

Berberine inhibited carotid atherosclerosis through PI3K/AKT/mTOR signaling pathway

Ting Song^a and Wei Da Chen^{b*}

^aDepartment of Neurology, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan City, Shandong Province, China;

^bHealth Care Department, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan City, Shandong Province, China

ABSTRACT

Atherosclerosis, a multifactorial vascular disease resulting from lipid metabolism disorders, features chronic inflammatory damage resulting from endothelial dysfunction, which usually affects multiple arteries. The carotid artery is a common site for clinical atherosclerosis evaluation. The aortic root is the standard site for quantifying atherosclerosis in mice. Due to the adverse reactions of first-line drugs, it is necessary to discover new drugs to prevent and treat atherosclerosis. Berberine (BBR) is one of the most promising natural products derived from herbal medicine *Coptidis Rhizoma* (Huanglian) that features significant anti-atherosclerosis properties. However, overall BBR mechanism against carotid atherosclerosis has not been clearly discovered. Our work aimed to investigate potential BBR mechanism in improving carotid atherosclerosis in ApoE knockout mice. Here, we proved that in ApoE^{-/-} mice receiving high-fat diet for 12 weeks, BBR can reduce serum lipid levels, improve intimal hyperplasia, and antagonize carotid lipid accumulation, which may be achieved through regulating the PI3K/AKT/mTOR signaling pathway, regulating autophagy, promoting cell proliferation and inhibiting cell apoptosis. In summary, these data indicate that BBR can ameliorate carotid atherosclerosis. Therefore, it could be a promisingly therapeutic alternative for atherosclerosis.

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1. Introduction

Atherosclerosis, a progressive chronic inflammatory and metabolic disease, features lipid deposition, focal intimal thickening, smooth muscle cell proliferation, and plaque formation [1]. It usually impacts various large and medium arteries, especially branches of the aorta, as well as coronary, femoral and carotid artery, and is the main reason for heart disease and stroke [2,3]. With the development of society, atherosclerosis and its complications have caused an increase in morbidity and mortality worldwide, accounting for only one third of the death toll in the world [4,5]. Although atherosclerosis pathogenesis is still not fully understood, it is found to participate in lipid metabolism disorders, endothelial dysfunction, inflammation along with oxidative stress [6]. It has been reported that endothelial dysfunction and the formation of neointima are the early key steps in atherosclerosis development, which are conducive to vasospasm, thrombosis, macrophage

penetration, cell growth, and inflammation that lead to atherosclerosis [7,8]. Therefore, it is vital to study the underlying mechanism of carotid atherosclerosis.

With complementary and alternative medicines increasingly popular with patients with atherosclerosis, natural derived compounds are increasingly used in Asian and Western countries. Berberine (BBR), an isoquinoline alkaloid and the main active ingredient in Chinese herbal medicine *Coptidis Rhizoma* (Huanglian), has been revealed to have a wide range of beneficial properties against antibiotics, anti-inflammatory, anti-apoptotic, anti-oxidant, and neuroprotective effects [9–14]. Recently, based on more and more *in vitro* and *in vivo* researches, BBR has protective influence in cardiovascular disease (CVD), like lowering serum lipid levels [15], and blood sugar [16], along with protecting vascular endothelium [17], with anti-inflammatory [18] and antioxidant [19] impacts. It is worth noting that BBR has

*CONTACT Wei Da Chen  183221241@qq.com  Health Care Department, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, No.42 Wenhua West Road, Lixia District, Jinan City, No.42 Wenhua West Road, Lixia District 250011, China

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presented beneficial impacts against atherosclerosis. BBR improves atherosclerosis in mice with hyperhomocysteinemia, which is relative to peroxisome proliferator-activated receptor γ (PPAR γ) activation as well as subsequent inhibition on endothelial cell oxidative stress [20]. The anti-atherosclerotic properties of BBR can also prevent the formation of foam cells through inhibiting cholesterol accumulating in macrophages [21]. However, the underlying mechanism by which BBR protects aorta and improves atherosclerosis is still not clear.

Here, we use ApoE knockout (ApoE^{-/-}) mice for experiments, which will develop hypercholesterolemia and spontaneously form complex plaques under a normal diet, and a high-fat diet can promote hyperlipidemia, which can form atherosclerotic lesions similar to humans [22]. It has been widely used in atherosclerosis research. We placed the perivascular collar around the carotid artery of ApoE^{-/-} mice receiving a high-fat diet to establish an animal model of carotid atherosclerosis, and investigated the regulation of BBR on serum lipid levels, carotid artery histology, endothelial function, cell proliferation and apoptosis levels and PI3K/AKT/mTOR signaling pathway, aiming to explore the protective impact of BBR on carotid atherosclerosis and its underlying mechanism [23,24]. This work provides an experimental basis for further research on the mechanism of BBR in preventing and treating carotid atherosclerosis, indicating that BBR may become a potential drug for the treatment of carotid atherosclerosis.

2. Materials & methods

2.1 Animals & treatment

Male 8-week-old wild-type (WT) C57BL/6 and ApoE^{-/-} mice on C57BL/6 genetic background from Beijing Vital River Laboratory Animal Technology Co., Ltd., which our team housed under specific-pathogen-free environment (20–25°C, 55–65% humidity, 12 h light/dark cycle), and had free access to water and food, all procedures in strict reference to the Guidelines for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology, China, as well

as approved by the Laboratory Animal Ethics Committee, Affiliated Hospital of Shandong University of Traditional Chinese Medicine.

Our team anesthetized ApoE^{-/-} mice via intraperitoneally injecting pentobarbital sodium (50 mg/kg), later performed carotid artery surgery under dissecting microscopes, gently separated the right common carotid artery, where placed narrow collars (length 5 mm; inner diameter 0.3 mm; outer diameter 0.6 mm) around, and fixed the collar with three surgical threads. In C57BL/6 J mice, our team isolated the right common carotid artery without placing the right neck [25]. One week after operation, our team allocated by random ApoE^{-/-} mice into 5 groups (n = 12) based on body weight: M group (ApoE^{-/-} model mice), ATO+PIO group (ApoE^{-/-} mice+ATO+PIO-4.6 mg/kg) (ATO and PIO, positive drug, Dalian, China), BBRL (ApoE^{-/-} mice + BBR-78 mg/kg) (BBR, A0151, Chengdu, China), BBRM (ApoE^{-/-} mice + BBR-117 mg/kg) and BBRH (ApoE^{-/-} mice + BBR-156 mg/kg), fed a high-fat diet (with fat content of 21% and cholesterol content of 0.15%). WT C57BL/6 mice served as the control group (C group, n = 12), receiving normal diet. Our team treated mice in C and M group with physiological saline, and others with doses. Our team intervened all mice via oral gavage for 12 weeks, measured their weight at 0, 1, 3, 6, and 12 weeks after experiment.

2.2 Sample collection and treatment

After experiment, our team anesthetized the mice with isoflurane, took the blood from abdominal aorta, later sacrificed the mice via decapitation, separated and collected the right carotid artery under operating microscopes for further analysis.

2.3 ELISA analysis

After centrifuging the blood sample at 1300 × g for 10 min at 4°C to collect serum, our team added corresponding reagents in reference to biochemical kits (Nanjing Jiancheng Bioengineering Institute) instructions and procedures for serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-

density lipoprotein cholesterol (HDL-C) content determination [25].

2.4 Morphological analysis

After fixing the right carotid artery in 4% paraformaldehyde, embedding it in OCT, and cutting it into frozen sections (6 μm), our team applied HE staining (Wuhan Servicebio Biological Technology Co., Ltd.) to observe carotid plaque area and neointima formation to assess carotid atherosclerotic lesions, stained oil red O working solution (Sigma-Aldrich, St. Louis, U.S.) for 20 min, later decolorized it with 60% isopropanol for 10 min, washed thoroughly in phosphate buffered saline (PBS) and later acquired images under common optical microscopes to evaluate lipid droplet accumulation [26].

2.5 Immunofluorescence staining

After blocking the frozen sections with 5% FBS for 30 min to incubate with Ki67 antibody (Cell Signaling Technology, U.S.) and TUNEL antibody (Cell Signaling Technology) at 4°C overnight, our team marked the target protein with red or green fluorescence for visualization, stained the nuclei with DAPI (blue fluorescence) (Cell Signaling Technology), and later performed image acquisition via confocal microscopes (Leica, Germany) to evaluate the proportion of positive cells [27].

2.6 Western blot analysis

After fully lysing mice carotid artery tissue in RIPA lysis buffer (Cell Signaling Technology) for 30 min, our team centrifuged them at 12,000 r/min at 4°C for 15 min, took the supernate as the total protein extract to be tested, applied BCA kit (Beyotime) to measure the total protein concentration, loaded each well with 50 μg protein and separated them via 8–15% SDS-PAGE. Our team transferred the protein to PVDF film (Millipore, U.S.), blocking the film in 5% skim milk, via following primary antibodies overnight at 4°C: p-PI3K p85, PI3K p85, p-AKT, AKT, p-mTOR, mTOR, Beclin-1, P62, and GAPDH antibodies (1:1000 dilution). Our team incubated the films with anti-mouse and -rabbit IgG peroxidase-conjugated secondary antibodies, visualized bands

with ECL kits (Millipore, U.S.) and quantified them via Quantity One Software (Bio-Rad, U.S.) [26].

2.7 Statistical analysis

With data being expressed as means \pm standard deviation (SD) from at least three independent experiments, our team proceeded statistical analysis between two groups with *t* test as well as among multiple groups via one-way analysis of variance (ANOVA), $P < 0.05$ regarded as statistically significant.

3. Results

In this study, we hypothesized that BBR plays a role in improving carotid atherosclerosis, which may be mediated by regulating the PI3K/AKT/mTOR signaling pathway. First, we evaluated the effect of BBR on serum lipid levels, carotid artery lipid accumulation and intimal hyperplasia in carotid atherosclerotic ApoE^{-/-} mice. To study the molecular mechanism, we tested the expression of cell proliferation and apoptosis, PI3K/AKT/mTOR and autophagy-related proteins.

3.1 BBR reduced serum lipid levels in ApoE^{-/-} mice

In order to confirm the blood lipid-lowering impact of BBR, our team measured blood lipid levels in each group. Compared with C group, serum TC, TG and LDL-C levels in M group increased signally (Figure 1(a–c)), while HDL-C levels were signally reduced ($P < 0.0001$) (Figure 1d). However, compared with M group, low-, medium- and high-dose BBR reduced serum TC, TG and LDL-C levels ($P < 0.0001$, $P < 0.05$, $P < 0.001$), of which BBRH group had a significant effect (Figure 1(a–c)). Only high-dose BBR increased HDL-C levels ($P < 0.05$) (Figure 1(d)). Throughout our experiment, the body weight of mice was recorded. All mice weight elevated in comparison with that before experiment, but there was no difference among the groups at each time point in ApoE^{-/-} mice (Figure 1e).

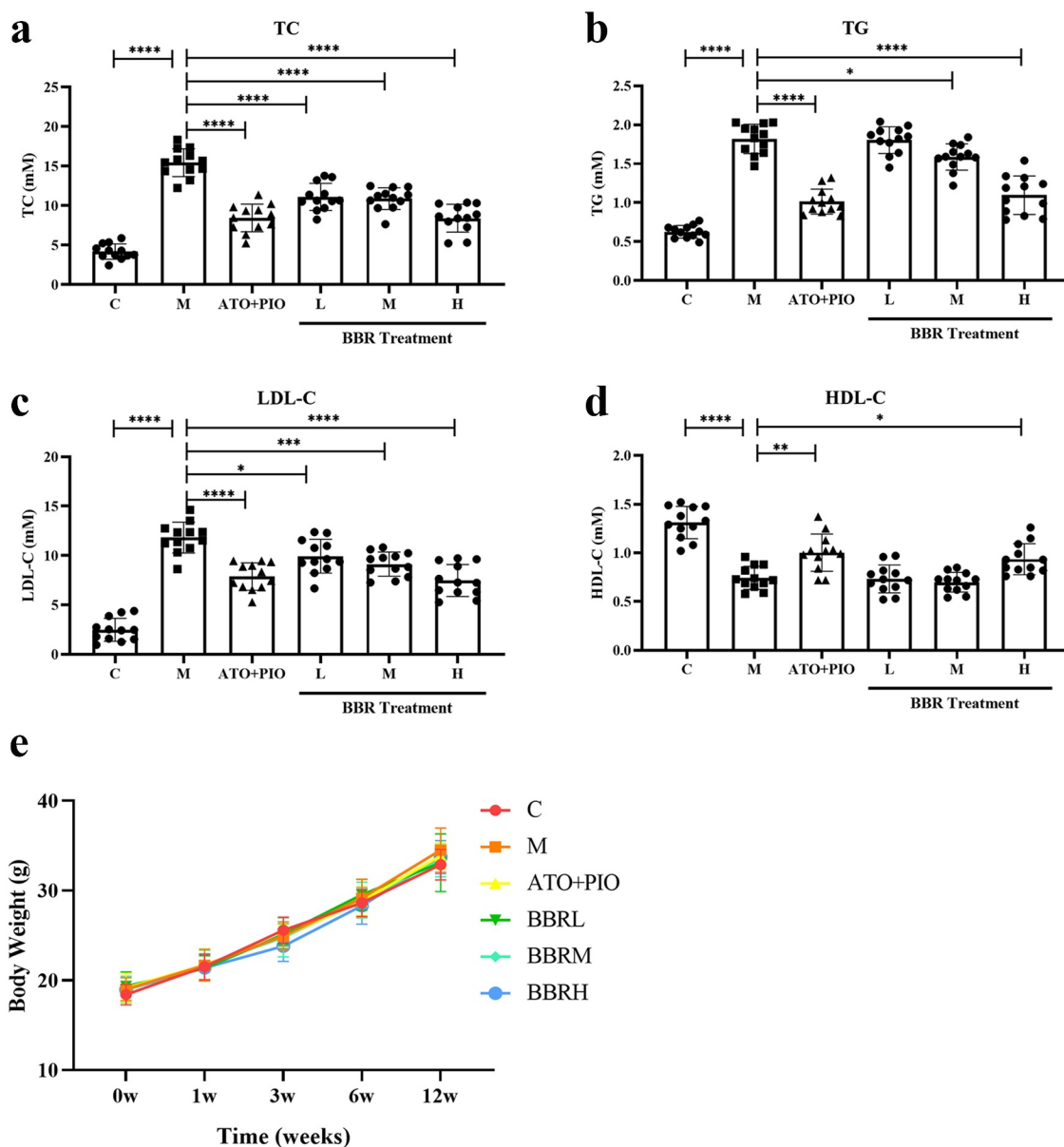


Figure 1. BBR reduced blood lipid levels in ApoE^{-/-} mice. (a) TC level; (b) TG level; (c) LDL-C level; (d) HDL-C level; (e) Body weight; the findings were presented as mean \pm SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.2 BBR reduced plaque area in carotid arteries in ApoE^{-/-} mice

The pathological diagnosis of carotid artery vascular tissue was significantly different between the groups. In C group, vascular intima, media, and adventitia were neat and intact, with no obvious structural damage being observed (Figure 2); while in M group, obvious thickening of the vascular intima

was observed near the intima, the smooth muscle in the vascular medium became less, and the atherosclerotic plaque increased ($P < 0.0001$) (Figure 2). Compared with the M group, low-, medium- and high-dose BBR showed a significant and dose-dependent inhibitory effect on these changes, the intima was visually thickened and relatively intact, atherosclerotic plaques were reduced, and

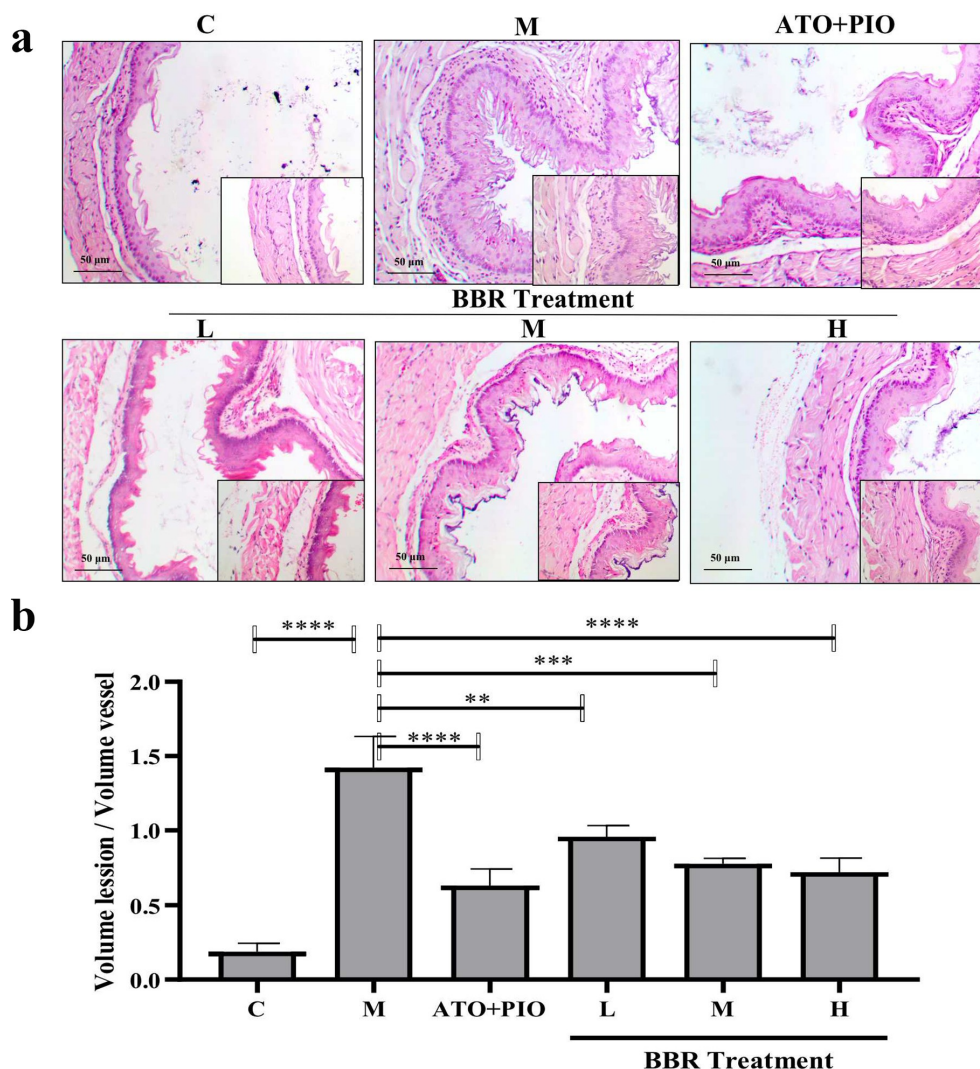


Figure 2. BBR reduced the plaque area in ApoE^{-/-} mice carotid arteries. (a) H&E staining representative image of the carotid artery (large image was magnified 40; small image was magnified 200); (b) the ratio of carotid artery atherosclerotic plaque area to lumen area; the findings were presented as mean \pm SD; ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

atherosclerotic plaques were reduced (Figure 2). The ratio of hardened plaque area to lumen area was visually reduced ($P < 0.01$, $P < 0.001$, $P < 0.0001$) (Figure 2(b)).

3.3 BBR inhibited neointima formation in carotid arteries of ApoE^{-/-} mice

Next, the impact of BBR on neointima formation was observed. Compared with C group, intimal hyperplasia was increased in M group ($P < 0.0001$) (Figure 3); while compared with M group, middle- and high-dose BBR could inhibit carotid artery neointimal hyperplasia in ApoE^{-/-}

mice ($P < 0.05$, $P < 0.01$), among which high-dose group had a significant impact (Figure 3).

3.4 BBR inhibited lipid accumulation in carotid arteries of ApoE^{-/-} mice

Oil red O staining results showed that compared with C group, carotid lipid droplets in M group elevated signally ($P < 0.0001$) (Figure 4); whereas in comparison with M group, medium and high-dose BBR could inhibit lipid droplets accumulating in ApoE^{-/-} mice ($P < 0.01$, $P < 0.001$), and high-dose group had a significant effect (Figure 4).

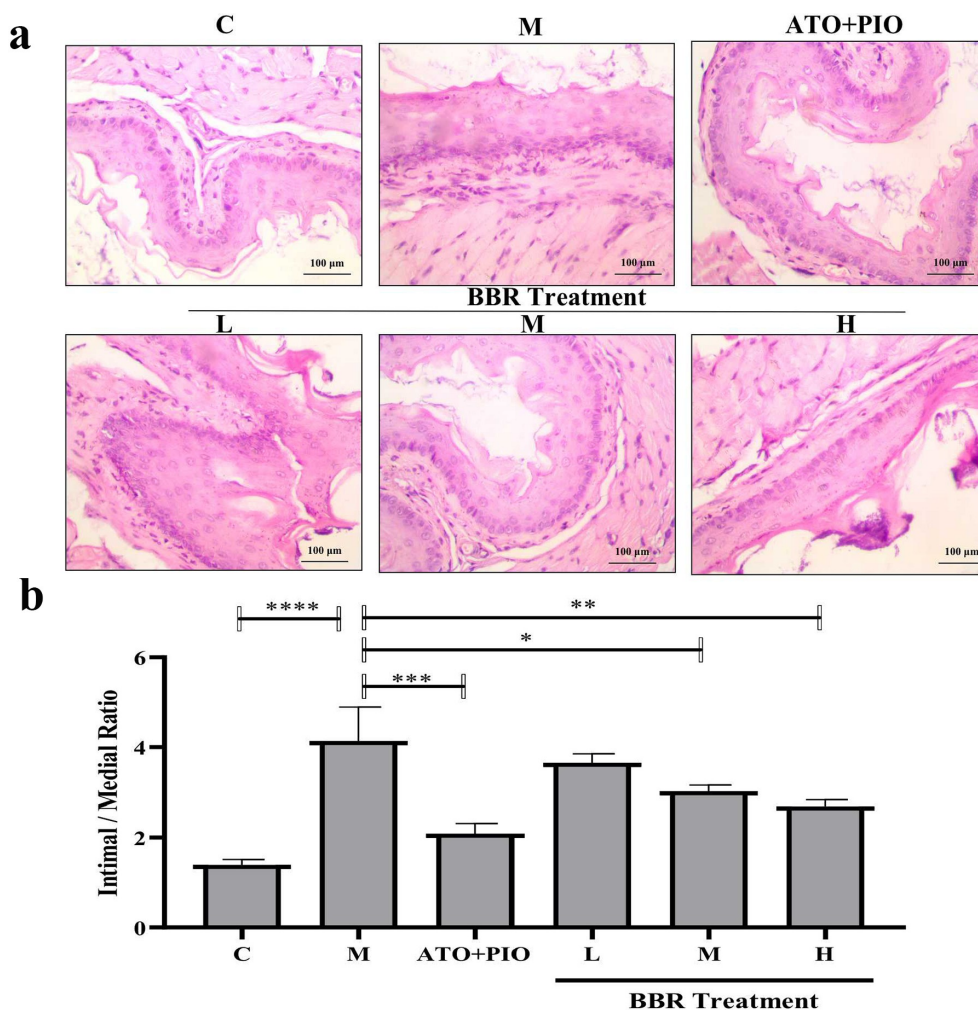


Figure 3. BBR inhibited neointima formation in $ApoE^{-/-}$ mice carotid arteries. (a) H&E staining representative image of carotid artery (magnification 200); (b) carotid artery intima/media thickness ratio; the findings were presented as mean \pm SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.5 BBR promoted cell proliferation and inhibited apoptosis in carotid arteries of $ApoE^{-/-}$ mice

Immunofluorescence results showed that compared with C group, the proportion of $Ki67^{+}$ cells in the carotid artery of M group decreased ($P < 0.0001$) (Figure 5), and the proportion of $TUNEL^{+}$ cells increased ($P < 0.0001$) (Figure 6), indicating that the cell proliferation rate reduced and the apoptosis rate was increased. Compared with M group, medium- and high-dose BBR promoted cell proliferation in a dose-dependent manner ($P < 0.001$, $P < 0.0001$) (Figure 5), medium- and high-dose BBR could inhibit cell apoptosis ($P < 0.05$, $P < 0.001$) (Figure 6), and high-dose group had a significant effect.

3.6 BBR regulated PI3K/AKT/mTOR signaling pathway in $ApoE^{-/-}$ mice carotid arteries

Aiming to confirm whether BBR regulatory mechanism was relative to PI3K/AKT/mTOR signaling pathway, our team measured relative protein expression via western blot. The findings revealed that compared with C group, p-PI3K and p-mTOR expressions elevated in M group ($P < 0.0001$), and p-AKT expression decreased ($P < 0.0001$); in comparison with M group, medium- and high-dose BBR reversed the changes in protein expression ($P < 0.0001$, $P < 0.001$, $P < 0.01$, $P < 0.05$) (Figure 7). Additionally, compared with C group, M group showed increased Beclin-1 and P62 protein expressions ($P < 0.0001$), while low-, medium-, and high-dose BBR reduced Beclin-1

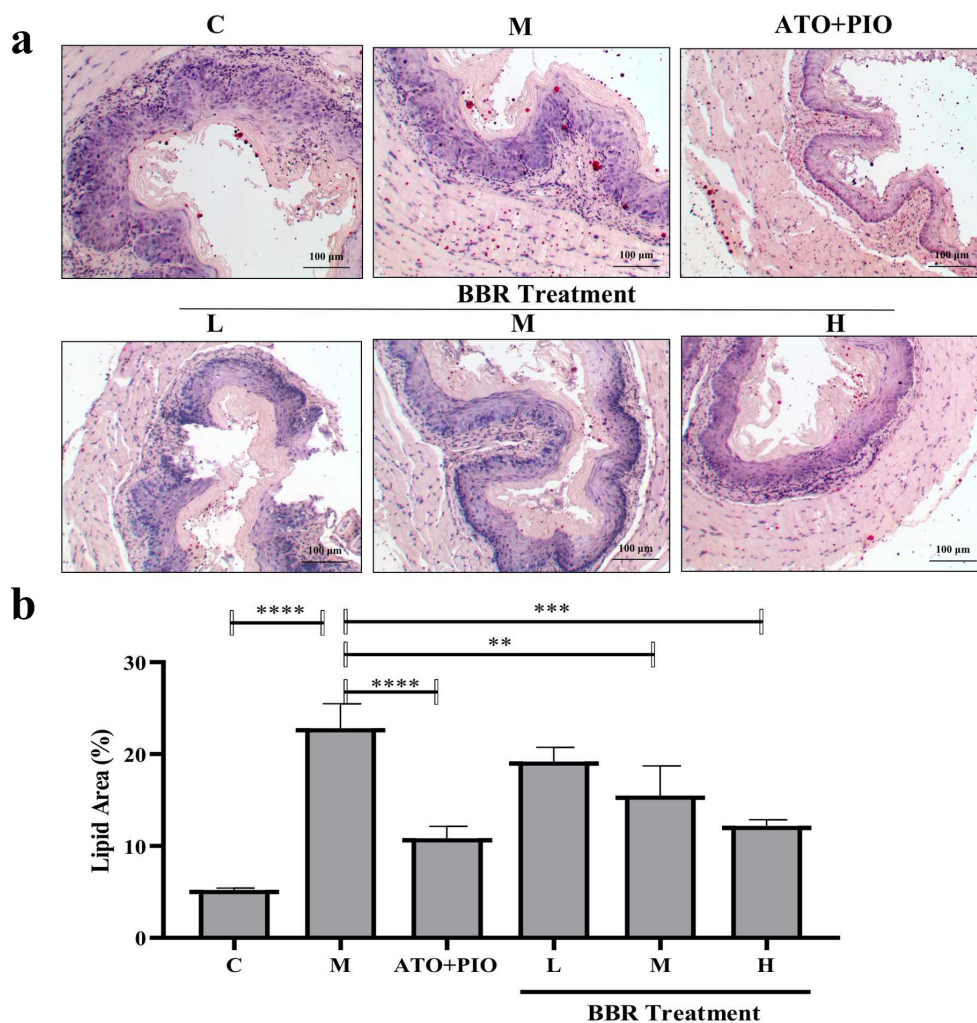


Figure 4. BBR inhibited lipid accumulation in ApoE^{-/-} mice carotid arteries. (a) oil red O staining representative image of carotid artery (magnification 40); (b) Carotid artery lipid droplet area; the findings were presented as mean \pm SD; ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

and P62 levels in a dose-dependent manner of ApoE^{-/-} mice ($P < 0.05$, $P < 0.0001$, $P < 0.01$) (Figure 7). These findings indicated that BBR modulated PI3K/AKT/mTOR signaling pathway in a dose-dependent manner to improve carotid atherosclerosis. The impact of BBR high-dose group is significant.

4. Discussion

It has been fully realized that carotid atherosclerosis is a complex pathophysiological process, which is related to hyperlipidemia, endothelial dysfunction, cell proliferation and autophagy [27,28]. In this study, ApoE^{-/-} mice were placed in a carotid artery neck ring combined with a high-fat diet to establish an animal model of carotid

atherosclerosis (survival rate after surgery was 100%) to explore the mechanism of BBR in improving the disease. Studies have shown that high-fat diet feeding increases the blood viscosity of mice, while the placement of the collar affects local blood circulation, which is based on local changes in the hemodynamic state of the carotid artery, which leads to carotid atherosclerosis [25]. Therefore, this animal model is similar to the pathophysiological conditions of human atherosclerosis and is considered a suitable research object [29–31].

For hundreds of years, Chinese herbal medicine has been widely applied to prevent and treat various diseases. Among them, BBR is a natural alkaloid isolated from the traditional Chinese herbal medicine *Coptidis Rhizoma* (Huanglian) [32].

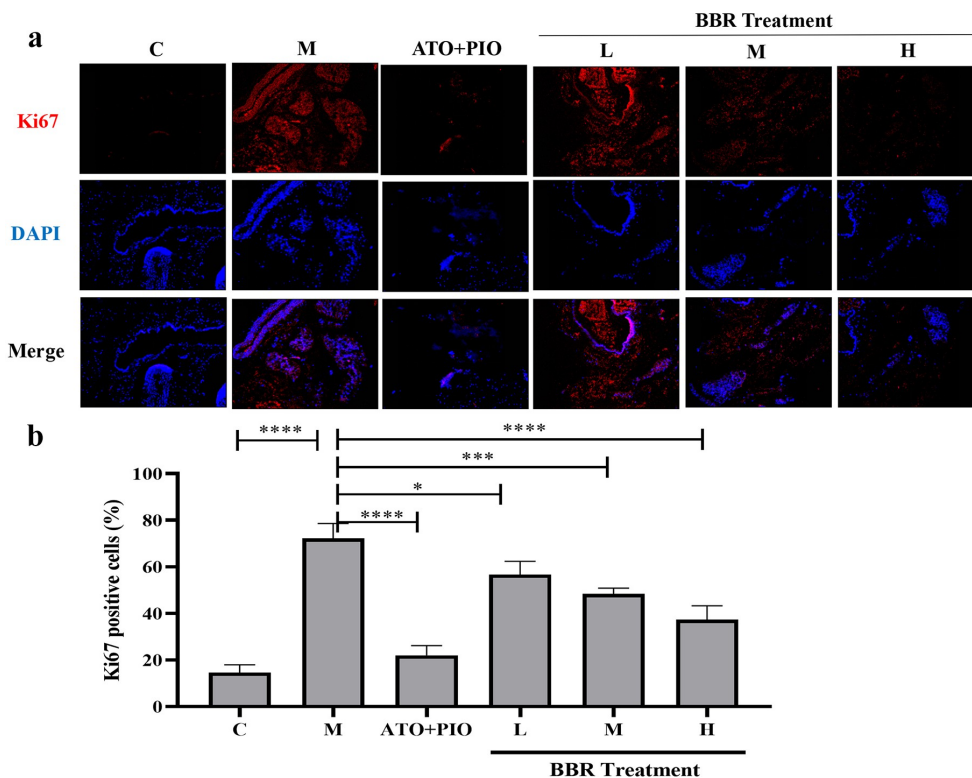


Figure 5. BBR promoted cell proliferation in ApoE^{-/-} mice carotid arteries. (a) Ki67 immunofluorescence staining representative image of carotid artery (magnification 100), the first row represented Ki67 staining of proliferating cells (red), the second row represented nuclear staining by DAPI (blue), and the third row represented the merged image; (b) The proportion of Ki67⁺ cells in carotid arteries; the findings were presented as mean \pm SD; ** $P < 0.01$, **** $P < 0.0001$.

Based on previous studies, BBR has various pharmacological properties, including microbial action, anti-inflammatory activity, and regulation of insulin secretion [33–35]. In recent years, BBR has a broader potential clinical significance in preventing and treating different human diseases [36–40]. Our study found that BBR can improve carotid atherosclerosis in a dose-dependent manner, manifested by lowering serum lipid levels, improving intimal hyperplasia, and antagonizing carotid lipid accumulation. In addition, this study clearly shows that the improvement effect of BBR on carotid atherosclerosis is achieved by mediating PI3K/AKT/mTOR signaling pathway, regulating autophagy, promoting cell proliferation and inhibiting cell apoptosis. Obviously, our research confirms and expands the potential mechanism of BBR in improving carotid atherosclerosis.

It has been previously reported that hyperlipidemia acts importantly in carotid atherosclerosis development [41,42]. Recent studies have shown that LDL-C promotes plaque formation, stimulates the proliferation of vascular smooth muscle,

and is related to inflammation and lipid accumulation. In our study, the histological observation of the carotid artery showed that BBR reduced the formation and growth of carotid artery plaque and the proliferation of neointima in ApoE^{-/-} mice, and inhibited lipid accumulation. In addition, cell proliferation and apoptosis have been proven to be one of the key steps in a variety of human diseases, including carotid atherosclerosis. Therefore, promoting cell proliferation and inhibiting cell apoptosis may be an effective strategy to prevent and treat carotid atherosclerosis. Our study found that BBR promoted carotid artery cell proliferation and inhibited cell apoptosis, which may be the key role of BBR in anti-carotid atherosclerosis.

PI3K/AKT/mTOR signaling pathway is relative to various cell functions, including cell survival, differentiation, invasion, proliferation, apoptosis and autophagy [43–45]. Studies have confirmed that PI3K/AKT/mTOR is an important signaling pathway of growth factors or cytokines involved in the proliferation and migration

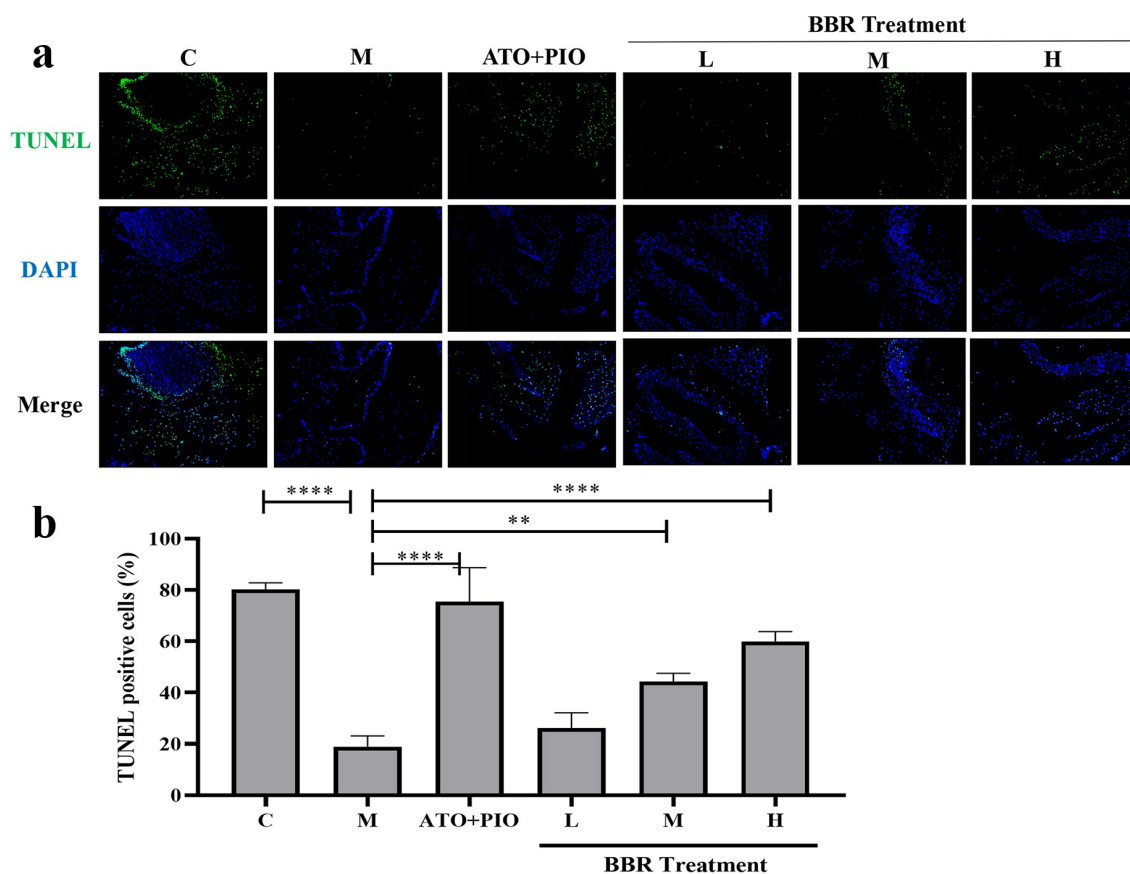


Figure 6. BBR inhibited cell apoptosis in ApoE^{-/-} mice carotid arteries. (a) TUNEL immunofluorescence staining representative image of carotid artery (magnification 100), the first row represented TUNEL staining of apoptotic cells (green), the second represented nuclear staining via DAPI (blue), and the third represented the merged images; (b) the proportion of TUNEL⁺ cells in the carotid arteries; the findings were presented as mean ± SD; * $P < 0.05$, **** $P < 0.0001$.

of vascular smooth muscle cells, and attenuates lipid accumulation, inflammation and apoptosis in ApoE^{-/-} mice by enhancing autophagy in atherosclerosis. While selective inhibition of mTOR plays a beneficial role in reducing the development of plaque lesions in ApoE^{-/-} mice [46–48]. PI3K is a substrate of tyrosine kinase receptor, and its activation leads to phosphorylation of AKT, which then promotes mTOR phosphorylation and activation to regulate cell function [49]. We found that BBR up-regulated p-PI3K and p-mTOR expressions but down-regulated p-AKT expression in a dose-dependent manner, confirming that BBR activates PI3K/AKT/mTOR signaling to improve atherosclerotic function. In addition, BBR also reduced autophagy activity markers Beclin-1 and P62 protein expression, confirming the effective effect of the mTOR-mediated negative control axis of autophagy [50].

5. Conclusions

The results of this study indicate that in ApoE^{-/-} mice receiving high-fat diet for 12 weeks, BBR can reduce serum lipid levels, antagonize carotid lipid accumulation, and improve intimal hyperplasia, which may be through regulating PI3K/AKT/mTOR signaling pathway, regulating autophagy, promoting cell proliferation and inhibiting cell apoptosis. In general, BBR is a promising anti-carotid atherosclerosis treatment strategy. In our future work, we can further study the treatment mechanism in order to provide a basis for clinical application and resource development and utilization.

Research highlights

1. BBR improves carotid atherosclerosis in ApoE^{-/-} mice.
2. BBR regulates autophagy, promotes cell proliferation and inhibits cell apoptosis.
3. BBR regulates the PI3K/AKT/mTOR signaling pathway.

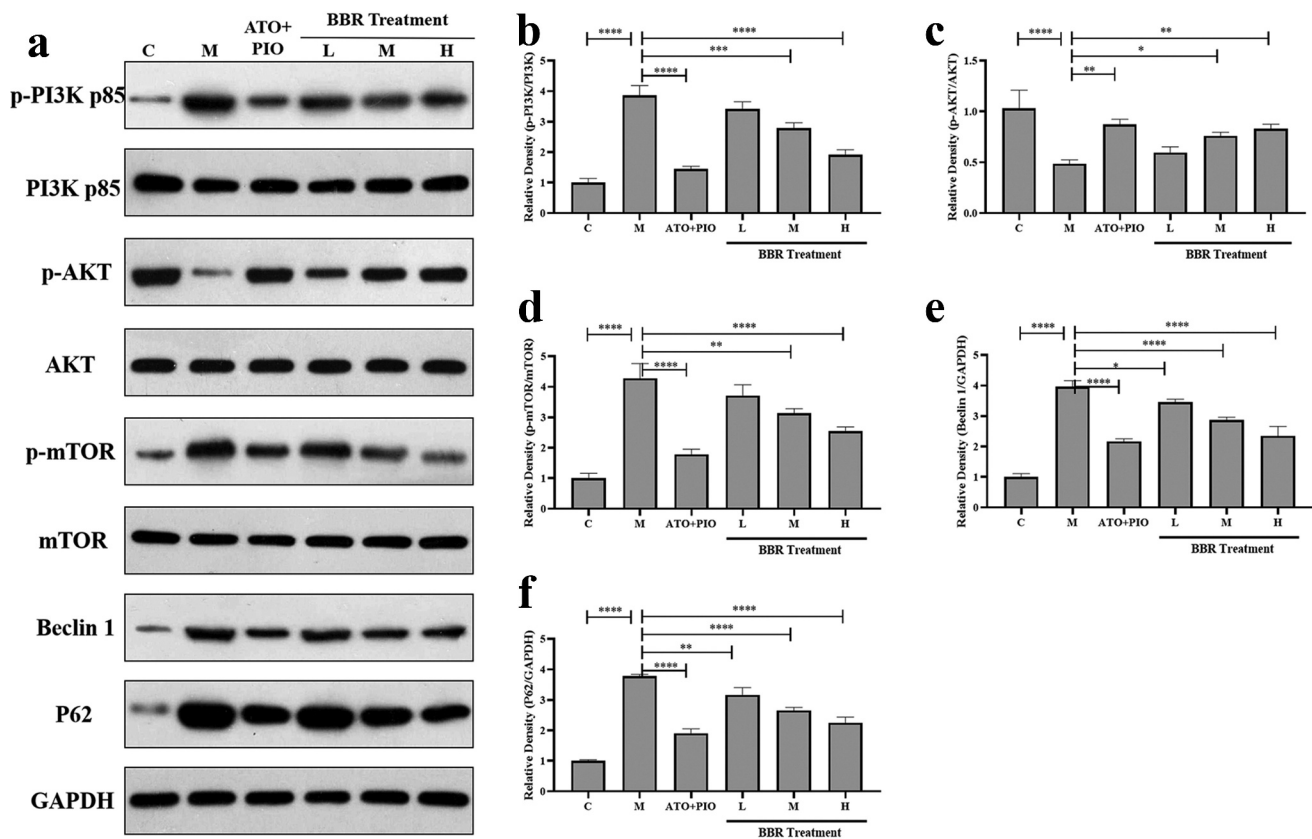


Figure 7. BBR regulated PI3K/AKT/mTOR signaling pathway in ApoE^{-/-} mice carotid arteries. (a) representative image of protein expression relative to PI3K/AKT/mTOR signaling pathway in carotid arteries; (b-f) p-PI3K/PI3K, (b) p-AKT/AKT, (c) p-mTOR/mTOR, (d) Beclin-1, (e-f) P62 protein expression levels in carotid arteries; the findings were presented as mean \pm SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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