

Epigallocatechin-3-gallate attenuates neointimal hyperplasia in a rat model of carotid artery injury by inhibition of high mobility group box 1 expression

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Abstract. Epigallocatechin-3-gallate (EGCG) is a kind of polyphenol compound, called catechin, and is extracted from green tea. EGCG has a wide range of biological activities. The present study aimed to evaluate the effect of EGCG on neointimal hyperplasia in a rat model of carotid artery balloon injury and to explore the molecular mechanisms involved. Various experiments were performed to assess the effects of EGCG on thickening of neointima, expression levels of high mobility group box 1 protein (HMGB1) and receptor of advanced glycation end products (RAGE), the inflammatory response, oxidative stress and activation of nuclear factor (NF)- κ B. Results demonstrated that EGCG decreased the intimal area and the ratio of intimal area/medial area compared with the balloon injury group. The expression levels of HMGB1 and RAGE induced by balloon injury were markedly inhibited by EGCG treatment. Furthermore, the inflammatory response and oxidative stress damage, which have close correlations with HMGB1, were restrained by EGCG. Finally, EGCG treatment markedly inhibited NF- κ B activation. The present data provided evidence that EGCG attenuates neointimal hyperplasia in a model of carotid artery balloon injury, which indicated that EGCG may serve as a potential drug for restenosis in clinics.

Introduction

Percutaneous coronary intervention (PCI) is an effective management for coronary heart disease (CHD). Restenosis, however, becomes the primary bottleneck for application of PCI (1). Increasing evidence has confirmed that neointimal proliferation following balloon dilatation and stent implantation-induced vascular injury is the primary mechanism of restenosis (2,3). Thus, inhibiting vascular smooth muscle abnormal hyperplasia to prevent restenosis is a strategy for therapy of CHD. For this reason, identification of an effective clinical treatment to restrain neointimal formation is the focus of research.

High mobility group box 1 (HMGB1) belongs to the high mobility group protein family, which was identified by Goodwin and Johns (4) in 1973 and named for its high electrophoretic mobility. It has been demonstrated that HMGB1 is an important inflammatory mediator and the molecule at the heart of initiating and maintaining inflammation responses (5). The expression of HMGB1 is closely associated with the pathogenesis of sepsis (6), arthritis (7), acute pneumonia (8), hepatitis (9) and arteriosclerosis (10). Receptor of advanced glycation end products (RAGE) mediates the effects of HMGB1 (11). Furthermore, HMGB1 is reported to be expressed in intimal smooth muscle cells and injured carotid arteries tissue (12). Inhibiting HMGB1 expression may alleviate neointimal hyperplasia (13,14).

Epigallocatechin-3-gallate (EGCG) is the most effective component of tea polyphenols and accounts for 45-65% of the catechinic acid content of tea (15). The metabolism products of EGCG have demonstrated strong biological activities, such as anti-inflammatory (16), antibacterial (17), anti-oxidative (18) and anti-tumor (19). Previous studies have also reported that EGCG inhibited the lipopolysaccharide (LPS)-induced inflammatory reaction by suppressing HMGB1 expression (20,21). Thus, the present study speculated that EGCG may alleviate balloon-injured carotid arteries via inhibiting the overexpression of HMGB1. Based on the above evidence, the present study aimed to evaluate the inhibitory effect of

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EGCG on carotid artery balloon injury in rats and explore the related molecular mechanisms.

Materials and methods

Drug. EGCG was purchased from Meilun Biotech Co., Ltd., (Dalian, China) with purity over 95% and dissolved in normal saline prior to use. The chemical structural of EGCG was demonstrated in Fig. 1.

Establishment of a rat model of carotid artery balloon injury. Male Sprague Dawley rats (weight, 250-300 g; age, 8 weeks) were purchased from Beijing Vital River Laboratory Animal Co., Ltd., (Beijing, China). The rats of clean grade were reared at 23°C and 45-55% humidity, under a 12-h light/dark cycle with access to food and water *ad libitum*. A total of 30 rats were randomly assigned to five groups (n=6 per group). The groups were as follows: Sham operation group; carotid artery balloon injury group; injury plus EGCG (1 mg/kg); injury plus EGCG (2 mg/kg); and injury plus EGCG (4 mg/kg). The procedure for rat carotid artery balloon injury model establishment has been described previously (22). Briefly, rats were anesthetized with intraperitoneal injection of chloral hydrate (350 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequently, the skin was incised along the anterior median raphe of neck and the left carotid artery was isolated. A balloon angioplasty catheter (Edwards Lifesciences Corp, Irvine, CA, USA; diameter, 1.5 mm; length, 20 mm) was inserted through the external carotid incision to the aortic arch. The balloon was then inflated to restrict blood flow for 30 sec, and gradually withdrawn three times. Following removal of the catheter, the external carotid branch was ligated. The rats in the sham operation group underwent the same procedures without balloon catheter insertion. Penicillin (2×10^5 U, Taizhou Pengchange Animal Pharmaceutical Co., Ltd, Xinghua, China) was intramuscularly injected twice a day for three days to prevent infection post-operation. The rats in the EGCG treatment groups were intraperitoneally injected with 1, 2 or 4 mg/kg EGCG once, 1 h prior to surgery and every day after surgery, respectively. Rats in the sham operation group and carotid artery balloon injury group were injected with an equal volume of normal saline at the same time points. A total of 14 days after surgery, rats were euthanized and carotid artery tissues and blood specimens were collected. All of the animal protocols were approved by and complied strictly with guidelines of the Institutional Animal Care and Use Committee of Shandong University (Jinan, China).

Histomorphometric analysis. The carotid artery tissues were fixed with 4% paraformaldehyde at 4°C overnight, embedded in paraffin and sectioned into 4- μ m thick slices. The thickening of neointima was observed by routine hematoxylin and eosin (H&E) staining under a light microscope (Olympus Corp, Tokyo, Japan; DP73) at a magnification of x400. The intimal, medial and luminal cross-sectional areas were measured. The neointima/media area (I/M) ratios were calculated.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from carotid tissues using a commercial RNA extraction kit (BioTeke Biotechnology, Co.,

Ltd., Beijing, China; catalogue no. RP1201) containing DNase to remove genomic DNA. Subsequently, RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (BioTeke Biotechnology, Co., Ltd.; catalogue no. PR6502) according to the manufacturer's protocol. qPCR was performed using 2x Power Taq PCR MasterMix (BioTeke Biotechnology, Co., Ltd.; catalogue no. PR6502), according to the manufacturer's instructions. PCR amplifications were performed using the Exicycler™ 96 (Bioneer Corp., Daejeon, Korea) as follows: 95°C for 10 min followed by 40 cycles at 95°C for 10 sec, 60°C for 20 sec, 72°C for 30 sec, and finally 4°C for 2 min. Relative expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (23) normalized to GAPDH expression. Specific primers used in the present study were as follows: HMGB1, forward 5'-AGG CTGACAAGGCTCGTTATG-3' and reverse 5'-TGTCAT CCGCAGCAGTGT TG-3'; RAGE, forward 5'-ACCGAGTCC GAGTCTACCA-3' and reverse 5'-GCTCTGACCGAAGCG TGA-3'; and β -actin, forward 5'-GGAGATTACTGCCCT G GCTCCTAGC-3' and reverse 5'-GGCCGGACTCATCGT ACTCCTGCTT-3'.

Western blot analysis. Western blot analysis was performed to assess the protein expression levels in carotid artery tissues. Tissue samples were lysed in lysis buffer (Wanleibio Co., Ltd., Shenyang, China) and then denatured. Equal amounts (40 μ g) of protein were separated by 11% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Subsequent to blocking with 5% skimmed milk for 1 h at room temperature, the membranes were incubated with primary antibodies against HMGB1 (Boster Biological Technology, Ltd., Wuhan, China; catalogue no. BA4277; 1:400), RAGE (Boster Biological Technology, Ltd.; catalogue no. PB0530; 1:400), nuclear factor (NF)- κ B (Boster Biological Technology, Ltd.; catalogue no. BA0610; 1:400) and β -actin (Santa Cruz Biotechnology, Dallas, USA; catalogue no. sc-47778; 1:400), respectively at 4°C overnight. After washing with 0.1% Tris buffered saline with Tween-20 three times for 5 min, the blots were incubated with Goat Anti-Rabbit IgG secondary antibody (Wanleibio Co., Ltd.; catalogue no. WLA023; 1:5,000) for 45 min at 37°C. Immunodetection was performed with an enhanced chemiluminescence detection system (Wanleibio Co., Ltd.). The optical densities of the bands relative to β -actin were quantified with a Gel Doc 2000 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

ELISA assay. Serum inflammatory cytokine levels were determined using commercial ELISA kits: Tumor necrosis factor (TNF)- α (catalogue no. EK0526), interleukin (IL)-1 β (catalogue no. EK0393), IL-6 (catalogue no. EK0412), intercellular adhesion molecule (ICAM)-1 (catalogue no. EK0372; all, BOSTER Biological Technology, Wuhan, China) and vascular cell adhesion molecule (VCAM)-1 (catalogue no. SEA547Ra; USCN Life Science Co., Ltd., Wuhan, China). The results were obtained at 450 nm using a microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA). The concentrations were calculated by creating a standard curve.

Malondialdehyde (MDA) determinations. MDA levels in carotid artery tissues were measured using a commercial Malondialdehyde assay kit (Nanjing Jiancheng Bioengineering

Institute, A003-1), according to the manufacturer's instructions. Briefly, homogenized carotid artery tissues were incubated at 95°C for 40 min with thiobarbituric acid to form a new complex. The results were measured on a microplate reader (Omega Bio-Tek, Inc.) at 532 nm and expressed as nM MDA/mg protein.

Measurement of reactive oxygen species (ROS) production. The tissue ROS levels were detected using a commercial Reactive oxygen species Assay Kit (Nanjing Jiancheng Bioengineering Institute, E004), according to the manufacturer's instructions. Briefly, the tissue homogenate was incubated with 1 mM oxidation-sensitive fluorescent probe, DCFH-DA, for 30 min at 37°C in the dark. The samples were washed twice for 5 min in phosphate-buffered saline and measured on a microplate reader.

Electrophoretic mobility shift assay (EMSA). Nuclear protein was prepared from carotid artery tissues using a nuclear and cytoplasmic kit (Wanleibio Co., Ltd., Shenyang, China), according to the manufacturer's instructions. Subsequently, the protein concentration was detected using a bicinchoninic acid assay protein estimation kit (Beyotime Institute of Biotechnology, Haimen, China). NF- κ B DNA binding ability was assessed by EMSA assay. Briefly, nuclear samples were incubated with a biotin end-labeled DNA probe (Viagen Biotech, Inc. Beijing, China). The DNA-protein complexes were electrophoresed on 6.5% non-denaturing polyacrylamide gels at 180 V for 80 min. By electroblotting, the complexes were transferred to a positively charged nylon membrane and detected by enhanced chemiluminescence (ECL) detection system (Wanleibio Co., Ltd.). The image was obtained and optical densities of the bands were quantified by gel imaging and analysis system (Liuyi Biotechnology, Co., Ltd., Beijing, China, WD-9413B).

Statistical analysis. All results were presented as the mean \pm standard deviation. Statistical analyses were performed by one-way analysis of variance followed by Bonferroni post hoc tests using GraphPad Prism, version 5 software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of EGCG on neointimal formation. H&E staining assay was performed to assess the degree of neointimal formation on day 14 after balloon injury (Fig. 2). As demonstrated in Fig. 2A, there was a single layer of endothelial cells in the carotid artery wall of sham-operated rats. In the balloon injury group, non-uniform thickening of the neointima and lumen stenosis were observed. EGCG treatment markedly reduced neointima hypertrophy. The intimal area was significantly reduced in the EGCG 2 mg/kg ($P < 0.05$) and 4 mg/kg ($P < 0.01$) groups compared with the injury group, and the I/M ratio was significantly reduced in the EGCG 4 mg/kg compared with the injury group ($P < 0.05$; Fig. 2B and E). A significant increase in luminal area was also observed in the EGCG 4 mg/kg group compared with the injury group ($P < 0.01$; Fig. 2C).

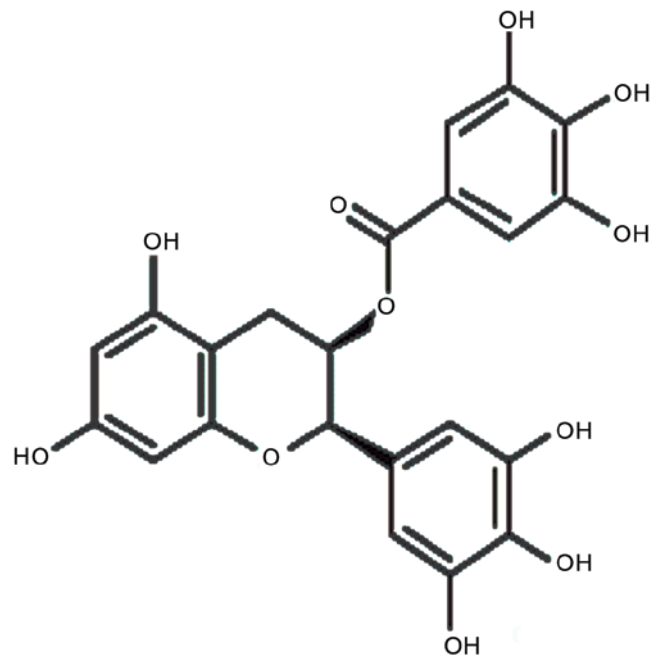


Figure 1. Chemical structure of EGCG. The molecular formula of EGCG is $C_{22}H_{18}O_{11}$ and its molecular weight is 458.38. EGCG, epigallocatechin-3-gallate.

Effect of EGCG on balloon injury-induced expression of HMGB1 and its receptor, RAGE. As demonstrated in Fig. 3A and B, compared with the sham-operated group, the mRNA expression levels of HMGB1 and RAGE were significantly increased in the balloon injury group ($P < 0.001$). EGCG treatment significantly inhibited the balloon injury-induced increase in HMGB1 and RAGE mRNA expression levels in a concentration-dependent manner ($P < 0.05$). Furthermore, the protein expression levels of HMGB1 and RAGE were also determined. As demonstrated in Fig. 3C and D, the levels of HMGB1 and RAGE were low in the sham-operated group, and these were significantly increased by balloon injury ($P < 0.001$). EGCG treatment significantly inhibited HMGB1 and RAGE protein expression levels compared with the injury group ($P < 0.05$).

Effect of EGCG on the inflammatory response. As HMGB1 is an important inflammatory mediator (5), inflammatory cytokine levels in serum were detected by ELISA. As demonstrated in Fig. 4, the production of TNF- α , IL-1 β , IL-6, ICAM-1 and VCAM-1 were significantly increased by balloon injury compared with the sham-operated group ($P < 0.001$), while treatment with EGCG significantly suppressed the excess production of these inflammatory cytokines ($P < 0.05$).

Antioxidant effect of EGCG in rats undergoing carotid artery balloon injury. Furthermore, the oxidative stress markers, MDA and ROS, were also assessed. As demonstrated in Fig. 5, the levels of MDA and ROS were significantly increased in the balloon injury group compared with the sham-operated group ($P < 0.001$). Following treatment with different concentrations of EGCG, the levels of MDA and ROS were significantly decreased compared with the balloon injury group ($P < 0.05$).

Effect of EGCG on NF- κ B activation. To further clarify the molecular mechanism of EGCG on alleviating carotid artery

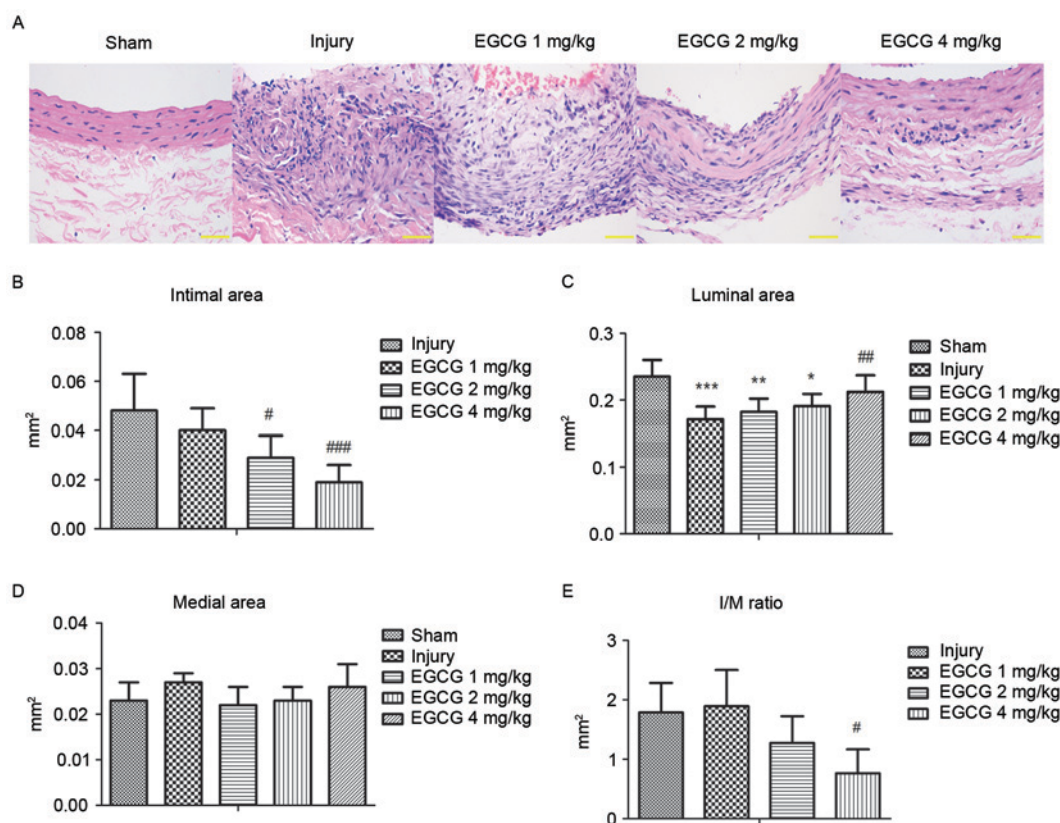


Figure 2. EGCG attenuates neointimal hyperplasia induced by carotid artery balloon injury. (A) Sections of carotid artery tissues 14 days after balloon injury were stained with hematoxylin and eosin (magnification, $\times 400$; scale bar, $50\ \mu\text{m}$). The areas of (B) intima, (C) lumen and (D) media were shown and the (E) I/M ratios were calculated. Data are presented as the mean + standard deviation ($n=6$). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. the sham group; # $P<0.05$, ## $P<0.01$ and ### $P<0.001$ vs. the injury group. EGCG, epigallocatechin-3-gallate; I/M, neointima/media area.

balloon injury in rats, the effects of EGCG on the expression and activity of NF- κ B were evaluated. As demonstrated in Fig. 6A, the expression of NF- κ B in the nucleus was significantly increased, while cytosolic NF- κ B was significantly decreased in the balloon injury group compared with the sham-operated group ($P<0.001$). Treatment with EGCG significantly inhibited the translocation of NF- κ B from cytosol to nucleus induced by balloon injury ($P<0.05$). Furthermore, the activity of NF- κ B binding to DNA was assessed by EMSA assay. As demonstrated in Fig. 6B, the activity of NF- κ B in the balloon injury group was enhanced significantly compared with the sham-operated group ($P<0.001$), while EGCG dose-dependently inhibited the activation of NF- κ B significantly compared with the balloon injury group ($P<0.05$).

Discussion

Although PCI has been widely used for treating vascular stenosis, restenosis after PCI has always been a difficult problem to overcome in therapy of CHD (1). Currently, the optimal treatment has not been demonstrated in clinics. Therefore, more effective treatments are required. EGCG, extracted from Chinese green tea, is used in a vast range of foods, pharmaceuticals, cosmetics and health products (24,25). The present study aimed to observe the effect of EGCG on intima thickening and investigate the related molecular mechanisms. According to the results of the present study, EGCG effectively ameliorated the morphological changes in the carotid artery following

balloon injury, decreased the neointimal area and I/M ratio, increased luminal area, and demonstrated a significant effect against intimal hyperplasia and restenosis of vessels.

HMGB1, an inflammatory cytokine, belongs to the alarmin family and has an important role in the progression of inflammation (26). Growing evidence has demonstrated that HMGB1 was involved in a variety of inflammatory diseases (27-29). Furthermore, HMGB1 has been indicated to be a powerful regulator of balloon injury-induced neointimal hyperplasia (14). Research has also demonstrated that plasma HMGB1 levels may serve as a prognostic biomarker in patients with ST-segment elevation myocardial infarction treated with PCI (30). RAGE, which is not highly expressed in normal tissues, was the first identified receptor of HMGB1 and is highly expressed when combined with its ligand (31). As the present study indicated that balloon injury-induced expression of HMGB1 and RAGE were significantly inhibited by EGCG, we probed the possibility that the suppression of the inflammatory response is related to the downregulation of HMGB1. The role of the inflammatory response in restenosis has attracted increased attention. A study by Libby *et al* (32) demonstrated that the release of inflammatory mediators facilitates neointimal formation and restenosis of angioplasty. Research has also provided evidence that leukocytes were persistently activated following aorta balloon injury in rabbits (33). HMGB1 is also reported to have cross-links with proinflammatory cytokines, such as TNF- α , IL-1 β and IL-6, which amplify the inflammation (34,35). According to

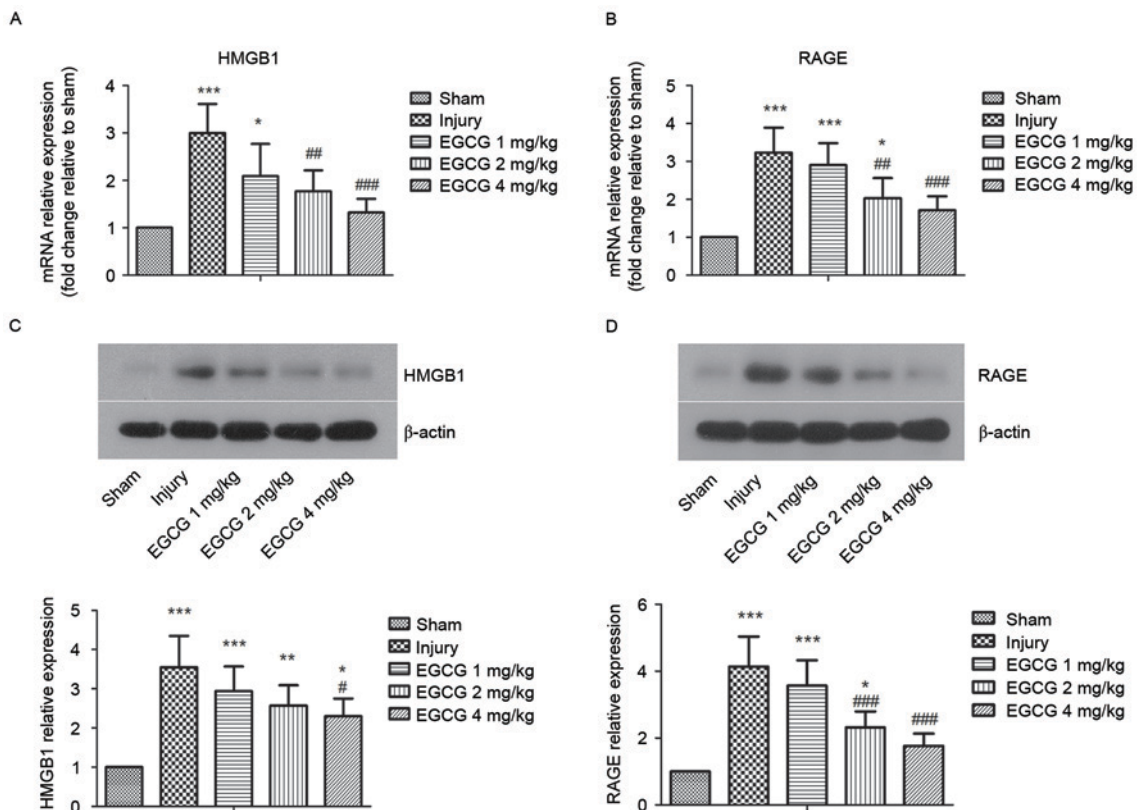


Figure 3. EGCG inhibits balloon injury-induced HMGB1 and RAGE expression levels. mRNA expression levels of (A) HMGB1 and (B) RAGE in artery tissues were determined by reverse transcription-quantitative polymerase chain reaction. Protein expression levels of (C) HMGB1 and (D) RAGE in artery tissues were detected by western blotting. β -actin was used as a loading control. The protein bands were quantified by gray scanning. Data are presented as the mean + standard deviation (n=6). * P <0.05, ** P <0.01 and *** P <0.001 vs. the sham group; # P <0.05, ## P <0.01 and ### P <0.001 vs. the injury group. EGCG, epigallocatechin-3-gallate; HMGB1, high mobility group box 1; RAGE, receptor of advanced glycation end products.

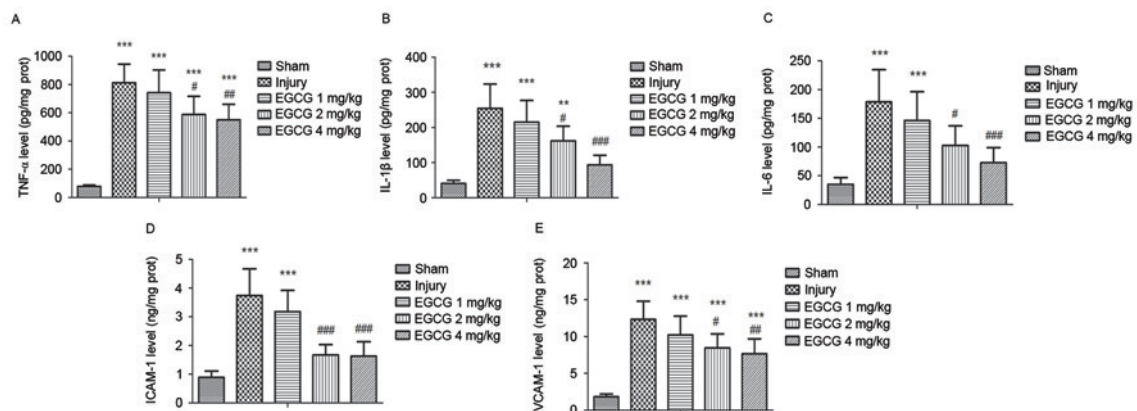


Figure 4. EGCG inhibits the balloon injury-induced inflammatory response. (A) TNF- α , (B) IL-1 β , (C) IL-6, (D) ICAM-1 and (E) VCAM-1 levels in peripheral blood were detected by ELISA. Three independent experiments were performed. Data are presented as the mean + standard deviation (n=6). ** P <0.01 and *** P <0.001 vs. the sham group; # P <0.05, ## P <0.01 and ### P <0.001 vs. the injury group. EGCG, epigallocatechin-3-gallate; TNF- α , tumor necrosis factor α ; IL, interleukin; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; prot, protein.

the present study, the effect of EGCG against arterial balloon injury should be related to its suppression of inflammation.

Prior studies have suggested that oxidative stress is closely related to restenosis following balloon injury (36,37). Oxidative stress is the key link of the development of atherosclerosis (38). A large number of ROS were produced in damaged endothelial cells, which induces endothelium dysfunction and vascular smooth muscle cell proliferation (39). As the

metabolite of oxidative stress, MDA may indirectly reflect the degree of vascular injury. Increasing evidence has reported that EGCG, as a powerful anti-oxidant, has pivotal roles in anti-cancer and anti-vascular disease (40-42). The results of the present study were consistent with previous studies and demonstrated that EGCG significantly inhibited oxidative stress induced by balloon injury by decreasing levels of MDA and ROS. Oxidative stress is always closely related to

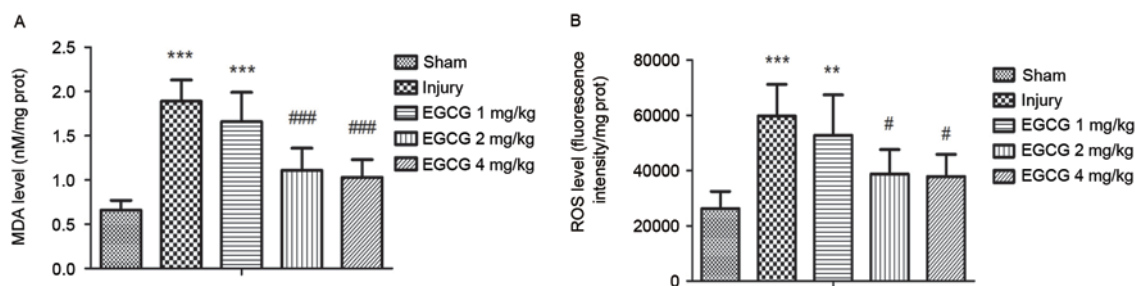


Figure 5. EGCG inhibits balloon injury-induced oxidative stress damage. (A) MDA and (B) ROS levels were assessed. Three independent experiments were performed. Data are presented as the mean + standard deviation (n=6). **P<0.01 and ***P<0.001 vs. the sham group; #P<0.05 and ###P<0.001 vs. the injury group. EGCG, epigallocatechin-3-gallate; MDA, malondialdehyde; ROS, reactive oxygen species; prot, protein.

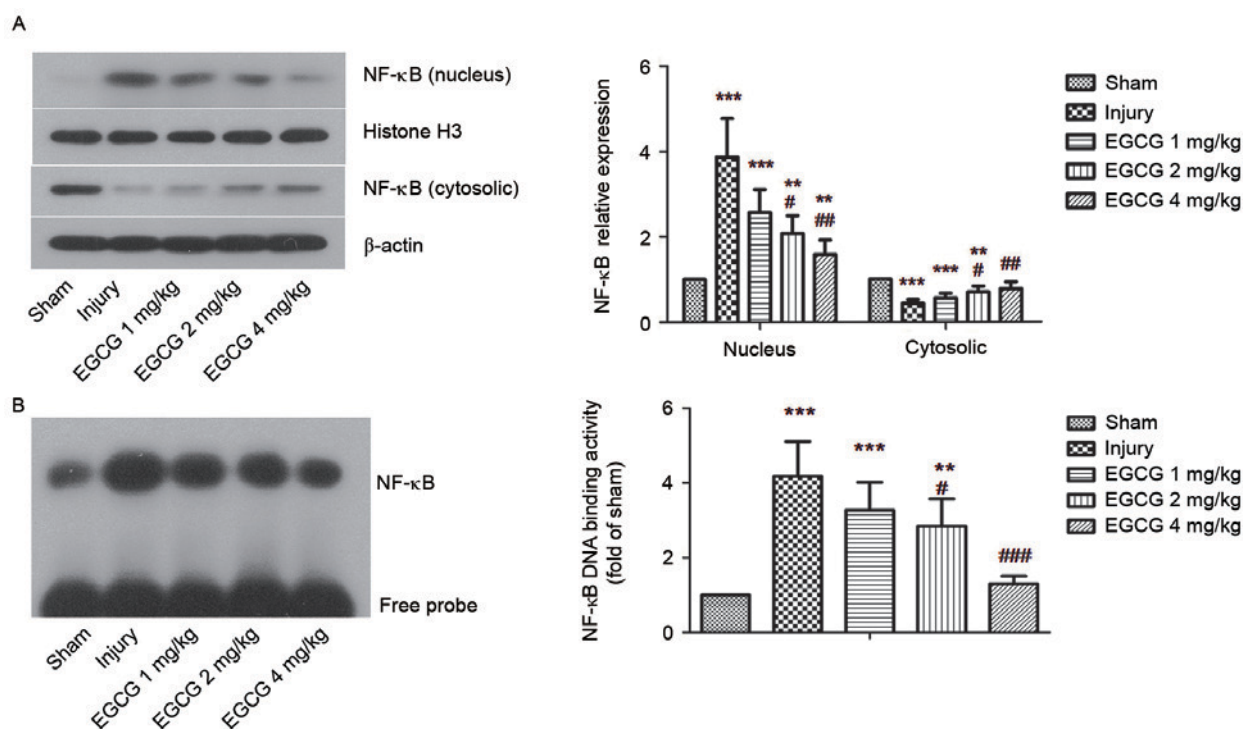


Figure 6. EGCG inhibits NF-κB activation induced by balloon injury. (A) The expression levels of NF-κB in the nucleus and cytoplasm were assessed by western blot assay. Histone H3 and β-actin were used as loading controls. The protein bands were quantified by gray scanning (n=6). (B) Electrophoretic mobility shift assay was performed to assess the DNA binding activity of NF-κB. Three independent experiments were performed. Data are presented as the mean + standard deviation (n=6). **P<0.01 and ***P<0.001 vs. the sham group; #P<0.05, ##P<0.01 and ###P<0.001 vs. the injury group. EGCG, epigallocatechin-3-gallate; NF, nuclear factor.

inflammation (43). Previous research has indicated that use of antioxidants may protect against inflammatory diseases (44). A previous study also demonstrated that there is a mutual enhancement between HMGB1 and oxidative stress (45). These results indicate that the inhibition of oxidative stress may suppress HMGB1 expression. In the present study, the results suggested that EGCG was able to restrain the oxidative stress induced by balloon injury to inhibit HMGB1 expression, which suppressed the inflammatory response and attenuated neointimal hyperplasia in a rat model of carotid artery injury.

NF-κB is an important transcription factor that is activated by nuclear translocation and induces the overexpression of multiple cytokines, including TNF-α, IL-1β and IL-6, and adhesion molecules, such as ICAM-1 and VCAM-1 (46,47). Thus, it has a central role in the inflammatory response (46,47).

Research has demonstrated that HMGB1 was induced by NF-κB activation, thus promoting the progression of acute lung injury (48). A study by Tan *et al* (49) indicated that suppression of NF-κB was able to reduce LPS-induced HMGB1. Therefore, inhibiting the activation of NF-κB may downregulate the level of HMGB1 to attenuate carotid artery injury. The findings of the present study demonstrated that EGCG significantly inhibited the activation of NF-κB, which subsequently reduced HMGB1 expression.

In conclusion, the present study provides evidence that EGCG effectively attenuates neointimal hyperplasia induced by carotid artery injury. Inhibition of HMGB1 expression, suppression of the inflammatory reaction and attenuation of oxidative stress were involved in the protective effects of EGCG. Therefore, EGCG may be a potential treatment for restenosis following PCI.

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