSMCs and reendothelialization

The apoptosis of VSMCs was not observed in the normal group. AI values in the Con4w, 3Exp4w, and 4Exp4w groups were $3.22\% \pm 1.13\%$, $2.64\% \pm 1.14\%$, and $3.03\% \pm 1.57\%$, respectively. No significant difference was observed among these groups. PCNA-positive staining was not observed in the VSMCs in the normal group but could be observed in neointimal in the Con and the Exp groups [**Fig. 2(A)**]. PI values of the tunica intima [**Fig. 2(B)**] and the tunica media [**Fig. 2(C)**] in the Exp4w group were comparable with those of the Con4w group, whereas PI values in the Exp2w and the Exp1w groups were significantly less than the corresponding Con groups ($P < 0.01$). PI values between the 3Exp and the 4Exp groups were not significantly different at the corresponding time points. The injured intima showed blue, whereas the intima that was repaired by endothelia cells did not show blue after Evans blue staining. No significant differences of the reendothelialization areas in the neointimal were observed among the three groups on the same observation point [**Fig. 3(A,B)**].

Expression of type-I and type-III collagens

Type-I and type-III collagens were stained primarily in the tunica externa in the normal group (data not shown). The IOD of type-I collagen began to increase in the neointimal of the Con1w group. The highest IOD value was observed in the Con4w group (0.20 ± 0.02). In the 3Exp4w and the 4Exp4w groups, the IOD values were 0.08 ± 0.02 and 0.07 ± 0.01, respectively, which were significantly less than that of the Con4w group ($P < 0.01$) [**Fig. 4(A,B)**]. Staining of type-III collagen in the neointimal was too weak to be calculated using the immunohistochemical methods in all the groups.

### Expression of p27^{Kip1} and PTEN

Western blot analysis indicated that the expression of p27^{Kip1} protein in the Con groups was similar to that of the
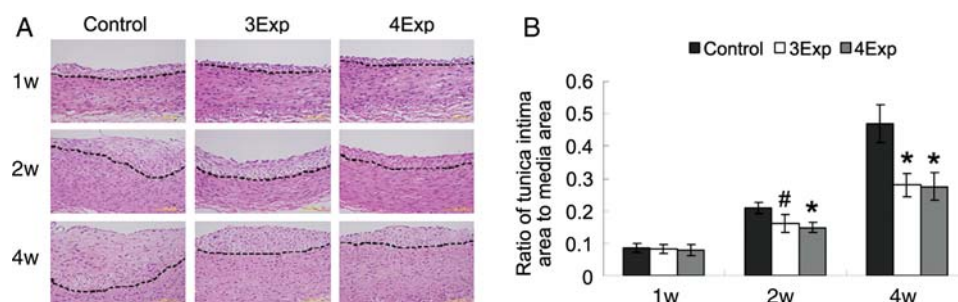
Figure 1 Neointimal formation of the abdominal aorta after balloon injury operation Neointimal formation with H&E staining (A, bars = 100 μm) and the ratio of the tunica intima area to media area of the neointimal (B). The dashed line in (A) shows the boundary of the neointimal. The thickest neointimal of the aorta was formed in the Con4w group, and it was significantly reduced after stimulation using EFs. $^{\#}P < 0.05$, $^{*}P < 0.01$ compared with the control group at identical time point.
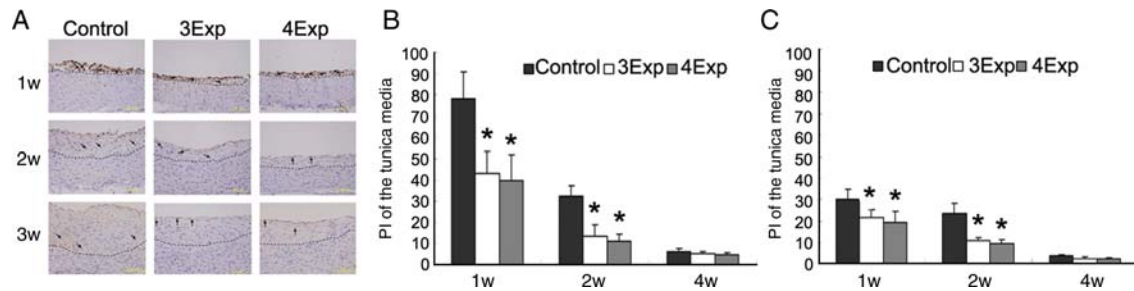
Figure 2 Immunohistochemical PCNA-positive staining The dashed line in the photomicrograph shows the boundary of the neointimal (A, bars = 100 μm). The black arrow examples show the PCNA staining positive cells in neointimal. The PI values of neointimal were calculated as the percentage of positive PCNA cell nuclei of VSMCs vs. the total amount of VSMCs in the neointimal. PI values of the tunica intima (B) and the tunica media (C) showed that the tunica intima and the tunica media in the Exp4w group were comparable with that of the Con4w group, whereas PI values in the Exp2w and Exp1w groups were significantly less than the corresponding control groups. *$P < 0.01$ compared with the control group at identical time point.
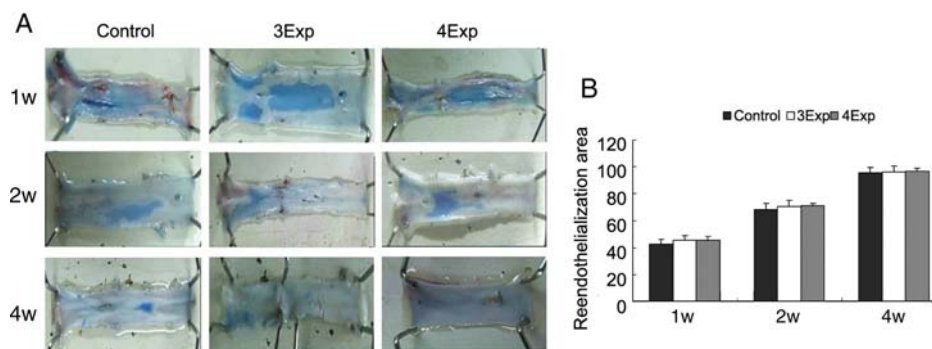

Figure 3 Reendothelialization in the neointimal (A, digital photograph) and IOD value (B) No significant differences of the reendothelialization areas in the neointimal were observed among the three groups on the same observation point.
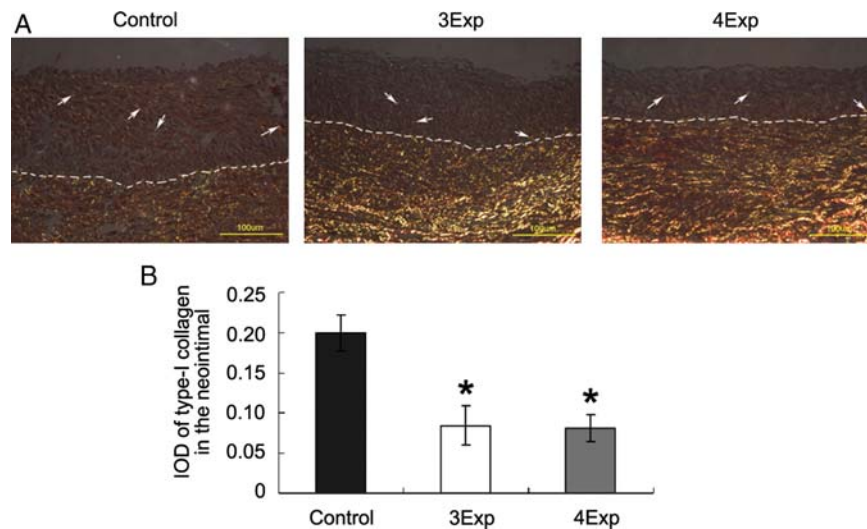
Figure 4 Expressions of type-I and type-III collagens with Sirius red dye staining Immunohistochemical staining of type-I and type-III collagens with Sirius red dye (A, bars = 100 μm) and the IOD value of type-I collagen (B) in the neointimal in different groups 4 weeks after surgery. The dashed line in the photomicrograph shows the boundary of the neointimal. The white arrow examples show the type-I collagens which were stained in red or orange in the neointimal, whereas type-III collagen was stained green. *$P < 0.01$ compared with the control group.

normal group [Fig. 5(A)]. It was significantly higher in all Exp groups compared with those of the corresponding Con groups, with the highest expression in Exp2w ($P < 0.01$).

Although the expression of p27^{Kip1} in the 3Exp1w and the 3Exp4w groups was comparable to the corresponding 4Exp groups, this level was much higher in the 3Exp2w
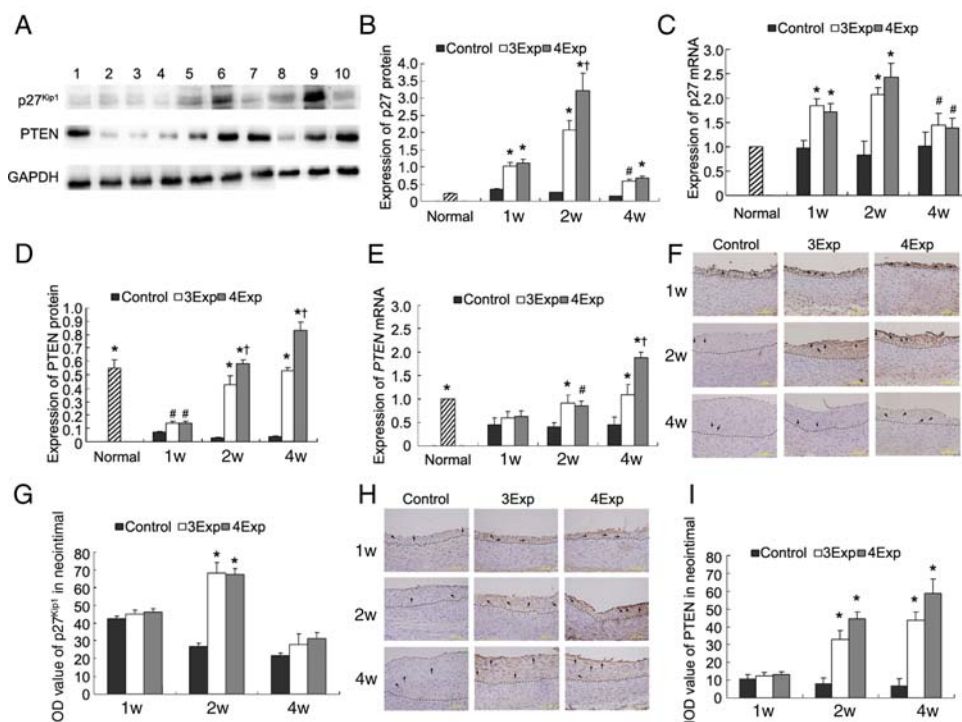
Figure 5 Expressions of p27^Kip1 and PTEN of the abdominal aorta after balloon injury operation Representative western blots of p27^{Kip1} (A and B) and PTEN (A and D) in protein samples isolated from rabbit abdominal aorta segments and the quantitative analysis of expressions of *p27^{Kip1}* mRNA (C) and *PTEN* mRNA (E). Lanes 1−10 show expressions of p27^{Kip1} and PTEN proteins in the normal, Con1w, Con2w, Con4w, 3Exp1w, 3Exp2w, 3Exp4w, 4Exp1w, 4Exp2w, and 4Exp4w groups, respectively. Expression of GAPDH was used as an internal control. The amount of protein used in each lane was 30 μg. $^{#}P < 0.05$, $*P < 0.01$ compared with the control group at the same time point; $^{†}P < 0.01$ compared with the 3Exp group at the same time point. Immunohistochemistry staining and the IOD values of p27^{Kip1} (F and G) and PTEN (H and I) showed that expressions of p27^{Kip1} and PTEN in the neointimal increased gradually after the EF application. The dashed line in the photomicrograph shows the boundary of the neointimal. The black arrow examples show the positive p27^{Kip1} or PTEN staining cells.

group [**Fig. 5(A,B)**]. qRT−PCR data showed that the pattern of expression of *p27^{Kip1}* mRNA was similar to that of western-blot analysis. The expression of *p27^{Kip1}* mRNA in the 3Exp and the 4Exp groups was significantly higher than that of the Con groups at the corresponding time points ($P < 0.01$ or $P < 0.05$) [**Fig. 5(C)**].

PTEN protein was more highly expressed in the normal group [**Fig. 5(A)**]. It began to decrease after the vessel was injured and reached the lowest level in the Con2w group. Although the expression of PTEN protein was significantly less in the Exp1w groups than that in the normal group ($P < 0.01$), this level increased and reached a normal level of expression in the Exp2w group and exceeded the normal level of expression in the 4Exp4w group ($P < 0.01$). At all time points, the expression of PTEN protein in the Exp groups was significantly higher than that of the Con groups, and the expression in the 4Exp2w and the 4Exp4w groups was higher than that of the corresponding 3Exp groups ($P < 0.01$) [**Fig. 5(A,D)**]. qRT−PCR analysis suggested that the expression of *PTEN* mRNA returned to its normal level in the 3Exp2w and the 4Exp2w groups. In the Con group, the expression of *PTEN* mRNA was significantly less than that of the normal group at corresponding

time points [**Fig. 5(E)**]. Immunohistochemistry results showed that the p27^{Kip1} and PTEN expressions in the neointimal were increased after the EF application compared with the control group at indicated time points [**Fig. 5(F−I)**].

Discussion

Studies have shown that the vessel injury can induce the proliferation and migration of VSMCs, and/or synthesis of the extracellular matrix (ECM) such as collagen in the tunica media and the tunica intima, which results in the formation of the neointimal [4]. In healthy organism, VSMCs in adult arteries are in the quiescent (G$_0$) phase of the cell cycle. After balloon injury, VSMCs most likely enter the growth cycle in 2 and 3 days, with most proliferation completed within 7 days [12]. In the present study, the neointimal was formed at 1 week and peaked at 4 weeks post-balloon injury and was accompanied by the proliferation of VSMCs and a high expression of type-I collagen. After the application of DC EFs, the neointimal thickness and the expression of type I-collagen were reduced significantly.

Some studies have suggested that the application of EFs can enhance neointimal hyperplasia, therefore resulting in stenosis in blood vessels [13]. The EFs used to produce such vessel injury were alternating-current (AC) EFs with electric current >1 mA. In addition, the stimulation electrodes in those studies were placed in direct contact with the tunica externa. We adopted low-voltage DC EFs with 100-μA stimulation current, and the stimulation electrodes were surgically implanted in the psoas major muscle instead of direct contact with vessels. The experimental application of EFs has been shown to exert a significant influence on cell migration and cell division. EFs can also affect cell growth by either decreasing cell proliferation [14,15] or increasing cell proliferation [16]. All these effects are depended on the field strengths (e.g. the precise stimulation parameters), the pattern of electric current (e.g. DC vs. AC), cell types and even the location where the stimulation electrodes were implanted. So we hypothesized that the EFs modifications applied in the present study may have contributed to the depression of cell proliferation and the reduction in neointimal formation.

Many cell types can be affected by EFs. Researchers have shown that EFs of 150–400 mV/mm can induce the direct migration, reorientation, and elongation of four types of vascular cells (including endothelial cell and VSMCs) [17], and also EFs can inhibit proliferation of cultured mammalian cells [14]. In our early experiments, we had observed that DC EFs could affect cell lamellipodia and cytoskeleton of VSMCs, meanwhile increase the expression of platelet-derived growth factor receptor in VSMCs and the polar asymmetrical distribution of VSMCs [18,19]. These results indicated that DC EFs could affect the migration and the growth of VSMCs. Nevertheless, recent studies have suggested that a reduction in apoptosis may be another important mechanism for restenosis formation [20]. And apoptosis is crucial for tunica intimal hyperplasia because VSMCs depletion is primarily due to apoptosis [21]. In the present study, apoptotic cells were not observed in the Con and the Exp groups throughout the post-operative period. Proliferative VSMCs were not observed in the normal vessel wall, but they were detected in the Con and the Exp groups after balloon injury in the abdominal aorta, and the levels were reduced by intervention using DC EFs. We also observed the reendothelialization of the injured intima by Evans blue staining in all groups. We found that there were no significant differences of reendothelialization areas in the neointimal among the three groups at the same observation points. So we hypothesized that the inhibition of neointimal hyperplasia under DC EFs in our present study may be resulted from the suppression of VSMC proliferation rather than the promotion of VSMC apoptosis or enhancement of endothelial cells proliferation.

Cell proliferation is regulated by various factors, including p27^{Kip1} and PTEN. p27^{Kip1} is a broad-spectrum, cyclin-dependent kinase inhibitor that has an important role in regulating the cell cycle [22]. It can inhibit cell proliferation by preventing cells entering the G_1 phase. The expression of p27^{Kip1} reflects cell proliferation [22]. Overexpression of p27^{Kip1} can reduce neointimal thickness in a balloon-injured artery model [23] and has a key role in vessel remodeling [24]. p27^{Kip1} can be regulated by the upstream gene PTEN via regulating the activity of the PI3K/Akt pathway [25]. PTEN can regulate the signals related to cell proliferation, therefore stopping cells crossing the G_1/S restriction point. As a potential endogenous inhibitor of VSMCs, overexpression of PTEN can indirectly inhibit the proliferation of VSMCs through upregulation of the expression of p27^{Kip1} [26] and suppression of neointimal formation [27]. The signal transduction via the PI3K/Akt pathway plays a pivotal role in regulating cell (including fibroblasts) proliferation, survival, migration, development, differentiation, apoptosis, etc. [25]. PTEN can negatively regulate the PI3K/Akt pathway and also is involved in the ECM remodeling and collagen expression. PTEN overexpression suppressed α-SMA expression, proliferation, and collagen production in myofibroblasts [28,29]. In our present study, we found collagen I in the neointimal was decreased accompanying with increased PTEN in neointimal. We supposed that there was a direct association between reduced collagen and increased PTEN/p27^{Kip1}.

Zhao *et al.* [30] suggested that PI3Kγ and PTEN mediated directional sensing of cell migration in response to electric signals and that they were crucial for the electrotaxis-regulated wound healing of whole tissue. In this study, p27^{Kip1} and PTEN were constitutively expressed in the normal group but decreased after the vessel was injured 1 week post-operation. After EF intervention, expressions of p27^{Kip1} and PTEN in the Exp groups were significantly increased compared with the Con group during the entire experimental period and were accompanied with the reduction in neointimal thickness. But the peak values of p27^{Kip1} expressions were in Exp2w instead of Exp4w whatever the experiment methods were used, which was contradictory with the changes of PTEN expressions. PTEN is one of the positive upstream regulators of p27^{Kip1}. But p27^{Kip1} is regulated by specific transcriptional induction via mitogenic and anti-mitogenic pathways and proteolysis by the ubiquitin-proteosome system [31]. It also may be regulated by renin angiotensin system and cyclin-dependent kinase 2 through many kinds of signal pathway. Otherwise, there are some other signaling mechanisms such as EGF receptors-ERK1/2 and integrins-Rac that mediate the electrotactic responds to the cells [32]. We hypothesized that the p27^{Kip1} degradation in our experiment exceeds the p27kip synthesis via some

special regulatory signal at 4w post-operation, thus resulting in that the p27^{Kip1} expression at Exp4w were less than that at Exp2w.

When a wound occurs, it disrupts the epithelial barrier, short-circuits the trans-epithelial potentials and then produces potential gradients. The potential gradients then form the laterally orientated wound EFs [32]. Endogenous EFs are regulated spatially and temporally. Endogenous EFs will rise to their peak immediately or shortly after wounding and decrease when the wound is healing [32]. In our present study, reendothelialization of the injured intima began to form obviously at 1w after the vessel injury. After 4 weeks following vessel injury, reendothelialization of the injured intima was almost completely formed, indicating the 'wound healing'. We speculated that this was the reason why PTEN was expressed in the normal group but decreased at 1 week after the vessel injury. But at this time, applied EFs could strengthen the electrotactic response to the cells through the PTEN/PI3K pathway

We therefore proposed that the elevation of p27^{Kip1} and PTEN, which might be induced by EF application, contributed to the inhibition of VSMCs proliferation and neointimal hyperplasia. But to our knowledge, the regulation mechanisms of PTEN under EFs remain unknown. EFs can alter cell membrane potential, cell membrane permeability, cell dielectrophoresis, and cell electrical coupling [33]. As a physical signal of transmembrane conduction, EFs influence the extracellular and intracellular signal such as Ca^{2+}, cyclic adenosine monophosphate, inositol triphosphate, etc. [34]. Molecules that couple the extracellular EFs to activation of those intracellular molecules are yet to be identified. Na^+/H^+ exchange 1 appears to be the good candidate molecules. Experiments have shown that directional Na^+/H^+ transport might relay the electric signal to PI3K/PTEN activation with subsequent directional migration [35].

DC EF which was used in our research is a resistance–capacitance electrical circuitry, which is constructed by the power source, electrodes, and the tissue between the electrodes [36]. Because of the complexity of the EF construction, AC EF has many differences with DC EF. It is very difficult to compare the EF interference parameters such as EF intensity and electric-current density. So we did not compare the effect of DC EFs with AC EFs on neointimal formation in our study.

In conclusion, the present study suggested that appropriate application of DC EFs can suppress the neointimal hyperplasia and the expression of ECM protein. This effect is probably induced by suppressing proliferation of VSMCs rather than promoting their apoptosis or enhancing endothelial cell proliferation. All these effects are probably achieved via upregulation of the p27^{Kip1} and PTEN expression in the balloon-injured vessel.

References

1 Bennett MR. In-stent stenosis: pathology and implications for the development of drug eluting stents. Heart 2003, 89: 218–224.

2 Bennett MR and O'Sullivan M. Mechanisms of angioplasty and stent restenosis: implications for design of rational therapy. Pharmacol Ther 2001, 91: 149–166.

3 Schwartz SM, Campbell GR and Campbell JH. Replication of smooth muscle cells in vascular disease. Circ Res 1986, 58: 427–444.

4 Liu MW, Roubin GS and King SB 3rd. Restenosis after coronary angioplasty. Potential biologic determinants and role of intimal hyperplasia. Circulation 1989, 79: 1374–1387.

5 Forrester JS, Fishbein M, Helfant R and Fagin J. A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies. J Am Coll Cardiol 1991, 17: 758–769.

6 Song B, Zhao M, Forrester J and McCaig C. Nerve regeneration and wound healing are stimulated and directed by an endogenous electrical field in vivo. J Cell Sci 2004, 117: 4681–4690.

7 Sawyer PN, Himmelfarb E, Lustrin I and Ziskind H. Measurement of streaming potentials of mammalian blood vessels, aorta and vena cava, in vivo. Biophys J 1966, 6: 641–651.

8 Ojingwa JC and Isseroff RR. Electrical stimulation of wound healing. J Invest Dermatol 2003, 121: 1–12.

9 Zhao M, Bai H, Wang E, Forrester JV and McCaig CD. Electrical stimulation directly induces pre-angiogenic responses in vascular endothelial cells by signaling through VEGF receptors. J Cell Sci 2004, 117: 397–405.

10 Kloth LC. Electrical stimulation for wound healing: a review of evidence from in vitro studies, animal experiments, and clinical trials. Int J Low Extrem Wounds 2005, 4: 23–44.

11 Chekanov VS. Low frequency electrical impulses reduce atherosclerosis in cholesterol fed rabbits. Med Sci Monit 2003, 9: BR302–BR309.

12 Clowes AW and Schwartz SM. Significance of quiescent smooth muscle migration in the injured rat carotid artery. Circ Res 1985, 56: 139–145.

13 Carmeliet P, Moons L, Stassen JM, De Mol M, Bouche A, van den Oord JJ and Kockx M, *et al.* Vascular wound healing and neointima formation induced by perivascular electric injury in mice. Am J Pathol 1997, 150: 761–776.

14 Wang ET, Reid B, Lois N, Forrester JV, McCaig CD and Zhao M. Electrical inhibition of lens epithelial cell proliferation: an additional factor in secondary cataract? FASEB J 2005, 19: 842–844.

15 Naegele RJ, Lipari J, Chakkalakal D, Strates B and McGuire M. Electric field stimulation of human osteosarcoma-derived cells: a dose-response study. Cancer Biochem Biophys 1991, 12: 95–101.

16 Binhi VN and Goldman RJ. Ion-protein dissociation predicts 'windows' in electric field-induced wound-cell proliferation. Biochim Biophys Acta 2000, 1474: 147–156.

17 Bai H, McCaig CD, Forrester JV and Zhao M. DC electric fields induce distinct preangiogenic responses in microvascular and macrovascular cells. Arterioscler Thromb Vasc Biol 2004, 24: 1234–1239.

18 Liu JP, He GX, Tang B and Jing T. Effect of electric field on the distribution of platelet derived growth factor receptor in vascular smooth muscle cells. Chin J Clin Rehabil 2006, 10: 81–85.

19 Tang B, He GX, Liu JP and Li D. Effects of electric field on cell lamellipodia and actin cytoskeleton organization of vascular smooth muscle cell. Chin J Clin Rehabil 2006, 10: 70–73.

20 Kearney M, Pieczek A, Haley L, Losordo DW, Andres V, Schainfeld R and Rosenfield K, et al. Histopathology of in-stent restenosis in patients with peripheral artery disease. Circulation 1997, 95: 1998–2002.

21 Bochaton-Piallat ML, Gabbiani F, Redard M, Desmouliere A and Gabbiani G. Apoptosis participates in cellularity regulation during rat aortic intimal thickening. Am J Pathol 1995, 146: 1059–1064.

22 Coats S, Flanagan WM, Nourse J and Roberts JM. Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. Science 1996, 272: 877–880.

23 Ueno H, Yamamoto H, Ito S, Li JJ and Takeshita A. Adenovirus-mediated transfer of a dominant-negative H-ras suppresses neointimal formation in balloon-injured arteries in vivo. Arterioscler Thromb Vasc Biol 1997, 17: 898–904.

24 Diez-Juan A, Castro C, Edo MD and Andres V. Role of the growth suppressor p27Kip1 during vascular remodeling. Curr Vasc Pharmacol 2003, 1: 99–106.

25 Sun H, Lesche R, Li DM, Liijental J, Zhang H, Gao J and Gavrilova N, et al. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3, 4, 5, - trisphosphate and Akt/ protein kinase B signaling pathway. Proc Natl Acad Sci USA 1999, 96: 6199–6204.

26 Garl PJ, Wenzlau JM, Walker HA, Whitelock JM, Costell M and Weiser-Evans MCM. Perlecan-induced suppression of smooth muscle cell proliferation is mediated through increased activity of the tumor suppressor PTEN. Circ Res 2004, 94: 175–183.

27 Huang JH, Niu XL, Pippen AM, Annex BH and Kontos CD. Adenovirus-mediated intraarterial delivery of PTEN inhibits neointimal hyperplasia. Arterioscler Thromb Vasc Biol 2005, 25: 354–358.

28 White ES, Atrasz RG, Hu B, Phan SH, Stambolic V, Mak TW and Hogaboam CM, et al. Negative regulation of myofibroblast differentiation by PTEN (phosphatase and tensin homolog deleted on chromosome 10). Am J Respir Crit Care Med 2006, 173: 112–121.

29 Teunissen BEJ, Smeets PJH, Willemsen PHM, De Windt LJ, Van der Vusse GJ and Van Bilsen M. Activation of PPARdelta inhibits cardiac fibroblast proliferation and the transdifferentiation into myofibroblasts. Cardiovasc Res 2007, 75: 519–529.

30 Zhao M, Song B, Pu J, Wada T, Reid B, Tai G and Wang F, et al. Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-gamma and PTEN. Nature 2006, 442: 457–460.

31 Quereda V and Malumbres M. Cell cycle control of pituitary development and disease. J Mol Endocrinol 2009, 42: 75–86.

32 Zhao M. Electrical fields in wound healing—an overriding signal that directs cell migration. Semin Cell Dev Biol 2009, 20: 674–682.

33 Chifflet S, Hernandez JA and Grasso S. A possible role for membrane depolarization in epithelial wound healing. Am J Physiol Cell Physiol 2005, 288: C1420–C1430.

34 McCaig CD, Rajnicek AM, Song B and Zhao M. Controlling cell behavior electrically: current views and future potential. Physiol Rev 2005, 85: 943–978.

35 Denker SP and Barber DL. Cell migration requires both ion translocation and cytoskeletal anchoring by the Na-H exchanger NHE1. J Cell Biol 2002, 159: 1087–1096.

36 Hu XM. Medicophysics, 6th edn. Beijing: People's Medical Publishing House Press, 2004, 170–180.