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Berberine attenuates inflammation and oxidative stress and modulates lymphocyte E-NTPDase in acute hyperlipidemia

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Abstract

Hyperlipidemia is a common clinically encountered health condition worldwide that promotes the development and progression of cardiovascular diseases, including atherosclerosis. Berberine (BBR) is a natural product with acknowledged anti-inflammatory, antioxidant, and metabolic effects. This study evaluated the effect of BBR on lipid alterations, oxidative stress, and inflammatory response in rats with acute hyperlipidemia induced by poloxamer-407 (P-407). Rats were pre-treated with BBR (25 and 50 mg/kg) for 14 days and acute hyperlipidemia was induced by a single dose of P-407 (500 mg/kg). BBR ameliorated hypercholesterolemia, hypertriglyceridemia, and plasma lipoproteins in P-407-adminsitered rats. Plasma lipoprotein lipase (LPL) activity was decreased, and hepatic 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) activity was enhanced in hyperlipidemic rats. The expression of low-density lipoprotein receptor (LDL-R) and ATP-binding cassette transporter 1 (ABCA1) was downregulated in hyperlipidemic rats. BBR enhanced LPL activity, upregulated LDL-R and ABCA1, and suppressed HMGCR in P-407-administered rats. Pre-treatment with BBR ameliorated lipid peroxidation, nitric oxide (NO), pro-inflammatory mediators (IL-6, IL-1β, TNF-α, IFN-γ, IL-4 and IL-18) and enhanced antioxidants. In addition, BBR suppressed lymphocyte E-NTPDase and E-ADA as well as NO and TNF-α release by macrophages isolated from normal and hyperlipidemic rats. *In silico* investigations revealed the binding affinity of BBR towards LPL, HMGCR, LDL-R, PSK9, ABCA1, and E-NTPDase. In conclusion, BBR effectively prevented acute hyperlipidemia and its associated inflammatory responses by modulating LPL, cholesterolgenesis, cytokine release and lymphocyte E-NTPDase and E-ADA. Therefore, BBR is an effective and safe natural compound that might be employed as an adjuvant against hyperlipidemia and its associated inflammation.

Keywords: Dyslipidemia; Inflammation; Oxidative stress; Berberine; E-NTPDase.

1. Introduction

Dyslipidemia, a lipid disorder involving derangements in circulating triglycerides (TG) and/or cholesterol (CHO), is a common clinically encountered health condition worldwide (Berberich & Hegele, 2022). Dyslipidemia promotes the development and progression of cardiovascular diseases (CVDs), including atherosclerosis (Berberich & Hegele, 2022). CVDs represent the leading cause of mortality, and the number of patients has nearly doubled during the period from 1990 to 2019 to reach 523 million and the mortality rate reached 18.6 million deaths in 2019 (Roth et al., 2020). Sedentary life style, obesity, smoking and suboptimal diet are among the main risk factors for dyslipidemia and the prevalence is much higher among patients with coexisting CVDs (Su et al., 2022). Hypercholesterolemia is mainly implicated in the pathogenesis of atherosclerosis, a CVD that may lead to myocardial infarction (MI), strokes, ischemic heart disease (IHD) and other serious disorders (Berberich & Hegele, 2022). Plasma CHO originates from the diet but the primary source is synthesis within the liver where 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) acts as the rate-limiting enzyme (Sato & Takano, 1995). Similar to CHO, both hepatic synthesis and dietary sources contribute to plasma TG. Dietary fatty acids (FAs) are synthesized into TG in the liver via a multistep process (Alves-Bezerra & Cohen, 2017). Hepatic free CHO is esterified and packaged together with TG for transit in lipoproteins (Kang & Davis, 2000). Lipid metabolism is regulated by transcription factors controlling the genes involved in TG and CHO synthesis, including HMGCR and low-density lipoprotein (LDL) receptor (LDL-R) (Horton et al., 2002). In addition, the pathways of CHO efflux inhibit its excessive accumulation within the cells. These pathways are mediated by ATP binding cassette (ABC) subfamily A member 1 (ABCA1) and ABCG5/8 that maintain homeostasis through the reverse CHO transport (RCT) pathway and direct excretion of CHO in the bile, respectively (Duong et al., 2006; Yu et al., 2002).

Oxidative stress (OS) and systemic inflammation are linked to hyperlipidemia (Bagley et al., 2015; Furukawa et al., 2004; Yang et al., 2008). OS is an important factor implicated in the development and progression of CVDs and hyperlipidemia is associated with redox imbalance mediated via excess reactive oxygen species (ROS) and declined antioxidants (Abduh et al., 2023; Yang et al., 2008). Intracellular lipids accumulation increases ROS that contribute to chronic inflammatory responses in atherosclerosis and can negatively impact body organs (Chisolm & Steinberg, 2000; Furukawa et al., 2004). Mitochondrial dysfunction, oxidized-LDL, NADPH oxidase, and oxidatively modified glucose and proteins represent the common contributors to OS in dyslipidemia (Singh et al., 2017; Yang et al., 2008). Excess ROS depletes antioxidant defenses and activate nuclear factor (NF)-kB, resulting pro-inflammatory cytokines release that can further increase ROS and together provoke cell death. Pro-inflammatory cytokines have been shown to influence abnormalities in lipid metabolism in atherosclerosis, obesity, steatosis, and diabetes (Hong et al., 2022; Popko et al., 2010). Therefore, agents with anti-hyperlipidemic, antioxidant, and anti-inflammatory activities could effectively manage dyslipidemia and prevent its associated complications and disorders.

The isoquinoline alkaloid berberine (BBR) showed a wide range of pharmacological activities, including antioxidant and anti-inflammatory effects (Feng et al., 2019; Germoush & Mahmoud, 2014; Mahmoud et al., 2017). BBR is the main bioactive constituent of *Rhizoma coptidis* that has been used traditionally in the treatment of inflammatory and metabolic disorders (Feng et al., 2019). It showed protective effects against chemotherapy-induced liver injury (Germoush & Mahmoud, 2014; Mahmoud et al., 2017), and anticancer, antidiabetic, antimicrobial, neuroprotective and nephroprotective properties (Qin et al., 2020; Rauf et al., 2021). The beneficial pharmacological actions of BBR in the management and/or treatment of metabolic disorders and CVDs have been well-acknowledged (Feng et al., 2019). *In vitro*, pre-clinical and clinical investigations revealed the benefits of BBR against diabetes, atherosclerosis,

thrombosis, IHD, hypertension, stroke, arrhythmias, and insulin resistance (Feng et al., 2019; Yin et al., 2008). It exerted positive effects on carbohydrate and lipid metabolism in steatohepatitis *in vivo* (Zhao et al., 2017) and prevented lipolysis in adipocytes *in vitro* (Zhou et al., 2011). In a rat model of poloxamer-407 (P-407)-induced hyperlipidemia, BBR inhibited neuronal apoptosis and improved short-term memory (Kim et al., 2019). Despite the reported effects, the efficacy of BBR against acute hyperlipidemia and its associated inflammation is not fully understood. This study investigated the effect of BBR on hyperlipidemia, OS, inflammation, and lymphocyte ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-adenosine deaminase (E-ADA) activities in P-407-administered rats.

2. Materials and Methods

2.1. Experimental groups and samples

Thirty adult male Sprague-Dawley rats (180-200 g) were housed under standard conditions of temperature (23 ± 1°C) and humidity (50-60%) on a 12h dark/light cycle. The animal study protocol was approved by the Ethics Committee of Al-Azhar University (Approval no. AZ-AS/PHREC/48/23). The rats were divided into five groups (n = 6) as follows:

Group I: received 0.5% carboxymethyl cellulose (CMC).

Group II: received BBR (50 mg/kg) (Mahmoud et al., 2017) in 0.5% CMC for 14 days.

Group III: received 0.5% CMC for 14 days and a single intraperitoneal (i.p.) dose of P-407 (500 mg/kg) (Zanwar et al., 2014) at day 15.

Group IV: received BBR (25 mg/kg) (Mahmoud et al., 2017) for 14 days and P-407 at day 15.

Group V: received BBR (50 mg/kg) (Mahmoud et al., 2017) for 14 days and P-407 at day 15.

BBR and CMC were administered via oral gavage. Groups I and II received saline (i.p.) on day 15. Blood was collected before P-407 administration and at 12, 24 and 48 h for the

136 determination of TG and total cholesterol (TC). After 48 h, the rats were anesthetized, and
137 blood was collected via cardiac puncture on EDTA.
138 2.2. Determination of plasma lipids, LPL, and HMGCR
139 TG, TC and high-density lipoprotein (HDL)-C were assayed using reagent kits supplied by
140 Biosystems (Spain) following the manufacturer's instructions. vLDL and LDL were calculated
141 as following:
142 $$vLDL = Trilycerides/5$$
143 $$LDL = Total\ Cholesterol - (HDL + vLDL)$$
144 The activities of LPL and HMGCR were determined in the plasma and liver of rats using
145 colorimetric assay kits supplied by Solarbio (China) and Sigma (USA), respectively, according
146 to the manufacturers' instructions.
147 2.3. Determination of serum thiobarbituric acid reactive substances (TBARS), NO, reduced
148 glutathione (GSH) and antioxidant enzymes
149 TBARS and NO were assayed according to the methods of Ohkawa *et al.* (1979) and Green *et*
150 *al*. (1982), respectively. GSH levels (Ellman, 1959) and the activities of superoxide dismutase
151 (SOD) (Marklund, 1985), catalase (Aebi, 1984), and glutathione peroxidase (GPx) (Flohé &
152 Günzler, 1984) were assayed in plasma of rats following the mentioned methods.
153 2.4. Determination of cytokines
154 Serum IL-4, IL-6, IL-1β, IL-18, IFN-γ, and TNF-α were assayed using ELISA kits (Cusabio,
155 China), according to the provided instructions.
156 2.5. Isolation of lymphocytes and determination of E-NTPDase and E-ADA activities
157 Lymphocyte-rich mononuclear cells were separated from the blood collected on EDTA using
158 sterile endotoxin-free Ficoll-Histopaque (Sigma, USA) density-gradient centrifugation
159 (Böyum, 1968). As previously reported, the integrity of lymphocyte preparation was checked
160 using trypan blue exclusion (Strober, 2015). Briefly, cell suspension was mixed with trypan

blue (Sigma, USA) at 1:1 ratio and the mixture was incubated for 3 min at room temperature (RT) followed by checking under a binocular microscope. The lymphocyte preparation was treated with 1% Triton X-100 and its protein content was assayed using Bradford reagent (Bradford, 1976). The activity of NTPDase was determined in the isolated cells as reported by Leal *et al* (2005). The reaction mixture consisted of 20 µl of the cell suspension preincubated for 10 min at 37 °C and 200 µl of a mixture containing 120 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 60 mM glucose, and 50 mM Tris–HCl buffer (pH 8.0). ATP or ADP (final concentration 2 mM) was added to initiate the reaction which was stopped with 200 µL trichloroacetic acid (10%). The released inorganic phosphate (Pi) was determined using KH_2PO_4 as a standard and malachite green as a colorimetric reagent. The absorbance was measured at 630 nm (Chan et al., 1986). The activity of E-ADA activity was assayed by mixing 25 µL of the cell preparation with 21 mM adenosine and the mixture was incubated for 1 h at 37 °C. The reaction was stopped using sodium nitroprusside and hypochlorite solution. 75 µM of ammonium sulfate was used as a standard (Giusti & Galanti, 1984).

2.6. Isolation of peritoneal MΦ and assay of NO and TNF-α

The peritoneal cavity was lavaged with complete minimum essential medium containing 5 U/ml heparin. The cell suspension was centrifuged at 250xg for 10 min and washed twice with the medium. The cells were suspended in the culture medium containing 10% fetal bovine serum and cultured in 6-well plates at 37°C under 95% CO_2 for 4 h for attachment and non-adherent cells were removed (Yassad et al., 1997). The cells were treated with 1µg/ml lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, USA) and incubated for 24 h. In another experiment, peritoneal MΦ from normal rats were incubated with 1µg/ml LPS in the presence and absence of 1, 5, or 10 µM BBR for 24 h (Shin et al., 2016). After incubation, NO and TNF-α in the culture medium were assayed.

2.7. qRT-PCR

Changes in the expression levels of ABCA1, LDL-R, ABCG5, and ABCG8 were determined using qRT-PCR. RNA was extracted from the liver using Trizol reagent and samples showed OD260/OD280 value ≥ 1.8 were reverse transcribed using transcription kit. cDNA was amplified using SYBR Green Master Mix and the primers listed in Table 1. The date were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and normalized to β-actin.

2.8. Molecular docking (MD)

The binding affinity of BBR with LPL (PDB ID: 6OB0), HMGCR (PDB ID: 1HWI), LDL-R binding domain of PCSK9 (PDB ID: 3GCX), PCSK9 (PDB ID: 6U26), ABCA1 (PDB ID: 7TBW), and E-NTPDase (PDB ID: 4BQZ) was carried out as previously reported (Antar et al., 2022).

2.9. Statistical analysis

All data are expressed as mean ± SEM. The statistical differences were analyzed by one-way ANOVA followed by Tukey's test using GraphPad Prism 8. A $p < 0.05$ was considered significantly different.

3. Results

3.1. BBR ameliorated dyslipidemia oxidative stress in P-407-induced rats

Changes in TG, TC, LDL-C, vLDL-C and HDL-C in P-407 and/or BBR-treated rats are shown in Figure 1A-E. Injection of P-407 increased circulating TG and TC significantly at 12, 24 and 48 h as compared to the control group ($P<0.001$). Both doses of BBR ameliorated TG and TC in P-407-administered rats at all time points. The effect of BBR on TG and TC was dose-dependent at 12 and 24 h time points. P-407-administered rats showed remarkable elevation in plasma LDL-C and vLDL-C, whereas HDL-C decreased after 48 h. These alterations were reversed in BBR-treated groups.

3.2. BBR modulated LPL and HMGCR activities in P-407-induced rats

The activities of LPL (Fig. 2A) and HMGCR (Fig. 2B) were assayed in plasma and liver, respectively, and MD was employed to explore the affinity of BBR toward these enzymes. Dyslipidemia in P-407-administered rats was associated with decreased LPL activity and increased activity of HMGCR ($P<0.001$). BBR at two doses ameliorated LPL and HMGCR activities ($P<0.001$).

MD revealed that BBR forms a polar bond with Ser418 and hydrophobic interactions with Tyr121, Ile221, Lys265, His268, Phe415, Trp417, Trp420, and Trp421 residues of LPL with a binding energy of -9.6 kcal/mol (Fig. 2C and Table 2). BBR interacts with HMGCR with hydrophobic interaction at the residues Tyr517, Val522, Gly532, Met534, Tyr533, Ile536, Val538, Ala556, Ile762, Ala763, Pro813, and Gln814 and the binding energy is -7 kcal/mol (Fig. 2D and Table 2).

3.3. Effect of BBR on hepatic LDL-R, ABCA1, and ABCG5/8 in P-407-induced rats

P-407 administration downregulated liver LDL-R mRNA (Fig. 3A) significantly ($P<0.001$) while its effect on ABCG5 (Fig. 3B) and ABCG8 (Fig. 3D) was not significant. Like LDL-R, ABCA1 mRNA was downregulated in P-407-administered rats (Fig. 3D). Both doses of BBR upregulated ABCA1 and LDL-R and had no effect on ABCG5 and ABCG8 mRNA. *In silico* analysis revealed the affinity of BBR to bind with LDL-R binding domain of PCSK9 (Fig. 4A), PCSK9 (Fig. 4B), and ABCA1 (Fig. 4C) with binding energies -7.9, -7.9, and -8.9 kcal/mol, respectively (Table 2). BBR forms polar bonds with the residues Arg476 and Arg485 of LDL-R binding domain of PCSK9. It shows hydrostatic interactions with ABCA1, LDL-R binding domain of PCSK9, and PCSK9 (Table 2).

3.4. BBR attenuated oxidative stress in P-407-induced rats

Circulating levels of TBARS (Fig. 5A) and NO (Fig. 5B) were elevated whereas GSH (Fig. 5C), SOD (Fig. 5D), catalase (Fig. 5E), and GPx (Fig. 5F) declined in P-407-administered rats.

BBR at 25 and 50 mg/kg doses decreased TBARS and NO and enhanced antioxidants in P-407-administered rats.

3.5. BBR ameliorated cytokines in P-407-induced rats

The pro-inflammatory cytokines IL-6 (Fig. 6A), IL-1β (Fig. 6B), and TNF-α (Fig. 6C) were elevated in the blood of P-407-administered rats. Likewise, P-407-administered rats showed high levels of IFN-γ (Fig. 6D), IL-4 (Fig. 6E), and IL-18 (Fig. 6F). BBR effectively ameliorated all assayed cytokines, and its effect on IL-6 and IL-18 was dose-dependent.

3.6. BBR modulated lymphocyte E-NTPDase and E-ADA activities in P-407-induced rats

To assess the effect of BBR on lymphocyte E-NTPDase and E-ADA, we measured the activities of E-NTPDase and E-ADA and investigated its binding with NTPDase using MD. The hydrolysis of both ATP (Fig. 7A) and ADP (Fig. 7B) representing E-NTPDase activity were elevated, and E-ADA activity (Fig. 7C) was increased in lymphocytes from P-407-administered rats. BBR significantly decreased the activities of E-NTPDase and E-ADA. *In silico* analysis revealed the ability of BBR to interact with E-NTPDase through hydrophobic interactions at the residues Asp45, Ser49, His50, Phe54, Asp201, Gly203, Gly204, Arg245 Ser346, Ala347, Tyr350, Arg394, Ala433, Val434, and Gly435, and showed -7.8 kcal/mol binding energy (Fig. 7D & Table 2).

3.7. BBR ameliorated NO and TNF-α release from LPS-induced macrophages

Peritoneal macrophages (MΦ) from P-407-adminsitered rats showed significant increase in NO (Fig. 8A) and TNF-α (Fig. 8B) upon exposure to LPS for 24 h. LPS failed to increase NO and TNF-α in MΦ from BBR-treated rats. Incubation of MΦ from normal rats with LPS and/or different concentrations of BBR revealed the ability of BBR to decrease LPS-induced NO (Fig. 8C) and TNF-α (Fig. 8D).

4. Discussion

Dyslipidemia is a major contributor to the development and progression of serious CVDs, including atherosclerosis (Berberich & Hegele, 2022). This study investigated the protective effect of BBR on acute dyslipidemia and its associated redox imbalance and inflammatory responses using *in vivo, in vitro* and *in silico* approaches. The results revealed that BBR is effective against acute hyperlipidemia and inflammation through its ability to modulate cholesterologenesis-related factors, cytokine release, and lymphocyte E-NTPDase and E-ADA. The non-ionic copolymer surfactant P-407 is commonly used to induce dyslipidemia in rodents and its administration resulted in elevated TG and TC in line with previous reports (Chaudhary & Brocks, 2013; Leon et al., 2006; J. Park et al., 2016; Yeom et al., 2018). It is non-toxic and its hyperlipidemic effect is related to promoting cholesterolgenesis and suppressing LPL activity and TG hydrolysis (Chaudhary & Brocks, 2013; Johnston & Palmer, 1993; Leon et al., 2006). The rats that received P-407 exhibited hypercholesterolemia and hypertriglyceridemia at 12, 24 and 48 h after receiving P-407. These effects were associated with elevated LDL-C and vLDL-C and decreased HDL-C. HDL-C plays a role in decreasing circulating CHO and its decline pinpoints an atherogenic lipid profile (O. Stein & Stein, 1999), and P-407 has been previously reported to induce early stages of atherosclerosis (Korolenko et al., 2016). Along with hyperlipidemia, plasma LPL activity was decreased and liver HMGCR was enhanced, demonstrating a decrease in TG hydrolysis and increased *de novo* cholesterologenesis, respectively. These effects represent the main mechanism of P-407-induced hyperlipidemia.

BBR effectively ameliorated TG, CHO, LDL-C and LDL-C and increased HDL-C, findings supported by previous studies showing the anti-hyperlipidemic activity of BBR. BBR decreased lipids in rat models of neuronal injury (Kim et al., 2019) and steatosis (Zhao et al., 2017), and diabetic mice (Wei et al., 2016). Studies have demonstrated several mechanisms that contribute to the positive impact of BBR on lipid metabolism. The anti-

hypercholesterolemia efficacy of BBR is associated with suppression of intestinal absorption, micellarization and uptake of CHO by enterocytes (Wang et al., 2014). In Caco-2 cells, BBR downregulated acetyl-CoA acetyltransferase 2 (ACAT2) and decreased permeability, resulting in inhibited CHO esterification (Wang et al., 2014). BBR suppressed hydrolase activity in bile and increased intestinal taurocholic acid levels. Subsequently, intestinal FXR pathway is upregulated leading to downregulated CD36 and hepatic uptake of long-chain FAs (Sun et al., 2017). The current study provided information supporting the anti-hyperlipidemic mechanism of BBR by showing its ability to upregulate LPL and suppress HMGCR, leading to increased TG hydrolysis and decreased hepatic CHO synthesis. The biochemical findings were supported by *in silico* MD that revealed the binding affinity of BBR toward LPL and HMGCR. P-407-induced hypertriglyceridemia in rodents is mainly attributed to LPL and hepatic lipase inhibition and reduced rate of TG hydrolysis (Johnston & Palmer, 1993; Wasan et al., 2003). LPL hydrolyses chylomicrons that contain approx. 90% TG and TG within vLDL, the main lipoprotein synthesized and secreted by the liver and the liberated fatty acids (FAs) are taken by peripheral tissues (Brahm & Hegele, 2015). HMGCR is the rate-limiting enzyme in hepatic CHO synthesis and its inhibition is an effective mechanism to treat hypercholesterolemia (Sato & Takano, 1995). Therefore, inhibition of HMGCR and activation of LPL are involved in the beneficial role of BBR against hypercholesterolemia and hypertriglyceridemia.

Next, we investigated changes in LDL-R, ABCA1, and ABCG5/8 and carried out *in silico* MD analysis to further understand the beneficial effect of BBR against acute hyperlipidemia. The results showed downregulation of LDL-R and ABCA1 in the liver of hyperlipidemic rats whereas ABCG1 and ABCG5 were not affected as we previously reported (Abduh et al., 2023). In support of our current and previous studies, P-407-induced mice exhibited a trend downregulation of liver ABCA1 (Leon et al., 2006). BBR upregulated both LDL-R and ABCA1 in dyslipidemic rat liver, effects that mediated, at least in part, LDL-C and CHO

homeostasis. LDL-R is a membrane glycoprotein that functions in the binding and internalization of LDL-C and is essential in maintaining CHO homeostasis. The endocytosis of LDL, mediated via LDL-R, reduces the expression of HMGCR and upregulate ACAT resulting in suppressed CHO biosynthesis and decreased free CHO (Go & Mani, 2012). The post-transcription by PCSK9 and the intracellular sterol levels are regulators of LDL-R transcription (Lambert et al., 2009). PSK9 can bind to and promote the degradation of LDL-R via two routes. Nascent PCSK9 binds to and directs LDL-R to the lysosomes for degradation (Poirier et al., 2009), and secreted PCSK9 can bind to LDL-R at the cell surface and prevents its endocytic recycling following internalization (Zhang et al., 2008). Therefore, inhibition of PCSK9 binding to LDL-R prevents its lysosomal degradation and results in lowered plasma LDL-C. Accordingly, anti-PCSK9 monoclonal antibodies lowered plasma LDL-C through blocking the PCSK9-LDL-R binding interface (Stein & Swergold, 2013).

Interestingly, the *in silico* findings of the current study revealed the affinity of BBR towards the LDL-R binding domain of PCSK9 as well as PCSK9 itself. These findings supported the upregulated LDL-R expression and the lowered LDL-C in BBR-treated rats. A Previous study has demonstrated that BBR can upregulate LDL-R expression in hepatoma cells *in vitro* by stabilizing its mRNA through a mechanism that is not related to sterol regulatory element binding proteins (SREBPs) or HMGCoA (Kong et al., 2004). Berberrubine, the main metabolite of BBR, exerted a hypolipidemic effect by upregulating LDL-R in HepG2 cells *in vitro* (Cao et al., 2019). Besides upregulation of LDL-R, BBR upregulated ABCA1, an ABC subfamily protein member, with an essential role in RCT and CHO homeostasis (Duong et al., 2006). Moreover, the *in silico* study revealed the binding affinity mediated via hydrophobic interactions between BBR and ABCA1. In RCT, ABCA1 exports excess cellular CHO and phosphatidylcholine to the circulating lipid-free ApoA-I, resulting in the generation of HDL-C (Duong et al., 2006). Hepatic ABCA1 is responsible for 70% of the biogenesis of HDL as

shown by murine tissue-specific knockout models (Brunham et al., 2006). Furthermore, ABCG5 and ABCG8 which are involved in hepatobiliary and trans-intestinal CHO secretion (Yu et al., 2002) were not affected by treatment with P-407 and/or BBR. These findings were in line with our recent study (Abduh et al., 2023) as well as the study of Leon *et al* (2006). In the same context, Wang *et al* (2010) have demonstrated that the BBR-induced lowering of blood CHO is mechanistically independent of the expression of ABCG5/8. Given that BBR upregulated LDL-R and ABCA1 and didn't affect ABCG5/8, its anti-hypercholesterolemia activity is therefore mediated via suppressed cholesterologenesis and increased HDL-C generation via CHO and phospholipid efflux.

Oxidative stress (OS) is linked to dyslipidemia and a positive association between redox imbalance and hypercholesterolemia/hyperlipidemia has been acknowledged (Singh et al., 2017; Yang et al., 2008). In P-407-treated rats in this study, OS has been marked by elevated TBARS and NO and decreased antioxidants as we previously reported (Abduh et al., 2023). OS is an imbalance between ROS levels and antioxidant defenses and together with hyperlipidemia is implicated in atherosclerosis and other CVDs (Abduh et al., 2023; Yang et al., 2008). In diseases linked to dyslipidemia such as atherosclerosis, OS is implicated in vascular damage, foam cell formation, and oxidation of LDL that activate the endothelium to recruit monocytes and T cells resulting in inflammatory and immune responses (Pirillo et al., 2013). Hyperlipidemia is a key contributor to the development of chronic inflammation caused by disrupted leukocyte activity and cytokine regulation (van Diepen et al., 2013). Extracellular adenine nucleotides and nucleosides play a centra role in inflammatory reactions where high extracellular ATP activate lymphocytes to release pro-inflammatory cytokines (Burnstock, 2017). E-NTPDase (CD39) is expressed on the surface of immune cells and hydrolyzes ATP into ADP then AMP which is then hydrolyzed by E-5′-nucleotidase (CD73). The produced adenosine is deaminated by E-ADA which is fundamental for T lymphocytes proliferation and

differentiation as well as monocytes maturation, resulting in the development of immune and inflammatory responses (Burnstock, 2017). Hyperlipidemic rats in this study exhibited an elevation in plasma IL-6, IL-1β, TNF-α, IL-4, INF-γ, and IL-18. The elevation in cytokines was associated with enhanced activities of E-NTPDase and E-ADA in lymphocytes. The elevated cytokines and activated E-NTPDase and E-ADA in hyperlipidemic rats coincided with previous studies attributed these effects to increased TG, CHO, and LDL-C (Braun et al., 2018; Klafke et al., 2016; Ruchel et al., 2021). INF-γ is a pro-atherogenic cytokine that contributes to inflammatory activities and promotes the adhesion of leukocytes to the endothelium, and studies on atherosclerosis supported the evidence that IL-4 is pro-atherogenic (M. L. Leon & Zuckerman, 2005; Walch et al., 2006). Both cytokines are increased in P-407-induced hyperlipidemia in rodent models (Braun et al., 2018; Ruchel et al., 2021). P-407-induced dyslipidemia has been associated with upregulated TNF-α, IL-6, and IL-1β, not only in the blood as reported by Saja et al (2018), but also in different tissues, including hippocampus (S.-S. Park et al., 2021). Moreover, IL-18 was elevated in dyslipidemic rats in the current study. IL-18 is a potent pro-inflammatory cytokine associated with the formation and progression of atherosclerotic plaque (Mallat et al., 2001). These findings pinpointed the role of dyslipidemia in promoting the release of pro-inflammatory cytokines. Additionally, isolated MΦ from P-407-adminsitered rats showed significantly enhanced release of NO and TNF-α upon LPS challenge, supporting the elevated plasma levels of pro-inflammatory cytokines.

BBR effectively attenuated OS and inflammation in P-407-admisniteerd rats as shown by the decreased TBARS, NO, and pro-inflammatory cytokines and enhanced antioxidants. These effects were attributed to the ameliorative effect of BBR on hyperlipidemia and agreed with previous studies revealed its anti-inflammatory and antioxidant activities (Song et al., 2020). BBR reduced oxLDL-mediated ROS release, upregulated adhesion molecules and leukocyte adhesion to endothelial cells (Hsieh et al., 2007; Huang et al., 2013). It suppressed ROS and

pro-inflammatory responses in LPS-challenged MΦ by modulating key signaling pathways (Jeong et al., 2009). The anti-inflammatory effect of BBR in oxLDL- and LPS-stimulated MΦ was evidenced by the suppressed IL-1β, IL-6, and other inflammatory mediators (Jeong et al., 2009). The anti-inflammatory and antioxidant activities of BBR are mediated via different mechanisms in different tissues and diseases (reviewed in (Feng et al., 2019; Song et al., 2020)). Our findings supported the anti-inflammatory activity of BBR be showing its ability to suppress the release of TNF-α by LPS-stimulated MΦ from both normal and dyslipidemic rats *in vitro*. Moreover, this study introduced new information that the anti-inflammatory mechanism of BBR involves the modulation of lymphocyte E-NTPDase and E-ADA. The reported suppressive effect of BBR on the activity of these enzymes was supported by *in silico* findings that revealed the binding affinity of BBR towards E-NTPDase. The lack of data showing the changes in protein expression of ABCA1 and LDL-R could be considered a limitation of this study, but the changes in mRNA of these proteins were in line with the biochemical findings and MD added further support. Although P-407-induced hyperlipidemia is one of the well-established models to evaluate the efficacy of therapeutics for hyperlipidemia, studies on diet-induced hyperlipidemia models are needed to demonstrate the mechanism of action of BBR.

5. Conclusion

This study demonstrated the protective effect of BBR against acute hyperlipidemia and its associated OS and inflammatory response. BBR ameliorated hypercholesterolemia and hypertriglyceridemia by suppressing CHO biosynthesis and stimulating TG hydrolysis through the modulation of LPL, HMGCR, LDL-R and ABCA1. OS marked by elevated TBARS and declined antioxidants, and altered cytokine secretion and the activity E-NTPDase and E-ADA were observed in rats with hyperlipidemia. Pre-treatment with BBR attenuated OS, enhanced antioxidants, suppressed cytokine secretion and the activities of lymphocyte E-NTPDase and

E-ADA. In addition, BBR attenuated LPS-stimulated NO and TNF-α secretion from macrophages. The modulatory effect of BBR on LPL, HMGCR, LDL-R, PSK9, ABCA1, and E-NTPDase was supported by *in silico* findings showed its binding affinity towards these proteins. Overall, the effective anti-hyperlipidemic efficacy of BBR is mediated through amelioration of plasma lipids, cholesterologenesis, TG hydrolysis and its modulatory effects on inflammatory responses.

Author Contributions

Conceptualization, A.M.M.; methodology, R.S.A., M.S.A., A.F.A., A.B., E.M.K., E.H.M.H., C.L., and A.M.M.; software, A.M.M. and E.M.K; validation, A.M.M., R.S.A., and C.L.; formal analysis, A.M.M., R.S.A., and E.M.K.; investigation, R.S.A., M.S.A., A.B., E.M.K., and A.M.M.; resources, R.S.A., M.S.A., A.F.A., and A.B.; data curation, R.S.A., M.S.A., A.B., E.M.K., and A.M.M.; writing—original draft preparation, R.S.A., M.S.A., A.B., C.L., and A.M.M.; writing—review and editing, A.M.M.; visualization, A.M.M.; supervision, A.M.M.; project administration, R.S.A. and A.M.M.; funding acquisition, R.S.A. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

All authors declare no conflict of interests in relation to the manuscript.

Availability of data and materials

The manuscript contains all data supporting the reported results.

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Tables:

Table 1. Primers used for qRT-PCR.

Gene	Genbank accession number	Sequence (5'-3')	Amplicon size (bp)
Abca1	NM_178095.3	F: GCAGCGACCATGAAAGTGAC R: GAGGCGGTCATCAATCTCGT	185
Abcg5	NM_053754.2	F: GGGAAGTGTTTGTGAACGGC R: GTGTATCTCAGCGTCTCCCG	121
Abcg8	NM_130414.2	F: TTCTGATGACGTCTGGCACC R: TTGCTGTAGCGAGACAAGG	97
Ldlr	NM_175762.3	F: CATTTTCAGTGCCAACCGCC R: TGCCTCACACCAGTTTACCC	127
Actb	NM_031144.3	F: AGGAGTACGATGAGTCCGGC R: CGCAGCTCAGTAACAGTCCG	71

685 Table 2. Binding interactions of BBR with LPL, HMGCR, LDLR binding domain of PCSK9,

686 PCSK9, ABCA1, and NTPDase2.

	kcal/mol	Polar bonds	Hydrophobic interactions
LPL	-9.6	Ser418	Tyr121, Ile221, Lys265, His268, Phe415, Trp417, Trp420 and Trp421
HMGCR	-7.0		Tyr517, Val522, Gly532, Met534, Tyr533, Ile536, Val538, Ala556, Ile762, Ala763, Pro813and Gln814
LDLR binding domain of PCSK9	-7.9	Arg476, and Arg458	Pro331, Arg357, Arg412, Val460, Trp461, Ala463, Ile474, Cys477 and Ala478
PCSK9	-7.9		Pro331, Arg357, Val359, Asp360, Arg458, Thr459, Val460 and Trp461
ABCA1	-8.9		Trp458, Phe466, Phe273, Trp278, Tyr482, Ala487 and Thr491
NTPDase2	-7.8		Asp45, Ser49, His50, Phe54, Asp201, Gly203, Gly204, Arg245 Ser346, Ala347, Tyr350, Arg394, Ala433, Val434 and Gly435

687

688

Figure legends:

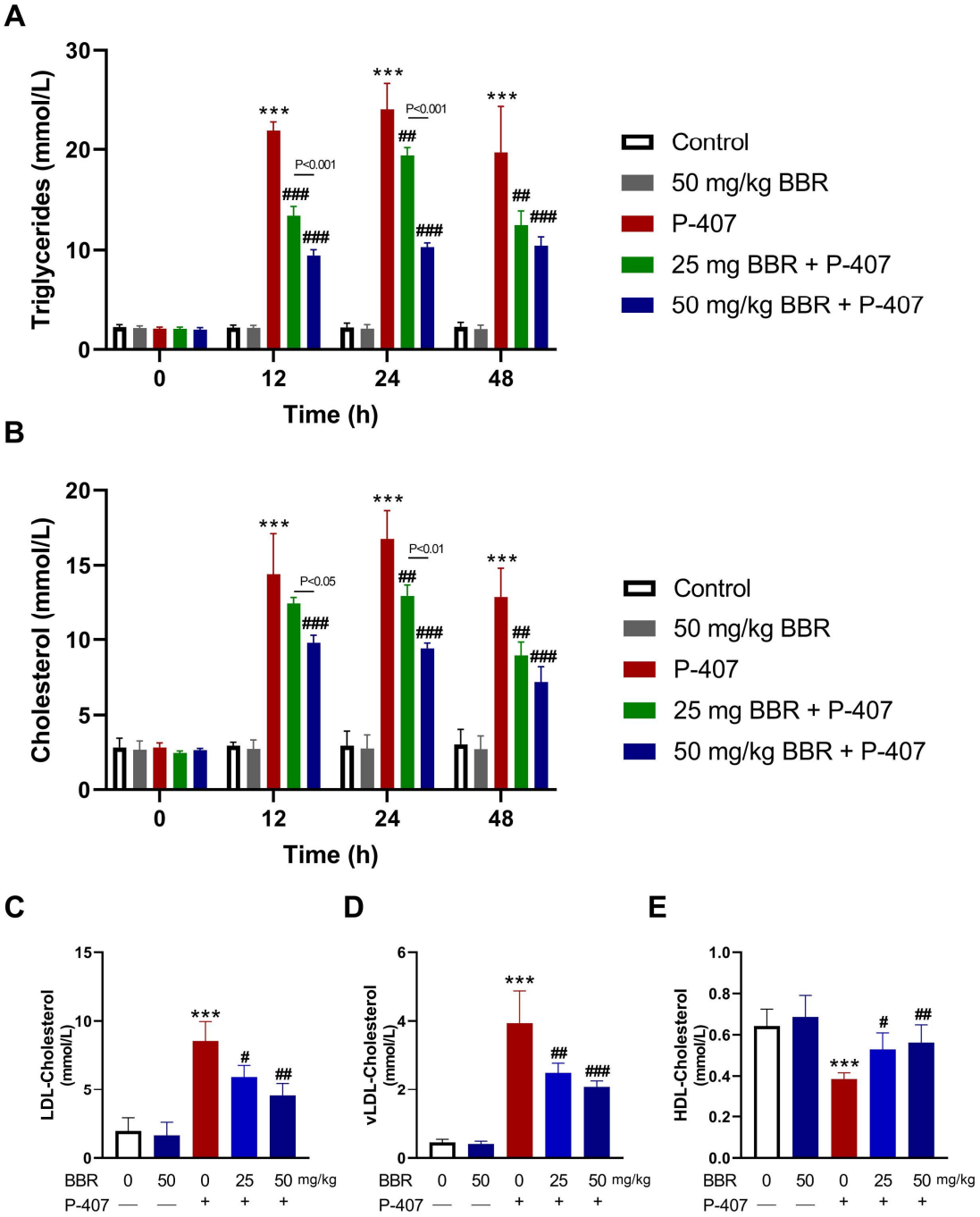
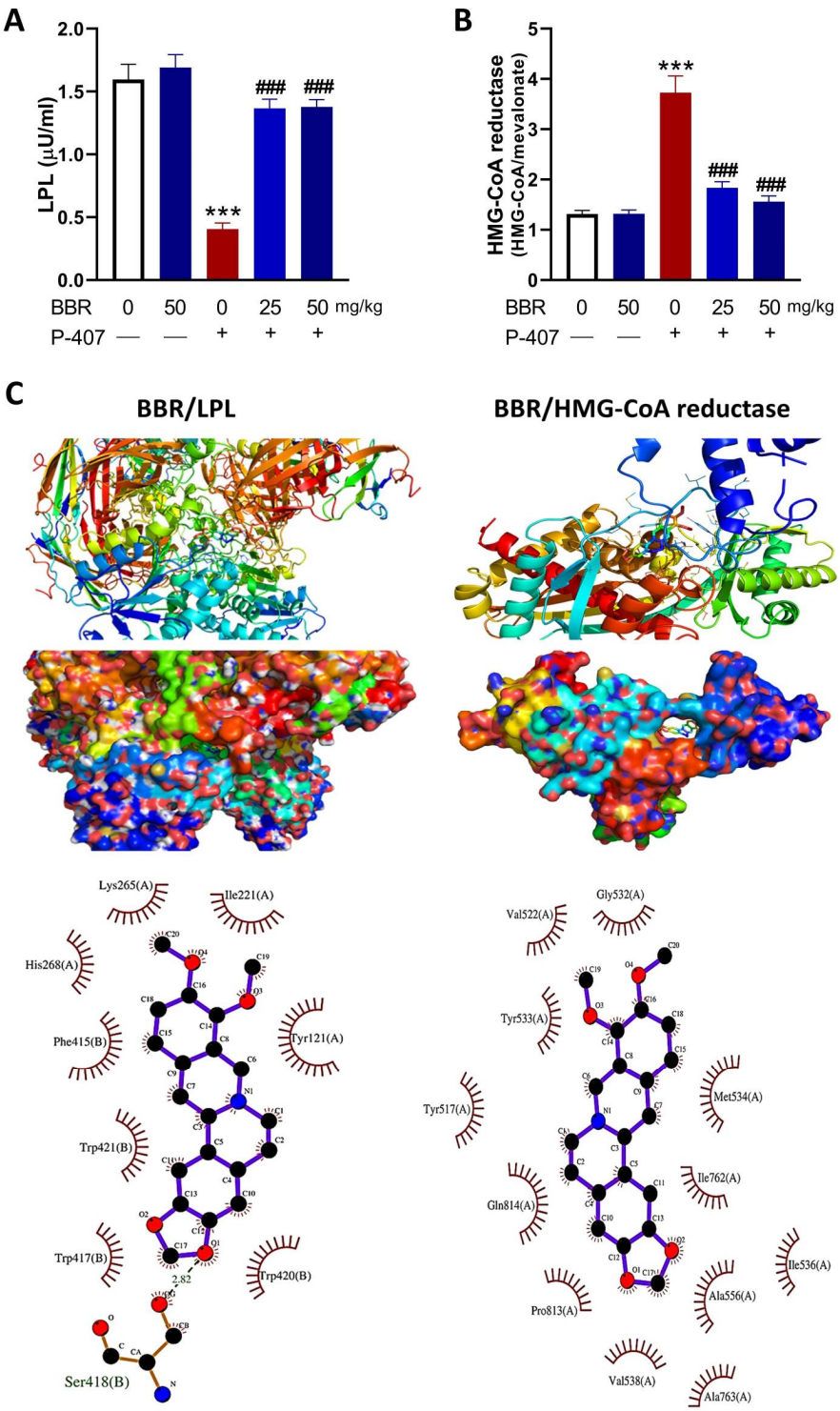


Fig. 1. BBR ameliorated TG (A) and CHO (B) levels at 12, 24 and 48 h, and LDL-C (C), vLDL-C (D) and HDL-C (E) at 48 h in P-407-administered rats. Data are Mean ± SEM, (*n* = 6). ***P<0.001 vs Control. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs P-407.



694

695 Fig. 2. BBR ameliorated plasma LPL (A) and liver HMGCR (B) activities in P-407-
696 administered rats. Data are Mean ± SEM, (*n* = 6). ***P<0.001 vs Control and $^{###}$P<0.001 vs P-
697 407. (C) Binding interactions of BBR with LPL and HMGCR.

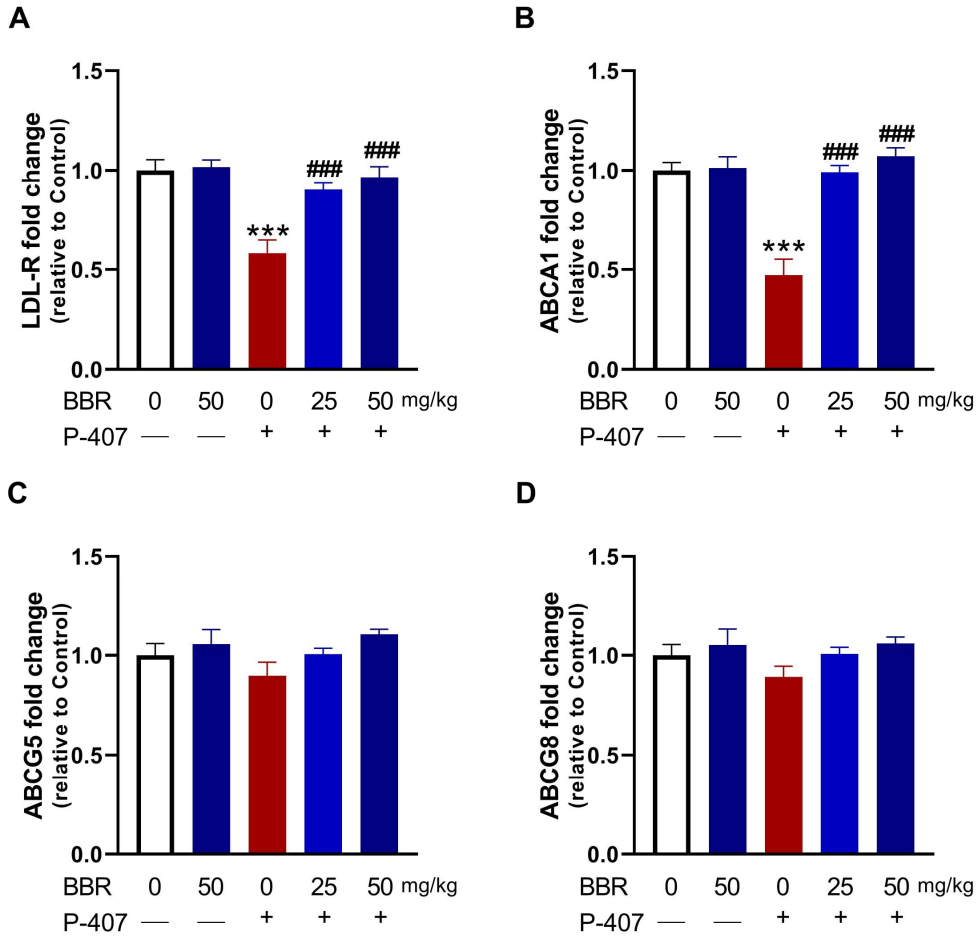


Fig. 3. Effect of BBR on LDL-R (A), ABCA1 (B), ABCG5 (C), and ABCG5 (D) mRNA in liver of P-407-administered rats. Data are Mean ± SEM, (n = 6). ***P<0.001 vs Control and $^{###}$P<0.001 vs P-407.

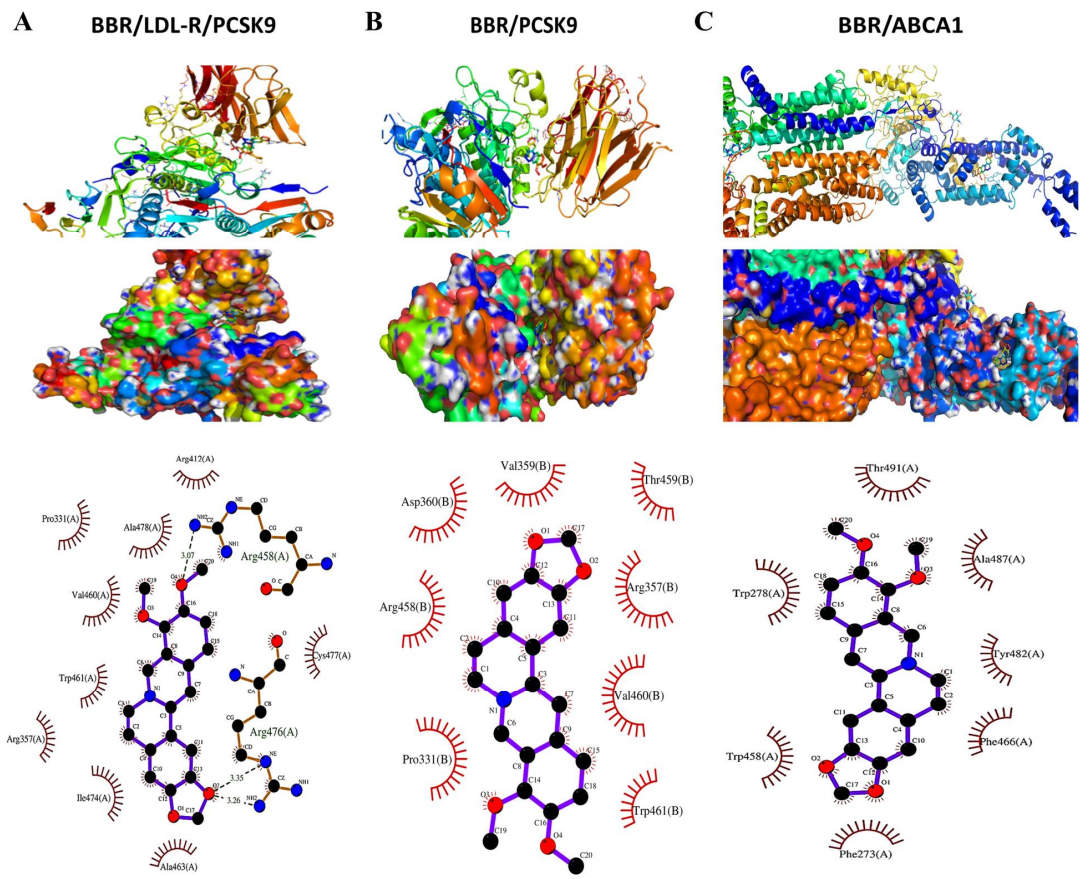
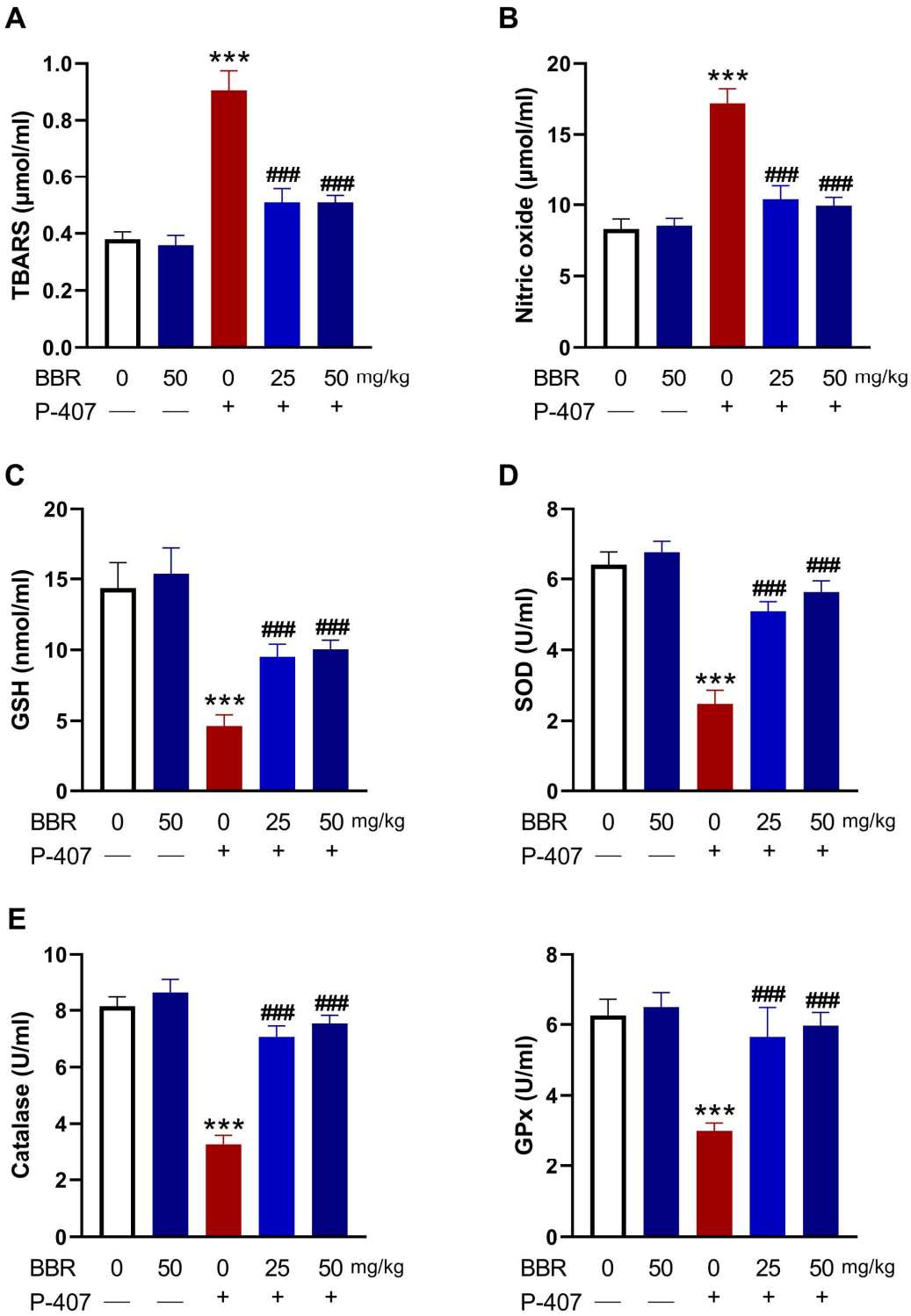
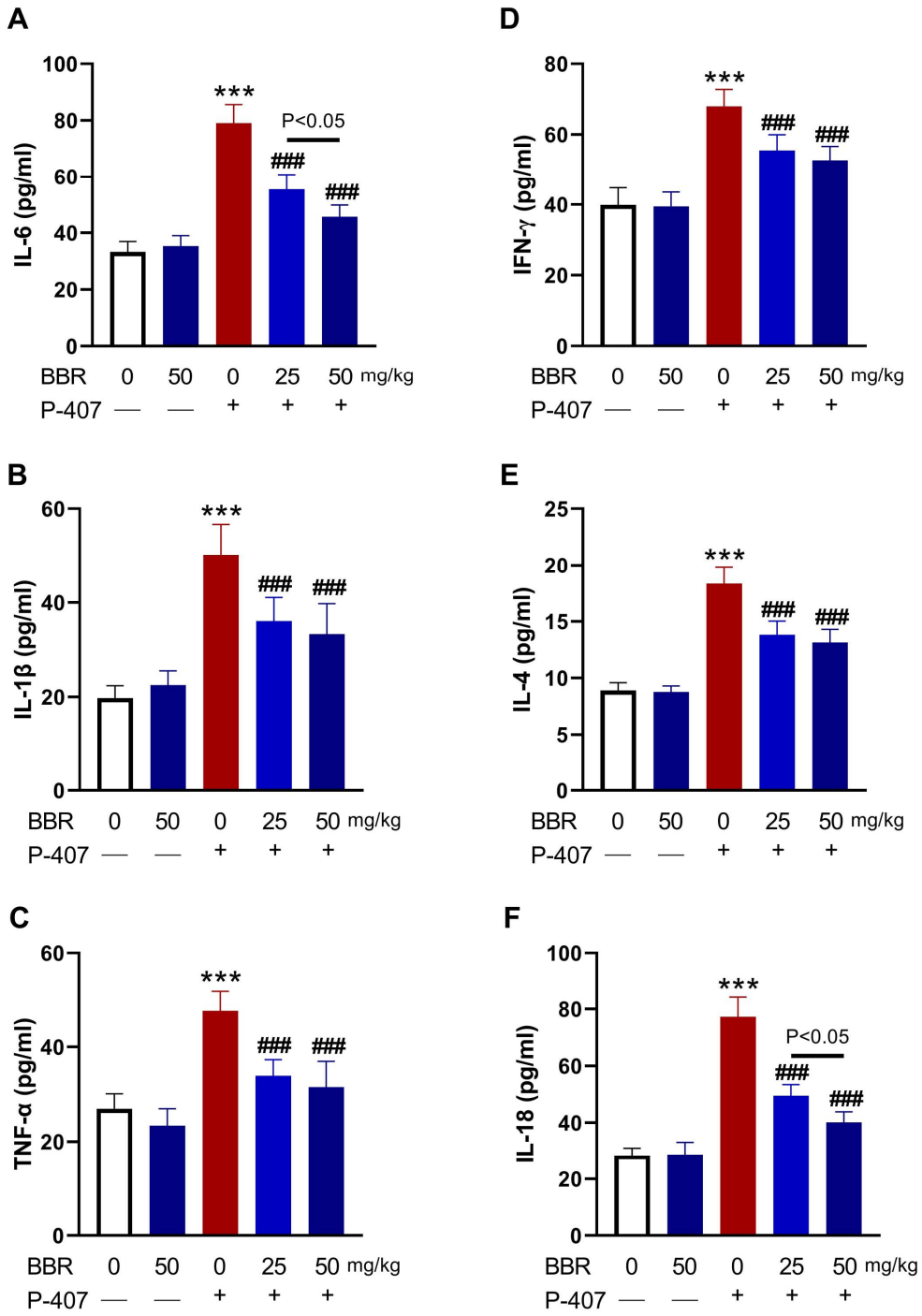


Fig. 4. Binding interactions of BBR with (A) LDLR binding domain of PCSK9, (B) PCSK9, and (C) ABCA1.



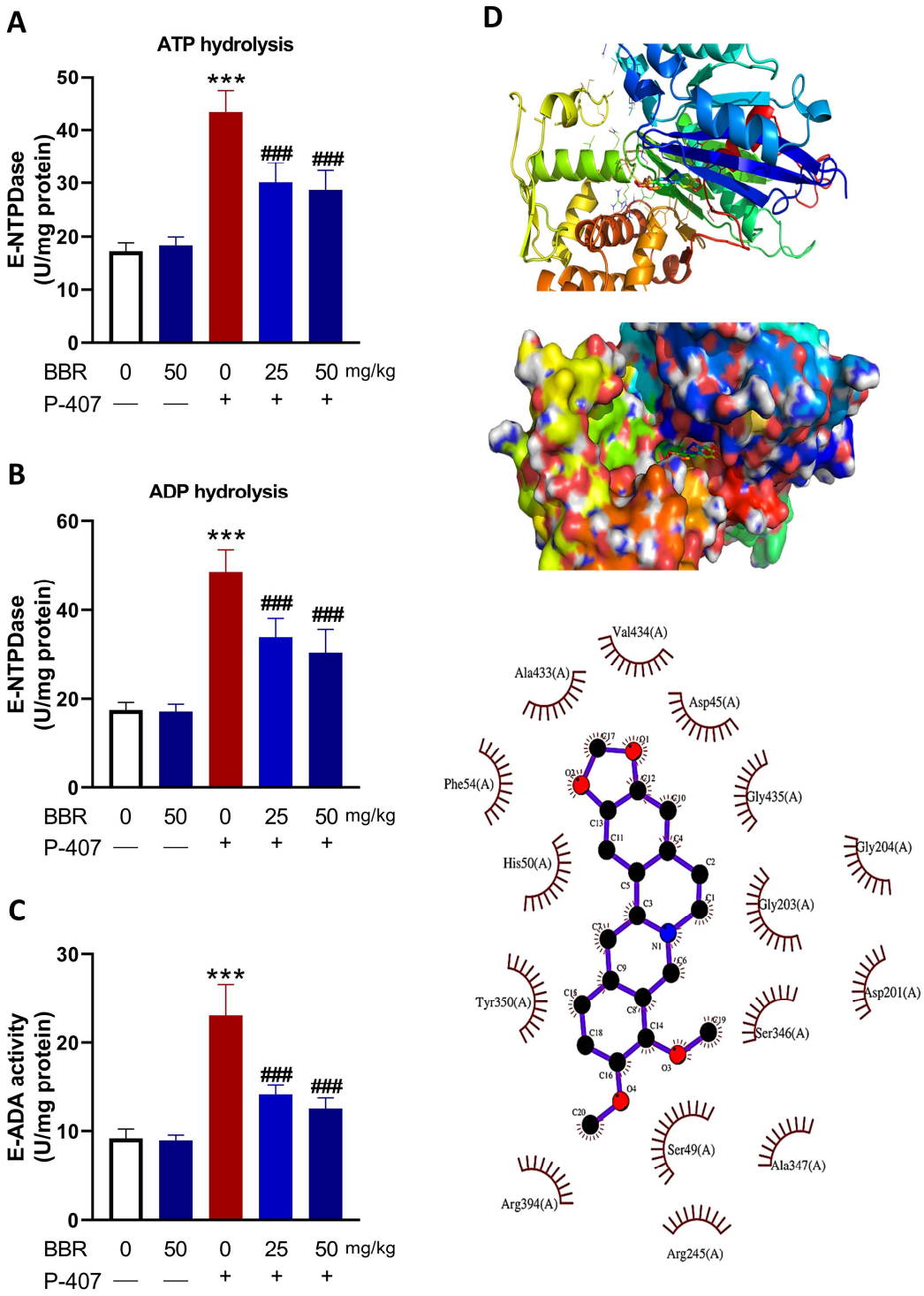
705

706 Fig. 5. BBR attenuated oxidative stress in hyperlipidemic rats. BBR decreased TBARS (A) and
707 NO (B) levels, and boosted GSH (C), SOD (D), CAT (E) and GPx (F) in P-407-administered
708 rats. Data are Mean ± SEM, (n = 6). ***P<0.001 vs Control and $^{###}$P<0.001 vs P-407.



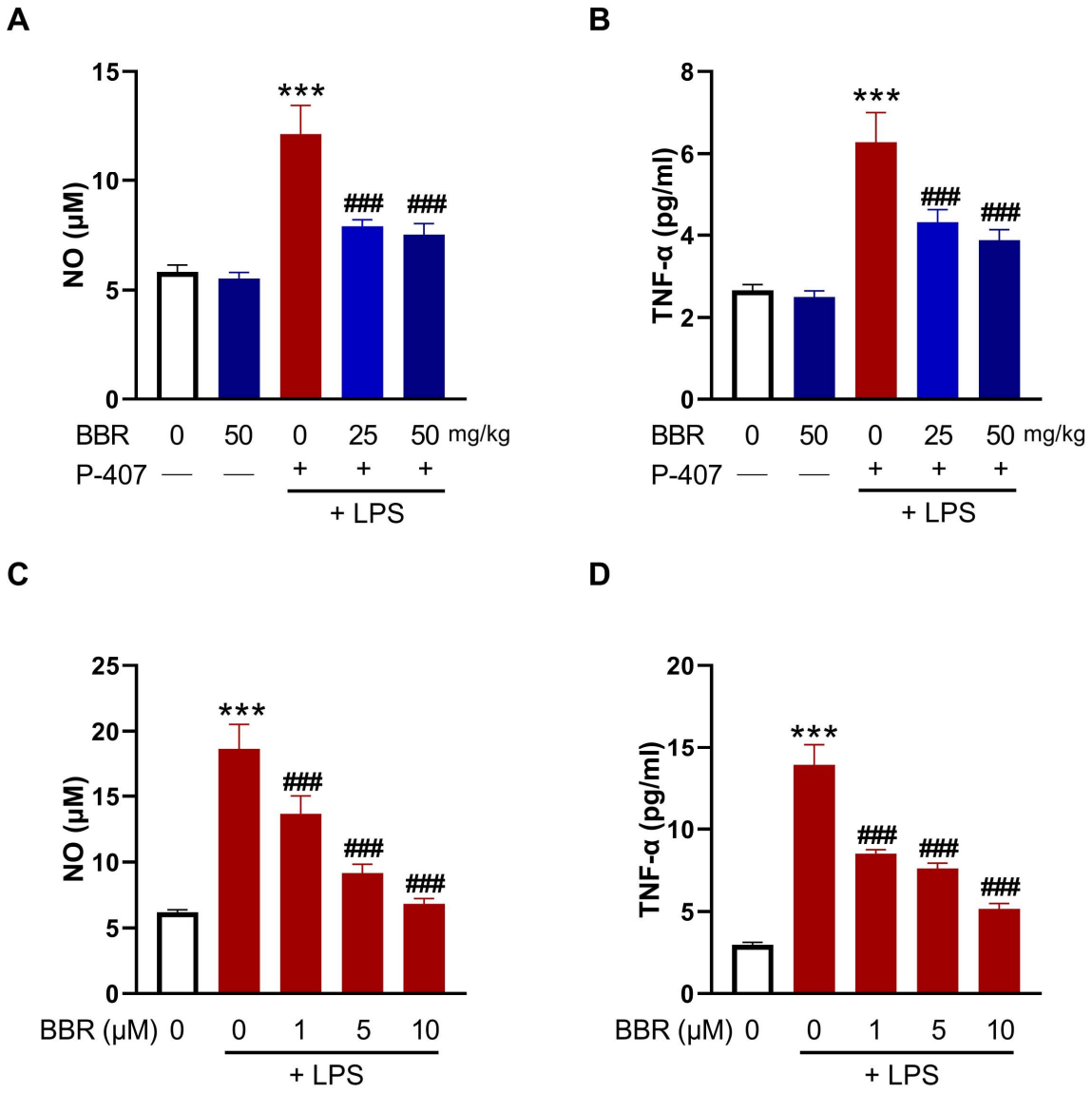
709

710 Fig. 6. BBR mitigated inflammation in hyperlipidemic rats. BBR decreased IL-6 (A), IL-1β
711 (B), TNF-α (C), IFN-γ (D), IL-4 (E) and IL-18 (F) in P-407-administered rats. Data are Mean
712 ± SEM, (n = 6). ***P<0.001 vs Control and $^{###}$P<0.001 vs P-407.



713

714 Fig. 7. BBR ameliorated E-NTPDase (A,B) and E-ADA (B) activities in lymphocytes of P-
715 407-administered rats. Data are Mean ± SEM, (n = 6). ***P<0.001 vs Control and $^{###}$P<0.001
716 vs P-407. Binding interactions of BBR with E-NTPDase.



717

718 Fig. 8. BBR decreased NO and TNF-α release from LPS-challenged macrophages from
719 dyslipidemic (A,B) and normal rats (C,D). Data are Mean ± SEM, (*n* = 6). (A,B) ***P<0.001
720 vs Control and ###P<0.001 vs P-407. (C,D) Data are Mean ± SEM, (*n* = 6). (A,B) ***P<0.001
721 vs Control and ###P<0.001 vs LPS.