Bortezomib Reduces Neointimal Hyperplasia in a Rat Carotid Artery Injury Model

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Introduction

Restenosis after coronary angioplasty and graft failure after vascular reconstruction remain unresolved problems for cardiologists and vascular surgeons. One of the major causes of restenosis or graft failure is intimal hyperplasia. However, the precise cellular mechanism of intimal hyperplasia remains unclear.1 Vascular smooth muscle cell (VSMC) proliferation is the normal reactive response after vascular injury. In this process, various types of transcriptional factors are activated and VSMCs migrate from the media across the internal elastic lamina to form a new intimal layer, called the neointima.2 VSMC proliferation contributes to an increase in vessel wall thickness, while decreasing the vascular lumen area.3 The exact mechanism of neointima formation is not fully understood. Many factors may be involved, including growth factors, cytokines, and adhesion molecules, which are regulated by nuclear factor-kappa B (NF-κB). NF-κB is a major anti-apoptotic factor, and the aberrant activation of NF-κB is one of the primary causes of a wide range of human diseases, including inflammation, rheumatoid arthritis, asthma, and atherosclerosis.4,5,6

Background and Objectives: The ubiquitin-proteasome system is the major intracellular protein degradation pathway in the eukaryotic cells. Bortezomib inhibits 26S proteasome-induced I-κBα degradation and suppresses nuclear factor-kappa B (NF-κB) activation. We examined the effect of bortezomib on neointima formation after a rat carotid artery balloon injury.

Materials and Methods: After carotid artery balloon denudation, bortezomib was immediately administered by tail vein injection (systemic treatment) and by using an F-127 pluronic gel (perivascular treatment). Two weeks after the injury, we compared the degree of neointima formation in the carotid artery and the tissue expression patterns of NF-κB and I-κBα.

Results: The systemic treatment group exhibited a 29% reduction in neointima volume at two weeks after the balloon injury. On the western blot analysis, the bortezomib group exhibited an increased I-κBα expression, which suggested the inhibition of I-κBα degradation. On immunofluorescence analysis, the nuclear import of NF-κB was clearly decreased in the systemic bortezomib group. The perivascular bortezomib treatment group exhibited a significant reduction in the neointimal area (0.21±0.06 mm² vs. 0.06±0.01 mm², p<0.05), the neointima/media area ratio (1.43±0.72 vs. 0.47±0.16, p<0.05) and the % area stenosis (45.5±0.72% vs. 14.5±0.05%, p<0.05) compared with the control group. In situ vascular smooth muscle cell proliferation at 2 days after the injury was significantly inhibited (24.7±10.9% vs. 10.7±4.7%, p<0.05).

Conclusion: Bortezomib suppressed NF-κB activation through the inhibition of I-κBα degradation, and significantly reduced neointima formation in a rat carotid artery injury model. These data suggested that bortezomib represented a new potent therapeutic agent for the prevention of restenosis. (Korean Circ J 2013;43:592-599)

KEY WORDS: Coronary restenosis; Angioplasty; Proteasome; Nuclear factor kappa B.

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the proteasome system play a crucial role. The ubiquitin attaches to
the inactive NF-κB complex and I-κBα is degraded by the 26S pro-
teasome. After I-κBα degradation, the activated NF-κB is freed up
to translocate to the nucleus and once there it participates in the
transcriptional regulation of multiple genes. Consequently, the
proteasome plays a central role in regulating the NF-κB signaling
pathway by eliminating I-κBα via protein degradation. Therefore,
proteasome inhibitors can exert potent anti-inflammatory and an-
ti-growth promotion actions. Bortezomib is a novel modified dipep-
tidyl boronic acid that inhibits 26S proteasome-induced I-κBα de-
gradation and suppresses NF-κB activation (Fig. 1).

Materials and Methods

Chemical structure of bortezomib

Bortezomib is a modified dipeptidyl boronic acid. The product is
provided as a mannitol boronic ester that, in a reconstituted form,
consists of mannitol ester in equilibrium with its hydrolysis prod-
uct, the monomeric boronic acid. The drug substance exists in its cy-
clic anhydride form as a trimeric boroxine. The chemical name for
bortezomib is \([(1R)-3-methyl-1-\{2S\}-1-15o xo-3-phenyl-2-\{py-
razinylcarbonyl\}amino\propyl\}amino\}butyl\] boronic acid, with a
molecular weight of 384.24 and its molecular formula is C_{19}H_{25}B-
N_{4}O_{4}. The solubility of bortezomib, as the monomeric boronic acid, in
water is 3.3 to 3.8 mg/mL in the pH range of 2 to 6.5 (Fig. 1).

Animals

Twelve- to fourteen-week-old male Sprague-Dawley rats (Charles
River Laboratory, Yokohama, Japan), weighing 300 g each, were fed
a normal chow diet and given water ad libitum. All the study proto-
cols were approved by the Chungbuk National University Animal
Care and Use Committee.

Balloon angioplasty

The animals were anesthetized with an intraperitoneal injection of
ketamine (50 mg/kg) and xylazine (6.7 mg/kg), and the right ca-
rotid artery was surgically exposed. The right carotid artery was de-
endothelialized as described previously. Briefly, a Fogarty 2 Fr bal-
loon catheter (Baxter Healthcare Corp, Round Lake, IL, USA) was in-
troduced through the right external carotid artery and advanced
into the common carotid artery. The balloon was then inflated with
0.15 mL saline and then withdrawn to the entry point. The entire pro-
cedure was repeated three times.

Systemic delivery of bortezomib

In order to evaluate the effect of bortezomib, 0.2 mg/kg of lyophi-
lized bortezomib diluted in sterile 0.9% saline was prepared, and a
total volume of 1 mL was administered as a bolus injection via a tail
vein immediately after balloon injury (n=10). An identical amount
of sterile 0.9% saline was used in the control (balloon injury alone)
group (n=10). No additional injection of bortezomib or saline was
administered, thereafter.

Perivascular delivery of bortezomib

Immediately after the balloon injury, a local perivascular polymer-
based delivery system was used to administer bortezomib to the
injured vessel wall in one group of animals (n=4). A separate cohort
of animals received an empty gel (n=4). Previous studies from our
laboratory and other studies have confirmed that an empty gel has
no effect on neointima formation. The delivery system consisted
of a 40% copolymer gel (F-127; Sigma Chemical Company, Munich,
Germany) that contained bortezomib at a concentration level of 4
μg/mL. This was topically applied in a circumferential manner to
the exposed adventitia of the carotid artery. F-127 gels exhibit re-
verse thermal behavior, that is, they remain liquid at refrigerator
temperature but become soft gels at body temperature.

Western blot analysis

The levels of NF-κB and I-κBα were quantified in the total extr-
acts of whole carotid arteries through western blot analysis, as pre-
viously described. Briefly, equivalent amounts of protein (20 μg)
from each sample were mixed with a gel loading buffer (50 mM
Tris, 10% sodium dodecyl sulfate, 10% glycerol, 10% 2-mercapto-
ethanol, and 2 mg bromophenol blue per mL) at a ratio of 1 : 1. This
mixture was boiled for 3 minutes, centrifuged at 10000 rpm for 10
minutes, and then electrophoresed on an 8% discontinuous poly-
acylamide minigel. The proteins were transferred onto nitrocellulose
membranes, according to the manufacturer’s instructions. The mem-
branes were saturated through incubation at 4°C overnight with
10% non-fat dry milk in phosphate-buffer saline (PBS), and were
then incubated with an anti-NF-κB monoclonal antibody or anti-I-
kBα (1 : 1000) monoclonal antibody for 2 hours at room tempera-
ture. The membranes were washed three times with 1% Triton X-100
in PBS, and then incubated with anti-mouse immunoglobulins
coupled to peroxidase (1 : 2000). The immune complexes were visu-
alized using the enhanced chemiluminescence method. Subsequent-
ly, the relative expression of the proteins was quantified by the den-
sitometric scanning of the X-ray films, with the scanning, analysis,
and calculation performed using the Scion Image System (version
1.01; Scion Corporation, Frederick, MD, USA).

Measurement of in situ vascular smooth muscle cell prolifera-
tion
The effect of the perivascular bortezomib treatment (n=3) versus
the control (empty gel alone) group (n=3) on in situ VSMC prolifera-
tion was measured by bromodeoxyuridine (BrdU) incorporation on
day 2 after the injury. Briefly, the perivascular bortezomib-treated
rats and control rats were injected subcutaneously with BrdU (30
mg/kg) at 30, 38, and 46 hours after the injury. The carotid artery sec-
tions were harvested at 48 hours after the injury and the histologi-
cal sections were incubated with mouse anti-BrdU monoclonal anti-
bodies (VECTOR, Burlingham, CA, USA). The fraction of BrdU-positive
medial VSMC nuclei per cross section was compared between the
perivascular bortezomib-treated group and the control (empty gel
alone) group.

Histomorphometric analysis
The carotid arteries were perfusion-fixed with 10% buffered for-
malin. Carotid artery sections (5 μm) were stained with hematoxy-
lin-eosin, and morphometric analysis was performed using 3 indi-
vidual sections from the middle of each injured arterial segment,
by an investigator who was kept blind to the experimental proce-
dure being undertaken. Cross-sectional areas (Aintima and Amedia),
the area ratios (Aintima/Amedia), and the percentage area stenosis
(%) were analyzed and calculated using the Scion Image System (version
1.01; Scion Corporation, Frederick, MD, USA).

Immunofluorescence analysis
Immediately following the balloon injury, the carotid arteries were
perfusion-fixed with 10% buffered formalin. Carotid artery sec-
tions (5 μm) were stained with 4′,6-diamidino-2-phenylindole. For
immunostaining, the tissue sections were incubated in anti-NF-
κB-p65 antibody for 24 hours at 4°C, washed three times in block-
ing buffer, incubated in an Alexa Fluor 568 anti-rabbit IgG anti-
body (Molecular Probes, Eugene, USA) for 1 hour, and then analyzed
using confocal fluorescence microscopy.

TdT-mediated dUTP nick-end labeling staining
TdT-mediated dUTP nick-end labeling (TUNEL) staining (In Situ
Apoptosis Detection Kit; Invitrogen, Carlsbad, CA, USA) was employed
for the detection of deoxyribonucleic acid fragmentation and apop-
totic bodies in rat carotid arteries. Briefly, after deparaffinizing the
carotid artery sections (5 μm), digesting protein using proteinase K,
and quenching endogenous peroxidase activity with 3% H2O2 in
PBS, slides were placed in an equilibration buffer, and then, in a work-
ing TdT enzyme, followed by a stop/wash buffer. Samples were incu-
bated with TdT in the presence of 11-digoxigenin-dUTP at 37°C for
60 minutes. Samples were then blocked with 2% bovine serum albu-
min and incubated with antidigoxigenin-peroxidase for 30 minutes
at room temperature. After the reaction with diaminobenzidine (Per-
oxidase Substrate Kit; VECTOR, Burlingham, CA, USA) for 4 minutes,
samples were visualized and counterstained with hematoxylin at
room temperature for 10 minutes. Samples were evaluated using
light microscopy, with apoptotic cells being labeled brown.

Statistics
Results are expressed as the mean±SD. Statistical analysis was
performed using the Student’s 2-tailed t-test. The level of statisti-
cal significance was defined as p<0.05.

Results
Histomorphometric analysis in systemic bortezomib treatment
We applied in vivo testing in a rat balloon injury model in order
to determine the capability of proteasome inhibition to prevent re-
stenosis. Systemic administration of bortezomib (0.2 mg/kg) resulted
in a 29% decrease (0.14±0.03 mm² vs. 0.10±0.03 mm², p<0.05) in
the neointima area and a 38% decrease (1.68±0.29 vs. 0.95±0.28,
p<0.05) in the neointima/media ratio (Fig. 2, Table 1); the dosage
administered did not cause mortality.

Histomorphometric analysis in perivascular bortezomib
treatment
The neointimal area of the bortezomib group was significantly
smaller than that of the control group (0.21±0.06 mm² vs. 0.35±
0.01 mm² p<0.05). The neointima/media area ratio (1.43±0.72 vs.
0.47±0.16, p<0.05) and percentage stenosis (45.5±22.8% vs. 14.5±
0.05%, p<0.05) were significantly lower in the bortezomib group
(Fig. 3, Table 2). However, the media and vessel wall areas did not dif-

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Measurement of in situ vascular smooth muscle cell proliferation

In vivo medial VSMC proliferation (as assessed by the BrdU proliferation assay) was significantly inhibited in the perivascular bortezomib-treated group at 2 days after the balloon injury (control vs. bortezomib: 24.7±10.9% vs. 10.7±4.7%, p=0.02) (Fig. 4).

Western blot analysis

The expression levels of NF-κB were higher in the control and bortezomib groups compared with the normal carotid artery group. Similar patterns of I-κBα expression were observed in the normal carotid artery and bortezomib groups. A low level of I-κBα expression was detected in the control group at 4 and 12 hours following the balloon injury. Conversely, the NF-κB expression did not differ in the control and bortezomib groups (Supplementary Fig. 1, Online). The bortezomib group exhibited an increased I-κBα expression compared with the control group, suggesting that the inhibition of I-κBα degradation took place in the bortezomib group.

Immunofluorescence analysis

The nuclear import of NF-κB-p65 clearly increased in the control group (n=3) at 1 hour and 4 hours after the balloon injury (Fig. 5, Supplementary Fig. 2, Online). However, the systemic bortezomib treatment group exhibited a clearly decreased nuclear import of NF-κB-p65 at 1 hour and 4 hours after the balloon injury. Similar expression patterns of NF-κB-p65 were observed in the normal carotid artery group (Fig. 5, Supplementary Fig. 2, Online). These results suggest that bortezomib suppressed the nuclear import and activation of NF-κB.

TdT-mediated dUTP nick-end labeling staining analysis

After the balloon injury, the process of cell death was detected at early time points within the innermost area of the media in the control group (n=3) and appeared to radiate in a wave of cell deaths in a time-dependent manner. At 4 hours after the balloon injury, a mass of TUNEL-positive cells (brown in color) was detected in the entire media area. In the bortezomib-treated group (n=3), a few TUNEL-positive cells were detected in the media at early stages and did not change until 4 hours after injury (Supplementary Fig. 3, Online). These findings suggest that bortezomib attenuated massive apoptosis early after vascular injury.

Discussion

Nuclear factor-kappa B is activated by numerous physiological sti-
muli, and it is commonly associated with immunological and inflammatory processes. In addition, it is also associated with the pathogenesis of proliferative disorders of the vasculature, including atherosclerosis and restenosis. After a vascular injury, vascular repair involves four fundamental cellular processes: programmed cell death (apoptosis), cell growth, migration, and matrix modification. NF-κB is one of the more important transcription factors that initiates the early vascular repair process, contributing to the development of restenosis. NF-κB is an inducible transcription factor complex that is composed of a heterodimeric complex, comprising p50 and p65 subunits. In unstimulated cells, NF-κB is held in an inactive form via sequestration in the cytoplasm by the I-κBα family of inhibitor proteins. Phosphorylation of I-κBα typically results in the ubiquitination of the protein and subsequent targeting for proteasomal degradation. As I-κBα is degraded, NF-κB is translocated to the nucleus, where it participates in the transcriptional regulation of multiple genes that are involved in the inflammatory process in multiple disease states. By blocking proteasomal activity, I-κBα cannot be degraded and NF-κB remains in the cytosol. In our results, western blot analysis showed that the bortezomib group exhibited increased levels of I-κBα expression at all times compared with the control.

Fig. 3. Representative cross sections of rat carotid arteries taken at 14 days after the balloon injury in the perivascular treatment group. The bortezomib group exhibited significant reductions in the neointimal area (71% decreases) and % area stenosis (68% decrease) at 14 days after the balloon injury. A: control (empty gel alone). B: bortezomib (perivascular treated, 4 μg/mL); original magnification ×100 (A and B); original magnification ×200 (C and D); bar represents 300 μm.

Table 2. Effects of perivascular bortezomib on the histomorphometric parameters at 2 weeks after treatment

<table>
<thead>
<tr>
<th></th>
<th>Control (n=4)</th>
<th>Bortezomib (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neointimal area (mm²)</td>
<td>0.21±0.06</td>
<td>0.06±0.01*</td>
</tr>
<tr>
<td>Medial area (mm²)</td>
<td>0.14±0.02</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>Lumen area (mm²)</td>
<td>0.25±0.09</td>
<td>0.36±0.05*</td>
</tr>
<tr>
<td>Vessel area (mm²)</td>
<td>0.61±0.06</td>
<td>0.56±0.05</td>
</tr>
<tr>
<td>Neointimal/media area ratio</td>
<td>1.43±0.72</td>
<td>0.47±0.16*</td>
</tr>
<tr>
<td>Area stenosis (%)</td>
<td>45.5±22.8</td>
<td>14.5±0.05†</td>
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Results are presented as mean±SD. *p<0.05, †p<0.01
group. These findings suggest that bortezomib effectively inhibited the proteasomal degradation of I-κBκ. In addition, upon immunofluorescence analysis, the systemic bortezomib treatment group exhibited a clearly decreased nuclear import of NF-κB-p65 compared with the control group at 1 hour and 4 hours after the balloon injury. The systemic bortezomib treatment group exhibited patterns of NF-κB-p65 expression similar to those of the group with normal carotid arteries. Consequently, NF-κB is to be considered an attrac-

![Graph showing in situ VSMC proliferation](image-url)

**Fig. 4.** Measurement of *in situ* VSMC proliferation as assessed by *in situ* BrdU labeling. A: *in situ* VSMC proliferation assay of the control group (empty gel alone, n=3) and the perivascular bortezomib-treated (n=3) group. VSMC proliferation was significantly inhibited in the bortezomib group (*p=0.02 vs. control). B: photomicrographs illustrating the BrdU assay at 2 days after injury. BrdU-positive VSMCs were significantly decreased in the bortezomib-treated group. BrdU: bromodeoxyuridine, VSMC: vascular smooth muscle cell; original magnification ×400; bar represents 100 μm.

![Immunofluorescence analysis](image-url)

**Fig. 5.** Immunofluorescence analysis of the nuclear import of NF-κB-p65 at 1 hour after the carotid artery balloon injury. The nuclear expression of NF-κB-p65 was clearly decreased in the bortezomib group, suggesting that bortezomib inhibited the nuclear translocation of NF-κB-p65. In contrast, the control group (balloon injury alone) exhibited a markedly increased nuclear expression of NF-κB-p65. These findings suggest that bortezomib effectively inhibited the nuclear translocation and activation of NF-κB. DAPI: 4',6-diamidino-2-phenylindole, NF-κB: nuclear factor-kappa B (original magnification ×6300; bar represents 4.0 μm).
Bortezomib is a cell-permeable dipeptide boronic acid that can reversibly inhibit 26S proteasome, inhibit I-κBα degradation, and suppress NF-κB activation. The reduction of NF-κB activity by bortezomib has been demonstrated as being correlated with antitumor activity in human solid tumors and hematologic malignancies, such as multiple myeloma, lung cancer, breast cancer, and lymphoma. Bortezomib is a clinically approved drug and its mechanisms of action, adverse effects, and clinically tolerable dosages are well reported. After parenteral injection, bortezomib is both rapidly removed from the vascular compartment and taken up by cells. More than 80% of the injected dosage cannot be detected in plasma after 10 minutes. The pharmacodynamic assay reported here indicated that proteasome inhibition occurs in a dose-dependent manner. This inhibition is reversible, and the rate of the return to the baseline (i.e., normal) activity is dose dependent. Bortezomib at a dose of 1.3 mg/m² was administered by intravenous bolus twice weekly in patients with relapsed multiple myeloma. The clinical dosage in humans (1.3 mg/m²) is equivalent to 0.15 mg/kg or 1.0 mg/m² in rats, based on body surface area. In rats, 0.2 mg/kg of bortezomib-induced blood proteasome inhibition was equivalent to that in human clinical trials for cancer treatment.

It is recognized, nevertheless, that proteasome is an important regulator for normal cellular functioning and cell cycling. Thus, the systemic use of proteasome inhibitors would surely be harmful in use in anti-inflammatory therapy. Furthermore, various cytotoxic side effects, such as gastrointestinal toxicity and hematologic toxicity, as well as cardiovascular side effects, may occur. In rats, the safe dose of bortezomib, which does not induce systemic toxicity, is 0.5 to 1.0 mg/kg. However, there is no information on how much bortezomib may be required to prevent restenosis.

In our experiment, we used systemic and perivascular bortezomib treatment models in order to evaluate the capacity of proteasome inhibition to prevent restenosis. The rats were treated with 0.2 mg/kg of bortezomib via tail vein injection immediately following carotid balloon injury. This dose produced a 29% decrease in neointima volume at 14 days after the balloon injury. However, although the dose did not cause mortality, hepatosplenomegaly was observed during autopsy procedures. Consequently, in order to reduce neointima formation and to avoid systemic toxicity, we used a lower dose (4 μg/mL) with the perivascular delivery of bortezomib. The dose of 4 μg/mL is approximately 1/15 of that used in systemic cancer treatment. For the perivascular administration of bortezomib, a 40% F–127 solution containing bortezomib (4 μg/mL) was applied immediately following the balloon injury.

In our study, we showed that systemic and perivascular bortezomib treatment groups significantly decreased the neointimal area, neointima/media area ratio, and % area stenosis compared with the control group. However, the systemic treatment group used an anticancer dosage of bortezomib. To minimize the potential systemic side effects, we used prevascular treatment. On BrdU assay performed 2 days after treatment, BrdU-positive cells were significantly decreased in the treatment group. In TUNEL staining analysis, massive apoptosis was detected in the entire media area in the control group. In the bortezomib-treated group, only a low frequency of apoptosis was detected in the media at an early stage. These findings suggest that bortezomib attenuates massive apoptosis in the early stages following a vascular injury. Taken together, it appears that bortezomib inhibited the proteasomal degradation of I-κBα and suppressed the nuclear import of NF-κB. Furthermore, bortezomib reduced early apoptosis, VSMC proliferation, neointimal hyperplasia, and restenosis. These findings indicate that bortezomib has the therapeutic effect of reducing neointimal hyperplasia and inhibiting restenosis after a balloon injury. Furthermore, bortezomib may be a possible candidate drug for the development of a new drug-eluting stent or drug-eluting balloon.

Limitations

The use of proteasome inhibitors has recently been widely investigated in experimental studies and during the treatment of clinical diseases. However, the safe and effective anti-inflammatory dose of bortezomib remains undetermined. Despite the fact that our study revealed, for the first time, that bortezomib inhibited neointimal formation in a rat carotid artery injury model, further studies are warranted for the following reasons. First, the effective, potent dose of bortezomib was not determined in this study. Second, we did not evaluate any adhesion molecules (i.e., ICAM-1 and VCAM-1), mediators of inflammation (i.e., TNF-a, COX-2, and iNOS), or cytokines (i.e., IL-1, IL-2, and IL-6) that are produced by NF-κB activation. Third, the level of NF-κB needs to be measured by electrophoretic mobility shift assay (EMSA) after nuclear extraction.

Conclusion

Our results demonstrated that the perivascular administration of bortezomib effectively reduced neointima formation after a carotid artery balloon injury in a rat model. Bortezomib inhibited the proteasomal degradation of I-κBα and it suppressed NF-κB activation. Thus, bortezomib represents an attractive drug for attenuating the vascular response after a balloon injury, and it may be useful for the development of a new drug-eluting stent, drug-eluting balloon, and in vascular surgery.
Acknowledgments

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Supplementary Materials

Accompanying Figs. 1, 2 and 3 can be found on the website [http://www.e-kcj.org].

References

Supplementary Fig. 1. Western blot analysis of NF-κB and I-κBα after rat carotid artery balloon injury. A low level of I-κBα expression was detected in the control group at 4 and 12 hours after balloon injury. Whereas, I-κBα expressions in the bortezomib group was changed at all times. These findings suggest that bortezomib effectively inhibited the proteasomal degradation of I-κBα. NF-κB: nuclear factor-kappa B.
Supplementary Fig. 2. Immunofluorescence analysis of the nuclear import of NF-κB-p65 at 4 hours after carotid artery balloon injury. The nuclear expression of NF-κB-p65 was clearly decreased in the bortezomib group, which suggests that bortezomib inhibited the nuclear translocation of NF-κB-p65. In contrast, the control group (balloon injury alone) exhibited a markedly increased nuclear expression of NF-κB-p65. These findings suggest that bortezomib effectively inhibited the nuclear translocation and activation of NF-κB. DAPI, 4',6-diamidino-2-phenylindole; original magnification ×6300; bar represents 4.0 μm. NF-κB: nuclear factor-kappa B.
Supplementary Fig. 3. Balloon injury induces acute VSMC apoptosis of carotid arteries at different time points after balloon injury. In the control group, a massive number of TUNEL-positive cells (brown color) were detected in the entire media area (A, C and E). In the bortezomib treated group, a few TUNEL-positive cells were detected in the media at early time points (B, D and F). These findings suggest that bortezomib attenuated massive apoptosis at an early stage after vascular injury. Original magnification ×400; bar represents 100 μm. TUNEL: TdT-mediated dUTP nick-end labeling, VSMC: vascular smooth muscle cell.