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**Original Research Article**

# Bergamot regulates oxidized low-density lipoprotein-induced inflammation and foam cell formation of human umbilical vein endothelial cells by regulating SIRT1/NF-κB pathway

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## *Abstract*

***Purpose:*** *To investigate the effects of bergamot (BGM) on the progression of atherosclerosis, and to unravel the mechanism of action.*
***Methods:*** *Oxidized low-density lipoprotein (Ox-LDL)-induced HUVECs were used as an in vitro model of atherosclerosis. CCK-8, flow cytometry (FCM), and enzyme-linked Immunosorbent assay (ELISA) assays were performed to confirm the effects of BGM on the viability and inflammation of ox-LDL-induced HUVECs. Oil-red staining and immunoblot tests were conducted to determine the effects of BGM on foam cell formation and macrophage polarization. Furthermore, The mechanism of action of BGM was examined by immunoblot studies.*
***Results:*** *BGM alleviated the ox-LDL-stimulated decline in HUVEC cell viability, and the ox-LDL-stimulated HUVEC inflammation, but inhibited ox-LDL-stimulated foam cell formation and macrophage polarization in vitro ($p < 0.05$). In addition, BGM regulated SIRT1/NF-κB pathway, thereby alleviating atherosclerosis ($p < 0.05$).*
***Conclusion:*** *BGM regulates OX-LDL-induced inflammation and foam cell formation of HUVECs by mediating SIRT1/NF-κB pathway, and therefore can potentially serve as a drug for the treatment of atherosclerosis.*

***Keywords:*** *Bergamot (BGM), Atherosclerosis, OX-LDL, HUVEC, Foam cell, SIRT1/NF-κB pathway, Macrophage polarization*

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## INTRODUCTION

Cardiovascular disease is one of the most common causes of disease-related death worldwide [1]. High blood pressure and hypercholesterolemia pose serious threats to the cardiovascular system, leading to inflammation of blood vessel and dysfunction [2]. Atherosclerosis is a systemic disease that primarily affects the aorta, carotid arteries, and coronary arteries [3]. As a major risk factor for atherosclerosis, hypercholesterolemia triggers the deposition of

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oxidized low-density lipoprotein (ox-LDL) under the intima of the blood vessel wall, and is one of the early pathogenesis of atherosclerosis [4]. In addition, during the formation of atherosclerotic lesions, polarization of macrophages leads to different phenotypes, and ox-LDL directly contributes to the polarization of macrophages, induces the formation of foam cells, and ultimately promotes the formation of plaque. Therefore, it is necessary to further investigate effective treatments and their mechanisms of action for the management of atherosclerosis and related conditions. Bergamot (BGM) is a bioactive furanocoumarin that is available in large quantities in grapefruit [5]. It initially attracted a lot of attention for its anti-cancer properties [6], and inhibition of the proliferation, invasion and migration of cancer cells, with antibacterial, anti-mutagenesis and other biological activities [7]. Recent studies have found that Bergamot has anti-inflammatory, antioxidant and adipogenic effects [7]. It inactivates many enzymes related to drug metabolism in cytochrome P450 enzyme system, thus affecting the absorption of drugs in the gastrointestinal tract and increasing the bioavailability of oral drugs [8]. In addition, Bergamot alleviates LPS-induced macrophage inflammation in RAW264.7 mice, inhibits NF-κB activation by inducing SIRT1, and alleviates lung injury [9]. It exerts its anti-lipogenesis effect through the activation of AMPK, inhibits the lipogenesis of 3T3-L1 cells, and regulates the body weight of diet-induced obese mice [10]. It is speculated that bergamot may inhibit ox-LDL-induced endothelial cell inflammation and foam cell formation by mediating SIRT1/NF-κB pathway. However, the potential impact of BGM on atherosclerosis remains unknown.

This study aims to examine the effect of BGM on atherosclerosis, and to evaluate its mechanism of action.

## EXPERIMENTAL

### Cell culture

Human HUVECs and RAW264.7 cells (Bluefbio, Shanghai, China) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) at 37 °C with 5 % $CO_2$. The cells were then treated with an increasing dose of ox-LDL (Sigma, purity > 98 %) or BGM (Sigma, purity > 98 %) for 24 or 48 h.

### Cell viability assay

The cells were plated in 96-well plates at a density of $1\times10^3$ cells/well. After treatment with ox-LDL or BGM at the indicated concentration, Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added to the cells after rinsing with phosphate-buffered saline (PBS). Then, the cells were incubated for 4 h and dissolved in 200 uL dimethyl sulfoxide (DMSO). and the absorbance measured spectrophotometrically at a wavelength of 490 nm.

### Determination of cell apoptosis

Annexin V/PI (Sigma Aldrich, USA) was used assess apoptosis in the cells. The cells were digested and mixed in a reaction buffer containing Annexin V and PI for 5 min. The proportion of cells were analyzed by flow cytometer (BD Biosciences).

### Immunoblot assay

The BCA assay method was used for protein concentration determination, after which proteins were separated (20 µg per lane) by SDS-PAGE. The proteins were separated by 9 % SDS-PAGE, and transferred onto PVDF membranes, followed by blocking with 5 % BSA. Subsequently, the membranes were conjugated with primary antibodies targeting Cd36 (ab252922, 1:1000, Abcam), Cd86 (ab220188, 1:1000, Abcam), SIRT1 (ab110304, 1:1000, Abcam), anti-p65 (ab32536, 1:500, Abcam), anti-p-p65 (ab76302, S536, 1:500, Abcam), anti-IκBα (sc-203, 1:1000, Santa Cruz), anti-p-IκBα (sc-101714, Tyr42, 1:500, Santa Cruz), and β-actin (ab8226, 1:3000, Abcam) for 1 h. Subsequently, the membranes were incubated with specific secondary antibodies for 1 h, and the blots analyzed using ECL kit. ImageJ (version 1.8.0; National Institutes of Health) was used for densitometry studies.

### Enzyme-linked immunosorbent assay (ELISA)

The MCP-1, IL-6, VCAM-1, and ICAM-1 levels were measured with the detection kits (Beyotime, Shanghai, China), and performed following the manufacturer's guidelines. Biotin-conjugated primary antibodies were added and followed by avidin conjugated HRP. Then, enzyme substrate was used for the color reaction.

### Oil-red O staining

To assess foam cell formation, RAW264.7 cells were plated into the 12-well plates and kept for 24 h for cell adhesion. After rinsing, the cells were incubated with 5 g/L Oil-red O (Sigma, USA) for 15 min at 37 °C, and observed under a ZEISS microscope.

### Statistical analysis

GraphPad 5.0 software (GraphPad Software, Inc.) was used for statistical analysis in the present study. Results were presented as the mean ± SD (n=3). Statistical differences between two groups were analyzed by paired student's t-test. ANOVA followed by Dunnett's post hoc test was used for multiple comparisons. $p$<0.05 was considered to indicate a statistically significant difference.

## RESULTS

### BGM alleviated OX-LDL-stimulated decrease in HUVEC viability

To investigate the possible effects of BGM on the progression of atherosclerosis *in vitro*, an OX-LDL-induced HUVEC model was constructed. High concentration of Ox-LDL (20, 40, and 80 ug/mL) suppressed the viability of HUVECs, unlike the low concentration (Figure 1 a). Therefore, 80 ug/mL of Ox-LDL was used in subsequent experiments. Furthermore, a high concentration of BGM (160 uM) suppressed the viability of HUVECs, whereas, a low concentration of BGM had modest effects on HUVEC viability (Figure 1 b). Also, 80 ug/mL of Ox-LDL treatment suppressed the viability of HUVECs, but BGM treatment reversed the suppressive effect (Figure 1 c).

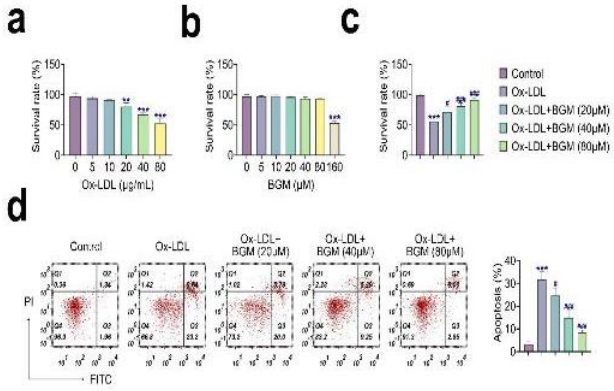


**Figure 1:** BGM alleviates OX-LDL-induced decrease in HUVEC viability. (a) Cell viability of HUVECs after treatment with Ox-LDL (5, 10, 20, 40, and 80 ug/mL) for 48 h; (b) effect of BGM (5, 10, 20, 40, 80, and 160 uM) on HUVEC viability; (c) effect of BGM cell viability of Ox-LDL-treated HUVECs (d) effect of BGM on Ox-LDL-treated HUVEC cell apoptosis. $^{**}P < 0.01$, $^{***}p < 0.001$ vs control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, Ox-LDL+BGM vs Ox-LDL

In FCM assay, 80 ug/mL Ox-LDL treatment stimulated the apoptosis of HUVECs, but BGM treatment reversed the action of Ox-LDL (Figure 1 d). Therefore, BGM alleviates OX-LDL-induced decrease in HUVEC viability.

### BGM alleviates OX-LDL-induced HUVEC inflammation

Ox-LDL increased the secretion of inflammatory factors (MCP-1, IL-6, VCAM-1, and ICAM-1) in HUVEC (Figure 2). However, BGM treatment reversed the secretion of these inflammatory factors caused by Ox-LDL (Figure 2). Thus, BGM alleviates OX-LDL-induced HUVEC inflammation.

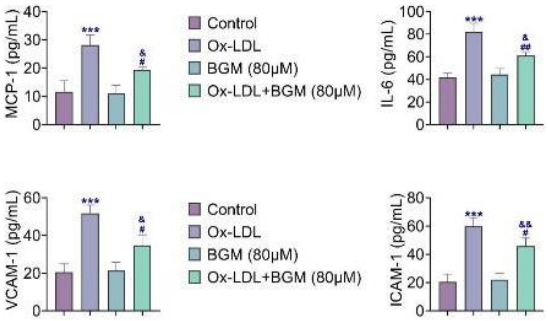


**Figure 2:** BGM alleviated Ox-LDL-induced HUVEC inflammation. Level of inflammation based on the secretion of MCP-1, IL-6, VCAM-1, and ICAM-1 in HUVECs. $^{***}P < 0.001$, vs control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, Ox-LDL+BGM vs Ox-LDL; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, Ox-LDL+BGM vs BGM

### BGM inhibits OX-LDL-induced foam cell formation and macrophage polarization *in vitro*

The formation of foam cells reflects the degree of atherosclerosis. Ox-LDL treatment increased the Oil-red O positive cell numbers of RAW264.7 cells (Figure 3 a). However, BGM treatment enhanced the increase in Oil-red O positive cell numbers of RAW264.7 cells caused by Ox-LDL treatment (Figure 3 a). Expression of two markers of macrophage polarization, Cd36 and Cd86, in RAW264.7 cells increased after Ox-LDL treatment (Figure 3 b), but BGM treatment reversed the increase in their expression (Figure 3 b). Therefore, BGM inhibits OX-LDL-induced foam cell formation and macrophage polarization *in vitro*.

### BGM regulates SIRT1/NF-κB pathway in HUVECs and RAW264.7 cells

To unravel the mechanisms involved of action of BGM in Ox-LDL-induced HUVECs and RAW264.7 cells, SIRT1 expression was analyzed. There was decreased expression of SIRT1 in Ox-LDL-induced HUVECs and RAW264.7 cells (Figure 4 a, c). However, BGM

treatment countered the increase (Figure 4 a, c). Similarly, the increased phosphorylation levels of p65 and IκBα in Ox-LDL-induced HUVECs and RAW264.7 cells (Figure 4 b, d) was reversed by BGM treatment (Figure 4 b, d). Therefore, BGM regulates SIRT1/NF-κB pathway in HUVECs and RAW264.7 cells.

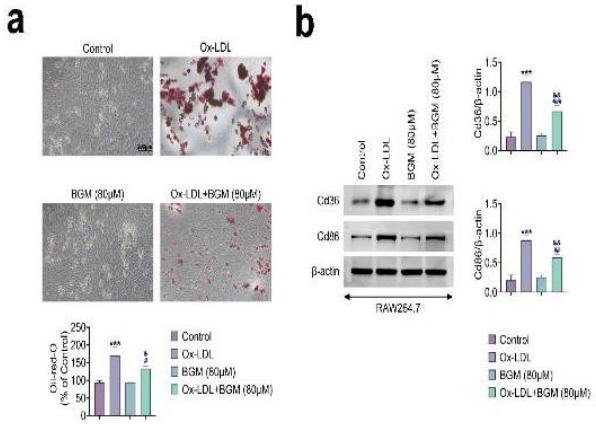


**Figure 3:** BGM inhibited Ox-LDL-induced foam cell formation and macrophage polarization *in vitro.* (a) Oil-red O staining showed the staining degree of Raw264.7 cells; (b) expression of Cd36 and Cd86 in Raw264.7 cells. ***$P$ < 0.001, vs control, $^{\#\#}p$ < 0.01, Ox-LDL+BGM *vs* Ox-LDL; $^{\&\&}p$ < 0.01, Ox-LDL+BGM *vs* BGM

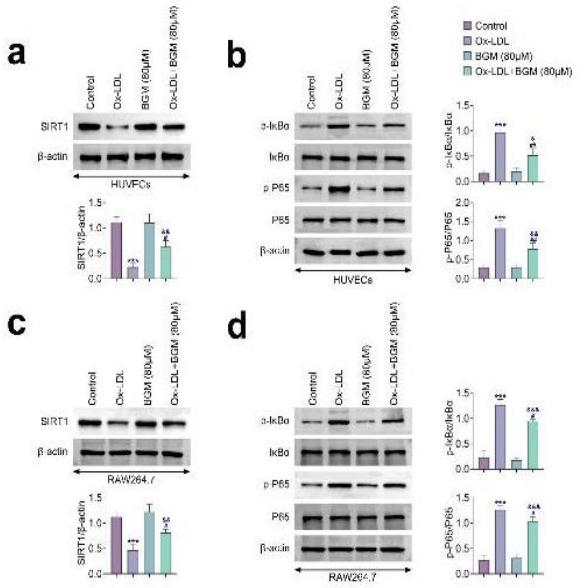


**Figure 4:** BGM regulated SIRT1/NF-κB pathway. (a) Expression of SIRT1 in HUVECs; (b) expression and phosphorylation levels of p65 and IκBα in HUVECs; (c) expression of SIRT1 in RAW264.7 cells pretreated with Ox-LDL; (d) expression and phosphorylation levels of p65 and IκBα in RAW264.7 cells pretreated with Ox-LDL. ***$P$ < 0.001, *vs* control, $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, Ox-LDL+BGM *vs* Ox-LDL, $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01, $^{\&\&\&}p$ < 0.001, Ox-LDL+BGM *vs* BGM

## DISCUSSION

Atherosclerosis is a multi-etiological disease, which exact cause is unknown. The main risk factors are dyslipidemia, hypertension, diabetes and genetic factors. Atherosclerosis is a chronic inflammatory reaction caused by the interaction of endothelial cells, lipids, mononuclear macrophages, VSMCs and platelets [11]. As the most common disease of the cardiovascular system, and the frequent pathological basis of many cardiovascular and cerebrovascular diseases, the disease has been observed to afflict increasingly younger persons [11]. The pathogenesis of atherosclerosis mainly involves endothelial injury reaction, inflammatory reaction, lipid infiltration, smooth muscle cell cloning and thrombosis [12,13]. In the present study, BGM alleviated OX-LDL-induced decline in HUVEC cell viability and inflammation, and inhibited OX-LDL-induced *in vitro* foam cell formation and macrophage polarization. Therefore, BGM is a potential drug for atherosclerosis.

Since BGM alleviated Ox-LDL-induced decline in HUVEC cell viability and inflammation, as well as macrophage polarization, it can be said that BGM alleviates atherosclerosis *in vitro*. The biological activities of BGM have been widely reported. It has anti-cancer, antibacterial, anti-mutagenesis and other biological activities [10]. A study indicates that it is a competitive inhibitor of CYP1A1, and it inhibits CYP1A1, CYP1A2, CYP2B1, and CYP2B2 with $IC_{50}$ of 0.192 ± 0.029, 5.077 ± 0.31 μM, 9.495 ± 0.979, and 4.535 ± 0.092 μM, respectively. Its anti-tumor effects have also been reported [10,14]. Bergamot (BGM) also has also been shown to have potent antiproliferative effects in A549 cells [9], like in HUVECs as has been demonstrated in the present study. In addition, bergamot also inhibits the clonogenic activity of the A549 cells by reducing the cancer colony forming cells [9].

BGM has been shown to have significant anti-inflammatory properties [15]. Inflammation is a self-protective response of the body's immune system to external stimuli. However, long-term chronic inflammation leads to the development of a variety of diseases, including atherosclerosis [15]. Studies have shown that BGM reduces inflammatory responses by inhibiting key molecules in the inflammatory pathway, thus reducing the degree of cell damage and tissue inflammation [15]. This has led to BGM being considered as a potential anti-inflammatory drug. Interestingly, its anti-inflammation activities in HUVECs have also been shown in the present.

It was also observed in the present work that BGM inhibited Ox-LDL-induced *in vitro* foam cell formation and macrophage polarization. When macrophages or smooth muscle cells consume excess fat, both become foam cells. Foam cells are one of the factors that cause arteriosclerosis [16].

In addition, BGM alleviated Ox-LDL-induced HUVEC viability and inflammation, but inhibited O-LxDL-induced *in vitro* foam cell formation and macrophage polarization via SIRT1/NF-κB pathway. SIRT1 is a histone deacetylase that acts as an essential mediated longevity gene, and has anti-atherogenic effects by regulating the acetylation of some functional proteins [17]. Also, the NF-κB pathway is involved in the development of some inflammatory diseases such as atherosclerosis by regulating the expression of inflammation-related factors [18]. Inhibiting these pathways lowers the burden of inflammation, thereby reducing inflammation and preventing atherosclerosis [18]. Thus, SIRT1/NF-κB pathway could may be a key target for atherosclerosis. However, the main limitation of this study was the lack of animal experiments and hence the absence of detailed analysis of the molecular mechanisms involved.

## CONCLUSION

BGM regulates cell inflammation and foam cell formation in Ox-LDL-induced HUVECs via SIRT1/NF-κB pathway. Thus, BGM is a potential therapy for atherosclerosis.

## DECLARATIONS

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### *Funding*

None provided.

### *Ethical approval*

None provided.

### *Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Fan Zhao and Taimin Liu designed the study and carried them out; Fan Zhao, Bo Liu, Taimin Liu and Jun Yin supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication. Fan Zhao and Taimin Liu contributed equally to the work and should be considered co-first authors.

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