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ORIGINAL ARTICLE

Green tea polyphenol epigallocatechin-3-gallate increases atherosclerotic plaque stability in apolipoprotein E-deficient mice fed a high-fat diet

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Abstract

Background: Epigallocatechin-3-gallate (EGCG), which is the principal component of green tea, has been shown to prevent atherosclerosis. However, the effect of EGCG on atherosclerotic plaque stability remains unknown.

Aim: This study aimed to assess whether EGCG can enhance atherosclerotic plaque stability and to investigate the underlying mechanisms.

Methods: Apolipoprotein E-deficient mice fed a high-fat diet were injected intraperitoneally with EGCG (10 mg/kg) for 16 weeks. Cross sections of the brachiocephalic arteries were stained with haematoxylin and eosin for morphometric analyses or Masson's trichrome for collagen content analyses. Immunohistochemistry was performed to evaluate the percentage of macrophages and smooth muscle cells (SMCs). Protein expression and matrix metalloproteinase (MMP) activity were assayed by Western blot and gelatin zymography, respectively. Serum inflammatory cytokine levels were quantified by enzyme-linked immunosorbent assays.

Results: After 16 weeks of feeding the high-fat diet, there were clear atherosclerotic lesions in the proximal brachiocephalic artery segments according to HE staining. EGCG treatment significantly increased the thickness of the fibrous cap. In the atherosclerotic plaques of the EGCG group, the relative macrophage content was decreased, whereas the relative SMC and collagen contents were increased. The expression levels of MMP-2, MMP-9, and extracellular matrix metalloproteinase inducer (EMMPRIN) were significantly decreased by EGCG treatment. In addition, EGCG treatment decreased the circulating tumour necrosis factor-α, interleukin-6, monocyte chemoattractant protein-1, and interferon-γ levels in apolipoprotein E-deficient mice.

Conclusions: EGCG promotes atherosclerotic lesion stability in apolipoprotein E-deficient mice. Potentially, these effects are mediated through the inhibition of inflammatory cytokine, MMPs and EMMPRIN expression.

Key words: epigallocatechin-3-gallate, atherosclerosis, plaque stability, inflammatory responses, matrix metalloproteinases

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INTRODUCTION

Coronary artery disease (CAD) is the leading global cause of death, morbidity, and disability. Among patients with CAD, acute coronary syndrome (ACS) is responsible for significant patient morbidity and mortality and is a frequent cause of hospital admissions. ACS refers to a range of acute myocardial ischaemic states, which include ST-segment elevation myocardial infarction (STEMI), non-STEMI, and unstable angina. Numerous reports have shown that coronary thrombosis, which is the immediate cause of ACS, results from vulnerable plaque

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rupture. Therefore, it is critical to improve atherosclerotic plaque stability in the treatment of ACS. Vulnerable plaques are characterised by a large necrotic core and a thin fibrous cap (< 65 μm) with macrophage and lymphocyte infiltration. There are few or no smooth muscle cells (SMCs) within the fibrous cap [1]. Growing evidence indicates that inflammation and matrix degradation by matrix metalloproteinases (MMPs) are the primary causes of atherosclerotic plaque instability [2]. Currently, the most appropriate therapeutic approach for preventing acute adverse coronary events is to modify the atherosclerotic plaque composition rather than bypass the lesion or reduce stenosis by using mechanical interventions [3].

Green tea is a popular drink around the world. Regular consumption of green tea has been suggested to protect against a variety of diseases, including cancer, stroke, and cardiovascular diseases [4–6]. The beneficial effects of green tea are attributed to its abundant and biologically active polyphenols. Catechins, the major constituents of green tea polyphenols, mainly comprise (–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin (EC), (–)-epigallocatechin (EGC), and (–)-epicatechin-3-gallate (ECG). Of these, EGCG is the most abundant catechin in green tea [6]. Numerous reports have demonstrated that the green tea polyphenol EGCG is a potent antioxidant that plays an important role in inhibiting inflammation [7, 8]. In our previous study, we showed that EGCG suppressed tumour necrosis factor-α (TNF-α)-induced monocyte chemoattractant protein-1 (MCP-1) expression in human umbilical vein endothelial cells (HUVECs) [9]. Additionally, EGCG inhibits the expression and gelatinolytic activity of MMPs in macrophages and SMCs [10, 11]. Furthermore, we showed that EGCG inhibited the upregulation of extracellular matrix metalloproteinase inducer (EMMPRIN) and MMP-9 expression in PMA-induced macrophages through 67LR [12]. These data all suggest that EGCG can enhance atherosclerotic plaque stability.

Chyu et al. [13] found that EGCG prevented the formation of atherosclerotic lesions. However, the effect of green tea polyphenols on atherosclerotic plaque stability remains unknown. Hence, in the present study, we aimed to elucidate the effect and underlying molecular mechanisms of the green tea polyphenol EGCG on atherosclerotic plaque stability in apolipoprotein E-deficient mice.

METHODS

Animals

Apolipoprotein E-deficient mice fed a high-fat diet were used to evaluate atherosclerotic plaque stability [14]. Thirty male apolipoprotein E-deficient mice (age: eight weeks; weight: 18–20 g) were obtained from the Model Animal Research Centre of Nanjing University (Nanjing, Jiangsu, China). All animals were maintained on a light/dark (12/12 h) cycle and at constant room temperature. The mice were fed a high-fat diet that contained 21% fat and 0.15% cholesterol (Medicine, Yangzhou, Jiangsu, China). The apolipoprotein E-deficient mice were randomly divided into a control group (n = 15) and an EGCG (Sigma, St Louis, MO, USA) group (n = 15). For 16 weeks, EGCG (10 mg/kg body weight/day, five days/week) [13] or 0.9% saline was injected intraperitoneally into the apolipoprotein E-deficient mice. Then, the animals were anaesthetised, and the blood, aorta, and brachiocephalic arteries were harvested. All the procedures were conducted following the regulations of the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Experimental Animal Ethics Committee of Nanjing Medical University (ethic approval no: IACUC-1601044, date: 2016-03).

Analysis of lipid and inflammatory cytokine levels

Blood samples were obtained from the retro-orbital plexus after the mice were anaesthetised. Serum lipid profiles were determined using an enzymatic assay (Roche, Shanghai, China) according to the manufacturer's instructions. Circulating levels of MCP-1, interleukin-6 (IL-6), TNF-α, and interferon-γ (IFN-γ) were measured using ELISA assay kits (CUSABIO, Wuhan, China) in accordance with the manufacturer's instructions.

Immunohistochemistry and histology

Brachiocephalic arteries were harvested and embedded in paraffin. Cross sections (4 μm) were stained with haematoxylin and eosin (HE) to evaluate the plaque morphologies and with Masson's trichrome to evaluate the collagen content in the plaques. Immunohistochemical staining was performed using an anti-Mac-2 mouse macrophage antibody (Abcam, Cambridge, UK) and an anti-α smooth muscle actin antibody (α-SMA, Sigma, St. Louis, MO, USA). The sections were imaged under a Nikon DS-Fi1 microscope (Nikon Corporation, Sendai, Japan). The integrated optical densities of the different indicators were analysed using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction

Total RNA was extracted from aortic tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA with the Reverse Transcription Kit (Takara, Dalian, Liaoning, China). Quantitative real-time polymerase chain reaction was performed using the ABI 7900 Sequence Detection System (Applied Biosystems, Foster, CA, USA) with Power SYBR green PCR Master Mix (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions. The primer sequences were as follows: MMP-2, Sense: 5′-GTTGCAACCTCTTTGTGCTG-3′, Antisense: 5′-TGATCTGGTTCTTGTCCCACT-3′; MMP-9, Sense: 5′-TCAAGGATTGCTCAGAGATT-3′, Antisense: 5′-GCAACCACAGTGAGTGAGTT-3′; EMMPRIN, Sense: 5′-CAGAAAACCCACCTGGAAGA-3′, Antisense: 5′-ATAAACCCCTAAGGAATGGA-3′. The expression levels of target genes were normalised against the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level; the $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression change.

Protein isolation and Western blot analyses

Protein isolation from the aorta and Western blot analyses were performed as previously described [12]. Briefly, equal amounts of protein were electrophoresed on 12% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked for 2 h with 5% bovine serum albumin in Tris-buffered saline and Tween-20 and incubated with primary antibodies for MMP-2, MMP-9, EMMPRIN (Abcam, Cambridge, UK), and GAPDH (Cell Signalling Technology, Boston, MA, USA) overnight at 4°C. The membranes were then incubated with horseradish peroxidase-labelled secondary antibodies (Santa Cruz, Dallas, TX, USA) for 1 h at room temperature. All signals were detected using a ChemiDoc XRS Imaging System (Bio-Rad, Hercules, CA, USA).

Gelatin zymography

MMP-2 and MMP-9 activities were determined by gelatin zymography [12]. The aorta protein supernatants were electrophoresed on 10% polyacrylamide gels containing 1 mg/mL gelatin. After washing twice for 15 min with 2.5% Triton X-100 at 37°C, the gels were incubated in a developing buffer (10 mM Tris base, 40 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl2, and 0.02% Brij 35) at 37°C for 11 h. Finally, the gels were stained with 0.5% Coomassie Blue R-250 for 2 h and then treated with a destaining buffer (50% methanol, 10% glacial acetic acid, and 40% water). Proteolysis was detected as a clear white band against a blue background. The MMP activity was quantified by using Image J (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The data were analysed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). All values are shown as the mean ± standard error of mean. Statistical significance was determined by Student t test. P-values < 0.05 were considered statistically significant.

RESULTS

Effects of EGCG on body weight and plasma lipid profile

To investigate the functional role of EGCG in atherosclerotic plaque stability, EGCG or 0.9% saline was administered to mice fed a high-fat diet for 16 weeks. We found no significant differences in the body weights after EGCG treatment. Similarly, there were no differences in the total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-C) or high-density lipoprotein cholesterol (HDL-C) levels (Table 1).

Table 1. Effects of epigallocatechin-3-gallate (EGCG) on body weight and lipid concentrations

	Control group	EGCG group
Body weight [g]	31.7 ± 4.08	29.9 ± 4.36
Total cholesterol [mM]	17.44 ± 2.13	16.89 ± 2.27
Triglyceride [mM]	2.67 ± 0.58	2.39 ± 0.62
LDL-C [mM]	7.84 ± 1.34	7.66 ± 1.11
HDL-C [mM]	1.63 ± 0.33	1.87 ± 0.41

The differences between the EGCG and control groups were not significant ($p > 0.05$). All values are shown as the mean ± standard error of mean ($n = 7$). HDL-C — high-density lipoprotein cholesterol; LDL-C — low-density lipoprotein cholesterol

Effects of EGCG on atherosclerotic plaque size and stability

After 16 weeks of high-fat diet feeding, atherosclerotic plaques were observed in the segment of proximal brachiocephalic arteries with HE staining (Fig. 1). The size of atherosclerotic plaques decreased significantly in the EGCG group (Fig. 2C). To determine whether EGCG enhanced atherosclerotic plaque stability in apolipoprotein E-deficient mice fed a high-fat diet, we compared the atherosclerotic plaque compositions in mice from the two groups. The relative collagen, SMC, and macrophage contents were detected by Masson's trichrome and immunohistochemical staining. We found that the collagen and SMC contents were significantly greater in the EGCG group than in the 0.9% saline group (Fig. 2B, E; 3B, D). The macrophage content was significantly lower in the EGCG group than in the 0.9% saline group (Fig. 3A, C). Moreover, EGCG treatment significantly increased the thickness of the fibrous cap (Fig. 2A, D). These results indicated that EGCG treatment enhanced atherosclerotic plaque stability.

Effects of EGCG on circulating inflammatory cytokine levels

Numerous reports have demonstrated that inflammatory cytokines play a pivotal role in the complex processes of atherogenesis and plaque destabilisation. To investigate the effects of EGCG on circulating inflammatory cytokines, the levels of the latter were measured with the use of ELISAs. MCP-1 and IL-6 levels were significantly lower in the EGCG group than in the 0.9% saline group. Moreover, EGCG treatment decreased serum TNF-α and IFN-γ levels by 53.5% and 39.7%, respectively (Fig. 4). These data showed that EGCG treatment inhibited the inflammatory responses in apolipoprotein E-deficient mice fed a high-fat diet.

Effects of EGCG on MMP-2, MMP-9, and EMMPRIN expression

Given that MMPs play a crucial role in the pathogenesis of vulnerable plaques, we examined whether EGCG regulates the expression levels of MMP-2 and MMP-9. The results showed that compared to 0.9% saline treatment, EGCG treatment significantly decreased MMP-2 and MMP-9 expression and activity levels (Fig. 5). Furthermore, we investigated the effects of EGCG on the expression of EMMPRIN, which induces MMP-2 and MMP-9 expression [15]. The results showed that

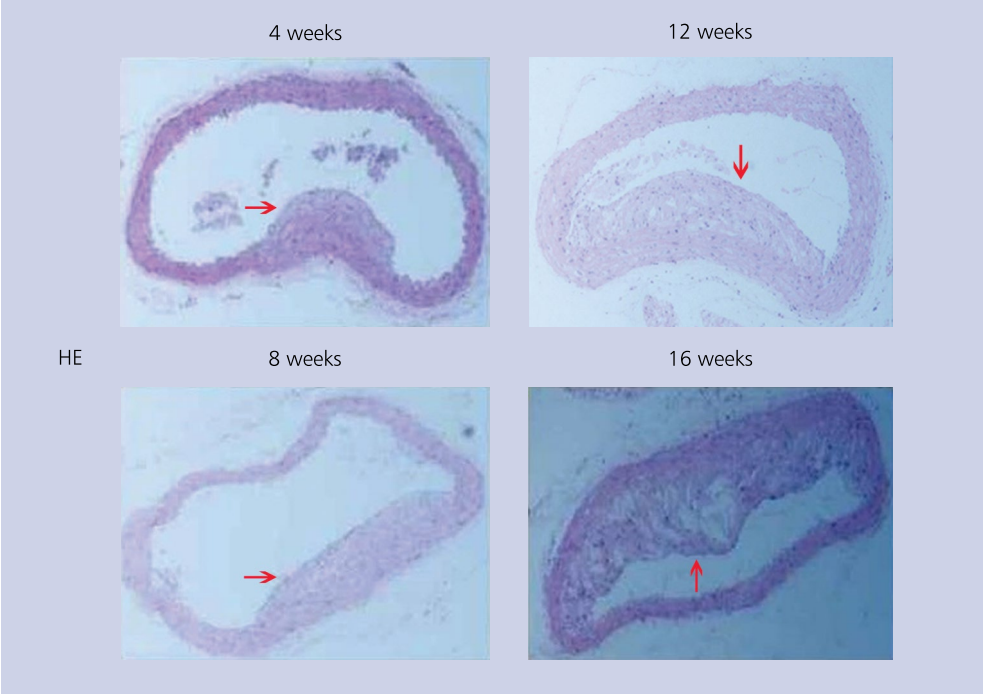

Figure 1. The formation of atherosclerotic plaques in the proximal brachiocephalic arteries. After 16 weeks of high-fat diet feeding, atherosclerotic plaques were observed in the segment of arteries with haematoxylin and eosin staining (arrows)

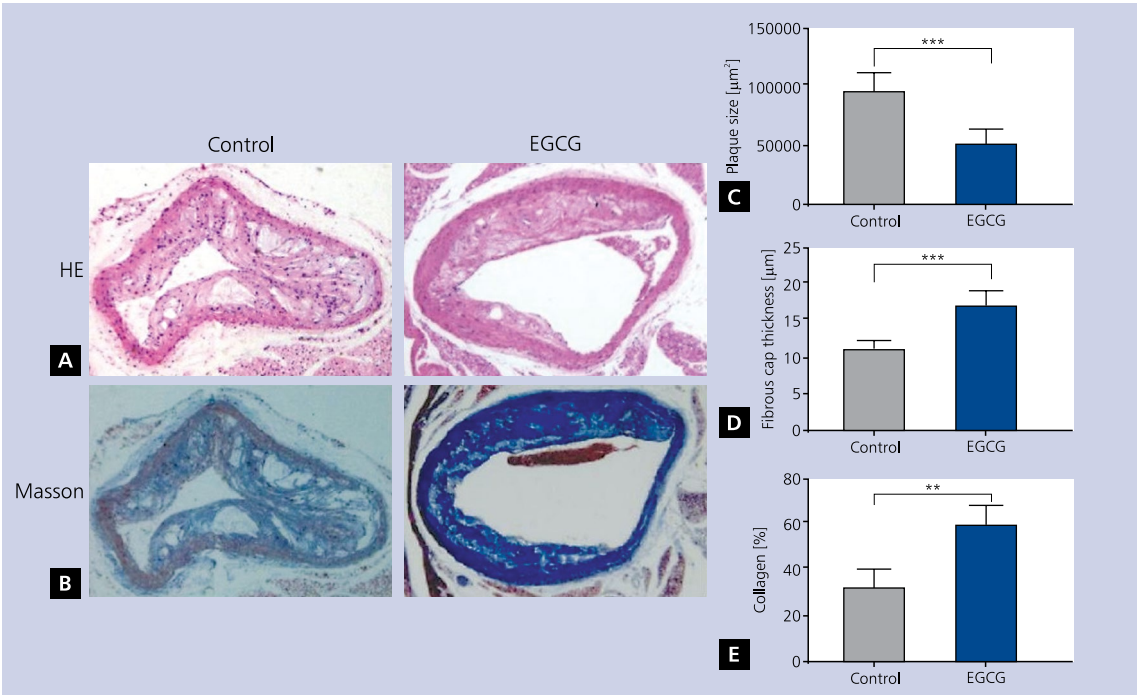

Figure 2. Effect of epigallocatechin-3-gallate (EGCG) on the plaque size, fibrous cap thickness, and collagen content of atherosclerotic plaques in the proximal brachiocephalic arteries. Representative photographs showing haematoxylin and eosin (HE) (**A**) and Masson's trichrome (**B**) staining of the arteries. **C.** Quantitative analysis of the results in panel A. **D.** Quantitative analysis of the results in panel A. **E.** Quantitative analysis of the results in panel B. All values are shown as the mean ± standard error of mean (n = 7; **p < 0.01 vs. control group; ***p < 0.001 vs. control group)

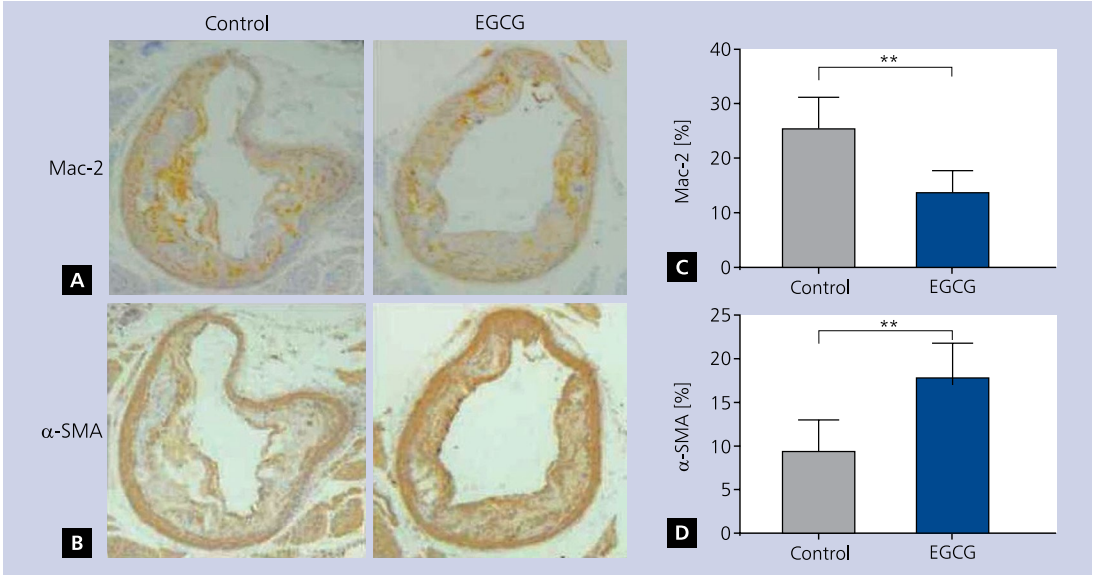

Figure 3. Effect of epigallocatechin-3-gallate (EGCG) on the macrophage and smooth muscle cell contents of atherosclerotic plaques in the proximal brachiocephalic arteries. Representative photographs showing anti-Mac-2 (**A**) and anti-α smooth muscle actin antibody (α-SMA) (**B**) staining of the arteries. **C.** Quantitative analysis of the results in panel A. **D.** Quantitative analysis of the results in panel B. All values are shown as the mean ± standard error of mean (n = 7; **p < 0.01 vs. control group)

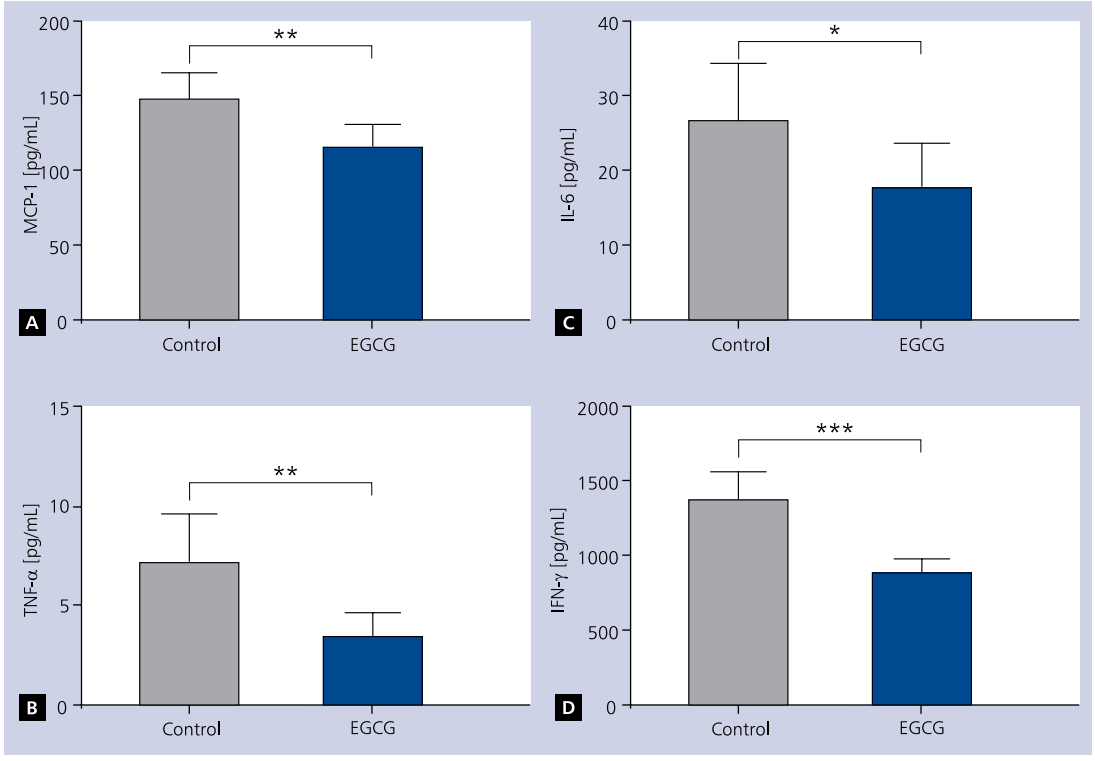

Figure 4. Effect of epigallocatechin-3-gallate (EGCG) on circulating inflammatory cytokine concentrations. Serum monocyte chemoattractant protein-1 (MCP-1, **A**), tumour necrosis factor-α (TNF-α; **B**), interleukin-6 (IL-6; **C**), and interferon-γ (IFN- γ; **D**) levels were significantly reduced by EGCG. All values are shown as the mean ± standard error of mean (n = 7; *p < 0.05 vs. control group; **p < 001 vs. control group; ***p < 0.001 vs. control group)

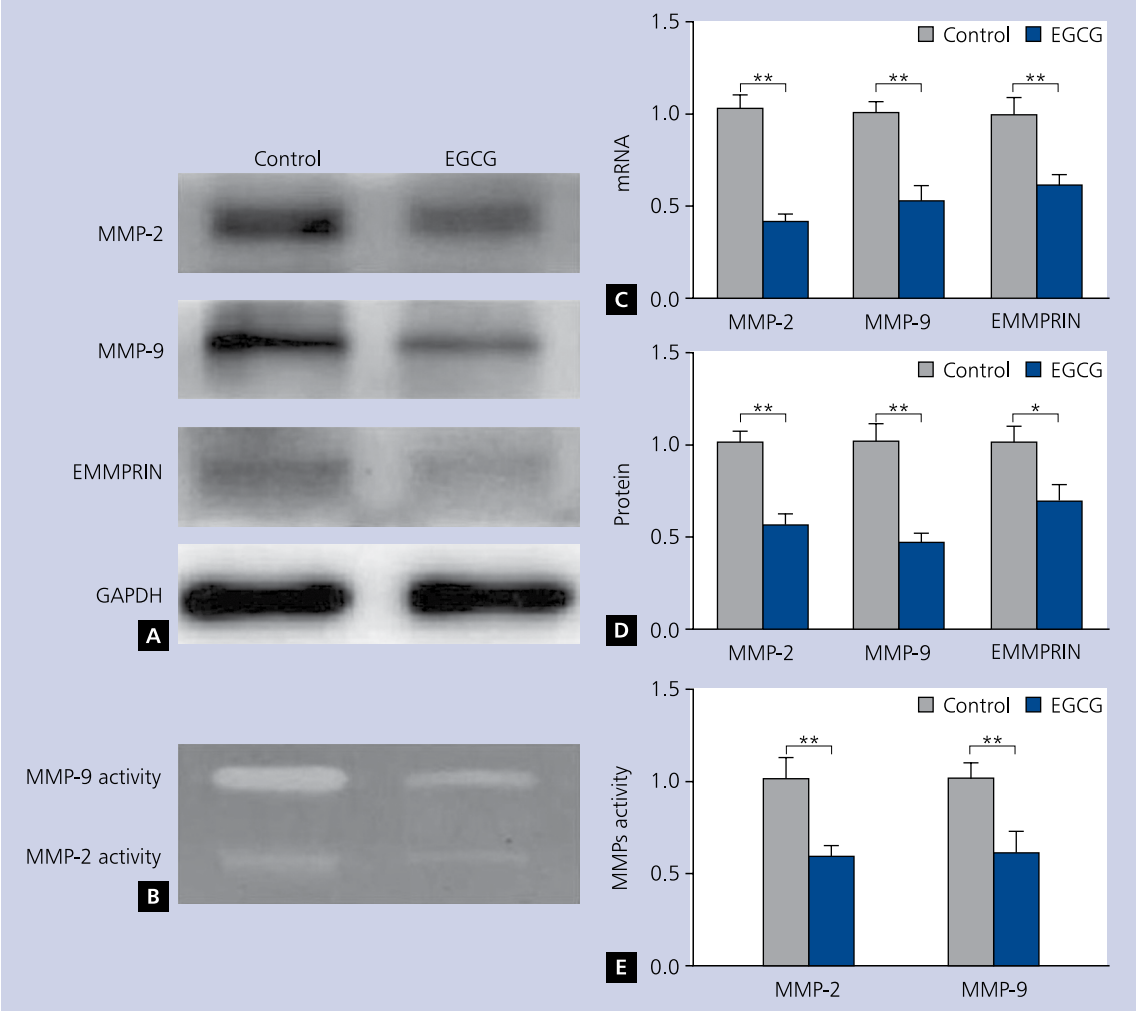

Figure 5. Effect of epigallocatechin-3-gallate (EGCG) on the expression of matrix metalloproteinase (MMP)-2, MMP-9 and extracellular matrix metalloproteinase inducer (EMMPRIN) in atherosclerotic plaques. **A.** Representative Western blots for MMP-2, MMP-9, and EMMPRIN; **B.** Representative gelatin zymography images showing MMP-2 and MMP-9 activity; **C.** Relative mRNA expression of MMP-2, MMP-9, and EMMPIRN; **D.** Quantitative analysis of the results in panel A; **E.** Quantitative analysis of the results in panel B. All values are shown as the mean ± standard error of mean (n = 7; *p < 0.05 vs. control group; **p < 0.01 vs. control group); GAPDH — glyceraldehyde 3-phosphate dehydrogenase

EMMPRIN mRNA and protein expression levels were markedly reduced after EGCG treatment (Fig. 5). This suggests that EGCG increases atherosclerotic plaque stability by suppressing MMP-2, MMP-9, and EMMPRIN expression.

DISCUSSION

It is common knowledge that coronary atherosclerotic plaque development is the underlying condition for ischaemic heart disease. On its own, atherosclerosis is a relatively benign disease. In contrast, atherothrombosis caused by atherosclerotic plaque rupture may lead to life-threatening ACS. The risk of plaque rupture depends more on the plaque composition and stability than on the severity of stenosis. In this study, the green tea polyphenol EGCG, which is the most abundant catechin in green tea, improved atherosclerotic plaque stability in apolipoprotein E-deficient mice fed a high-fat diet. Furthermore, this effect was mediated through the inhibition of inflammatory responses as well as MMP-2, MMP-9, and EMMPRIN expressions. To the best of our knowledge, the present study is the first to evaluate the effects of a green tea polyphenol on the stability of atherosclerotic plaques.

Our epidemiological studies have demonstrated that green tea consumption reduces the risk of CAD [16, 17]. In addition, the consumption of green tea has been associated with a lower risk of acute myocardial infarction (AMI) [18]. Furthermore, EGCG can prevent the formation of atherosclerotic plaques [13]. However, the effect of green tea polyphenols on atherosclerotic plaque stability remains unknown. As previously mentioned, the lipid core size, fibrous cap thickness, plaque cell composition, and local MMP expression levels are key determinants of

plaque stability. Collagen is the primary structural component of the fibrous cap of atherosclerotic plaques [1]. Accumulating evidence has emphasised the critical role of collagen synthesis and breakdown in the maintenance of fibrous cap strength and integrity. The amount and organisation of matrix collagen have been widely used as indirect indicators of plaque stability [19]. In this study, EGCG treatment significantly increased the thickness of the fibrous cap. Moreover, collagen content in the plaques was remarkably increased by EGCG treatment. Vascular SMCs are the only cells capable of synthesising extracellular matrix collagen in atherosclerotic plaques. Therefore, we also used immunohistochemical staining to determine the composition of cell types present in atherosclerotic plaques. The results showed that the relative macrophage content decreased, whereas the relative SMC content increased, in the atherosclerotic plaques of the EGCG group. These findings indicate that EGCG administration can promote atherosclerotic plaque stability in apolipoprotein E-deficient mice fed a high-fat diet.

MMPs are a group of similar-structured proteases involved in the degradation of collagen and most other extracellular matrix components [20]. Therefore, the overexpression of MMPs can promote atherosclerotic plaque instability [2]. Numerous reports have indicated that MMP-2 and MMP-9, also known as gelatinases, participate in plaque destabilisation and rupture [21, 22]. In the present study, protein expression and activity levels of MMP-2 and MMP-9 were determined by Western blot and gelatin zymography, respectively. The results showed that EGCG treatment significantly inhibited the protein expression and activity levels of MMP-2 and MMP-9. EMMPRIN, also called CD147 or basigin, has been reported to induce the production of various MMPs in tumour studies [23]. Similarly, EMMPRIN has been shown to regulate MMP-2 and MMP-9 expressions in monocytes, macrophages, and SMCs [20]. In recent years, a growing body of evidence has suggested that EMMPRIN plays an important role in the complex processes of atherosclerotic plaque rupture and atherothrombosis [24]. Previously, we found that EGCG significantly suppressed the expression of EMMPRIN and MMP-9 in a dose-dependent manner in PMA-induced macrophages [12]. Our current data our data showed that protein levels of EMMPRIN were markedly reduced after EGCG treatment in apolipoprotein E-deficient mice fed a high-fat diet. Taken together, these results suggest that EGCG improves atherosclerotic plaque stability by inhibiting MMP-2, MMP-9, and EMMPRIN expressions.

Furthermore, MCP-1, IL-6, and TNF-α have been shown to upregulate the expression of MMPs. IFN-γ was found to inhibit collagen synthesis in human vascular SMCs and trigger apoptosis in vascular SMCs [2]. Our previous study showed that EGCG suppressed TNF-α-induced MCP-1 expression in HUVECs via inhibiting the activation of the nuclear factor-κB [9]. In addition, in vivo studies showed that EGCG could inhibit the production of MCP-1, TNF-α, IL-6, and IFN-γ [25]. To investigate the effects of EGCG on MCP-1, TNF-α, IL-6, and IFN-γ expression, the levels of these circulating inflammatory cytokines were measured by ELISA. The results showed that serum MCP-1, TNF-α, IL-6, and IFN-γ levels were significantly reduced by EGCG. These findings indicate that the administration of EGCG increases atherosclerotic plaque stability through suppressing inflammatory responses.

In our study, we found that there were no differences in the total cholesterol, triglyceride, LDL-C, or HDL-C levels between the EGCG (10 mg/kg/day) and control groups. Consistent with our findings, Chyu et al. [13] found that EGCG (10 mg/kg/day) reduced cuff-induced evolving atherosclerotic plaque size, but no difference was observed in total cholesterol level. However, other studies found that supplementation with EGCG (40 mg/kg/day, 100 mg/kg/day) dramatically increased HDL-C levels and decreased total cholesterol and LDL-C levels. These disparate findings may be explained by different doses of EGCG used. Our results suggest that EGCG enhances atherosclerotic plaque stability independently of circulating lipid levels [26, 27].

In conclusion, we reported for the first time that the green tea polyphenol EGCG could enhance atherosclerotic plaque stability in apolipoprotein E-deficient mice fed a high-fat diet. Potentially, these effects of EGCG were mediated through reduction of MMP and EMMPRIN expression levels and suppression of inflammatory responses. Our results indicate that EGCG might be a novel therapeutic agent for stabilising atherosclerotic plaques. In other studies the consumption of green tea has been associated with a reduced risk of AMI. Our findings may help to better understand the protective function of green tea against AMI in human epidemiological studies.

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Conflict of interest: *none declared*

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WHAT IS NEW?

The rupture of unstable atherosclerotic plaque is the main underlying cause of acute coronary syndrome. Currently, the most appropriate therapeutic approach for preventing acute coronary events is to modify the atherosclerotic plaque composition rather than bypass the lesion or reduce stenosis by using mechanical interventions. In this work, we evaluated the effect and underlying molecular mechanisms of the green tea polyphenol epigallocatechin-3-gallate (EGCG) on atherosclerotic plaque stability in apolipoprotein E-deficient mice. To our best knowledge, we were the first to show that EGCG could increase atherosclerotic plaque stability by reducing matrix metalloproteinase expression levels and suppressing inflammatory responses. Our findings provide a new insight into the pharmacological role of EGCG in stabilising atherosclerotic plaques.