Original Paper

ROS Promote Ox-LDL-Induced Platelet Activation by Up-Regulating Autophagy Through the Inhibition of the PI3K/AKT/mTOR Pathway

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Abstract

Background/Aims: Oxidized low-density lipoprotein (oxLDL) promotes unregulated platelet activation in patients with dyslipidemic disorders. Although oxLDL stimulates activating signaling, researchers have not clearly determined how these events drive accelerated thrombosis. Here, we describe the mechanism by which ROS regulate autophagy during ox-LDL-induced platelet activation by modulating the PI3K/AKT/mTOR signaling pathway. ***Methods:*** For *in vitro* experiments, ox-LDL, the ROS scavenger N-acetylcysteine (NAC), the mTOR inhibitor rapamycin and the autophagy inhibitor 3-MA were used alone or in combination with other compounds to treat platelets. Then, platelet aggregation was evaluated on an aggregometer and platelet adhesion was measured under shear stress. The levels of a platelet activation marker (CD62p) were measured by flow cytometry, reactive oxygen species (ROS) levels were then quantified by measuring DCFH-DA fluorescence intensity via flow cytometry. Nitric oxide (NO) and superoxide ($O_2^{\cdot-}$) levels were determined by the nitric acid deoxidize enzyme method and lucigenin-enhanced chemiluminescence (CL), respectively. Transmission electron microscopy was used to observe the autophagosome formation, immunofluorescence staining was employed to detect LC3 expression and western blotting was used to measure the levels of PI3K/AKT/mTOR pathway- and autophagy-related proteins. ***Results:*** Ox-LDL-induced platelets showed a significant increase in platelet aggregation and adhesion, CD62p expression, ROS level and $O_2^{\cdot-}$ content, with an elevated LC3II/LC3I ratio and Beclin1 expression, but a dramatic reduction in the levels of p62 and pathway-related proteins (all $P < 0.05$). However, platelet activation and autophagy were aggravated by the Rapamycin treatment, and decreased following treatment with NAC, 3-MA, or NAC and 3-MA, together with increased activity of the PI3K/AKT/mTOR pathway. Additionally, decreased platelet activation and autophagy were observed in platelets treated with NAC and Rapamycin or Rapamycin

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and 3-MA compared with platelets treated with Rapamycin alone, suggesting that both NAC and 3-MA reversed the effects of Rapamycin. **_Conclusion:_** Inhibition of ROS production may reduce autophagy to suppress ox-LDL-induced platelet activation by activating PI3K/AKT/mTOR pathway.

Introduction

Atherosclerosis (AS), a leading cause of death in developed countries, may result in myocardial ischemia, myocardial infarction, stoke and other cardio-/cerebrovascular diseases, seriously affecting human health and patients' quality of life [1, 2]. AS is a complex disease associated with various genetic and environmental factors, and is mainly triggered by oxidative stress and the inflammatory response [3]. Reactive oxygen species (ROS) generated by oxidative stress, which are well-known important initiating factors required for AS development, oxidize low-density lipoprotein (LDL) to form oxidized-LDL (ox-LDL) [4, 5]. Moreover, ox-LDL, a main factor contributing to endothelial dysfunction, induces the autophagy of vascular endothelial cells, thereby leading to AS, as reported by several researchers [6, 7]. For example, according to Peng N *et al.*, ox-LDL inhibits mammalian target of rapamycin (mTOR) and induces autophagy and apoptosis in vascular endothelial cells (VECs), which play very critical roles in cardiovascular homeostasis [8]. More importantly, ox-LDL has been implicated in the early phase of atherosclerosis by recruiting inflammatory cells in the sub-endothelium and eliciting changes in endothelial function that favor the thrombotic process [7]. Ox-LDL also stimulates platelet activation to induce the expression of pro-inflammatory cytokines, such as adhesion molecules and thrombogenic tissue factor, which play crucial roles in pro-atherogenic mechanisms [9-11]. Thus, the protection of endothelial cells against ox-LDL-induced platelet activation and injury is a very important strategy to prevent and treat AS-related diseases.

Autophagy, a highly conserved pathway mediated by lysosomes that degrades cytosolic components in eukaryotic cells [12], is essential for maintaining cell survival and protein homeostasis during normal development, and its dysfunction is involved in many human diseases [13, 14]. Recently, the role of autophagy in platelets has gradually been recognized; for example, some unidentified forms of platelet dysfunction might be caused by abnormal autophagy, resulting in severe hemostasis and thrombosis defects, as illustrated by Ouseph *et al.* [15]. Additionally, Feng *et al.* also reported the induction of autophagy in platelets by cell starvation or rapamycin (mTOR inhibitor) treatment [16]. However, the specific mechanism of autophagy in platelets has not yet been elucidated. On the other hand, the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, a classic autophagy pathway [17], is well established as a regulator of a wide range of cellular processes that mediate cell survival and death, and this pathway is inhibited by malnutrition, hypoxia, external pressure or other stress conditions to suppress cell proliferation and enhance cell autophagy, eventually leading to cell death [16, 18]. Moreover, suppression of PI3K and Akt signaling inhibits mTOR phosphorylation at Ser2448, thereby inducing autophagy and increasing the levels of autophagy-related proteins [18, 19]. Recently, ROS was shown to play a regulatory role in autophagy in various cells by modulating the PI3K/AKT/mTOR pathway. For instance, in the study by Jiang *et al.*, hydroxysafor yellow-mediated sonodynamic therapy not only repressed the PI3K/AKT/mTOR signaling pathway to trigger an autophagy response but also suppressed ROS-induced inflammation in THP-1 macrophages [20]. Furthermore, the important influence of the ROS/PI3K/AKT/mTOR signaling pathway was also highlighted by Han *et al.* in photodynamic therapy-induced autophagy in THP-1 and peritoneal macrophage-derived foam cells [21]. Therefore, we aim to explore whether ROS regulate platelet autophagy by modulating PI3K/AKT/mTOR signaling pathway. Notably, N-acetylcysteine (NAC), a well-known ROS scavenger that is widely utilized to identify and test ROS inducers and to decrease the intracellular ROS levels, has shown therapeutic potential as an antiplatelet agent due to its inhibitory effect on platelet

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hyperaggregability in patients with type-2 diabetes [22]. Meanwhile, 3-methyladenine (3-MA), a classic autophagy inhibitor, blocks autophagosome formation by inhibiting type III phosphatidylinositol 3-kinases in platelets [23], and the specific mTOR inhibitor, rapamycin, the most frequently used trigger of autophagy, has been used to inhibit mammalian target of rapamycin (mTOR) function in platelets [24]. Thus, in this study, ox-LDL, NAC, Rapamycin, or 3-MA was administered alone or in combination with other compounds to platelets to improve our understanding of the potential molecular effects of these compounds on ROS-induced autophagy and platelet activation.

Materials and Methods

Ethics statement

All subjects provided written informed consent to participate in this study, which was approved by the Ethics Committee of Clinical Laboratory in our hospital. All procedures performed in this study adhered to the guidelines of the Declaration of Helsinki [25].

Preparation of ox-LDL and washed platelets

Whole blood was collected from healthy human volunteers at The Third Xiangya Hospital of Central South University. We prepared ox-LDL using a previously reported method [26]. The blood was mixed with 0.109 M sodium citrate in 1:9 ratio as an anti-freeze. One hundred fifty grams of the mixture were centrifuged for 20 min to obtain platelet-rich plasma (PRP). We only removed the upper two-thirds of the PRP fraction to prevent contamination with other cell types. Based on the results from a previous study, the gel was filtered using a Sepharose 4B column in HEPES-Tyrode's buffer supplemented with 0.5% human serum albumin [3]. Then, the gel or PGI2 (1 μM; Sigma-Aldrich, USA) was used to centrifuge (3000 × g, 2 min) platelets.

Grouping and treatment of washed platelets

The washed platelets were divided into 8 groups in equal volumes: the Control group (platelets treated with the vehicle, dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA)); ox-LDL group (platelet suspension treated with 50 μg/ml ox-LDL for 5 min) [27]; N-acetylcysteine (NAC) group; Rapamycin group; 3-methyladenine (3-MA) group; NAC + Rapamycin group; NAC + 3-MA group; and Rapamycin + 3-MA group. The suspended platelets in each group were pre-treated with a 1-mM dose of the ROS scavenger N-acetylcysteine (NAC) (Sigma-Aldrich, USA) [28], a 0.2-μM dose of the mTOR inhibitor Rapamycin (Sigma-Aldrich, USA) [16] or a 1-mM dose of the autophagy inhibitor 3-MA (Sigma-Aldrich, USA) [16] for 30 min, and then 50 μg/ml ox-LDL was added to platelets and incubated for 10 min.

Scanning electron microscopy

Platelets (4×10^7/ml) were fixed with 4% paraformaldehyde to examine the platelet morphology in solution. Subsequent procedures (including dehydration and critical-point drying) were performed using standard methods, and the coated specimens were examined under a JEOL 6400 scanning electron microscope (JEOL, Tokyo, Japan) with automated image digitization and archiving.

Determination of platelet aggregation

A total of 4 μM adenosine diphosphate (ADP) was added to trigger platelet aggregation. Then, an aggregometer (Lumi-Aggregometer, Canada) was used to measure changes in turbidity to determine the degree of platelet aggregation at 37 °C with stirring at 900 rpm. The quantification of aggregation is presented as the ratio of the treated group to the control.

Platelet adhesion under shear stress

After platelets were diluted with PBS at a 1:10 ratio, platelets were perfused over collagen IV-coated channel slides (iBiDi, Martinsried, Germany) for 2 min and monitored by capturing real-time phase contrast images using a microscopy. We set the flow rate to 5 ml/min to obtain a shear rate of approximately 1000 s^{-1}. Next, slides were washed with PBS for 5 min and 5 isolated fields along the centerline of the channel

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were observed under the microscope and analyzed using ImagePro Software (Version 5.0, United Kingdom, 2003) and ImageJ (Version 1.45s, USA, 2012). Platelet adhesion was calculated as the ratio of the treated group to collagen (fold changes).

Flow cytometry

Platelets were incubated and detected using flow cytometry. Two Falco sample tubes were labeled A and B: A was the control tube for the same type and B was the determination tube. Then, 20 μl of CD61 Per CP antibody (BD Biosciences, USA) were separately added to tubes A and B. We added 20 μl of the same species of Ig G control antibody (BD Biosciences, USA) to tube A and 20 μl of CD62p PE (BD Biosciences, USA) to tube B. Next, 5 μl of platelets and 200 μl of Tyrode's buffer were added to each tube and mixed with the aforementioned liquids in the bottom of the tube. After a 20 min incubation at room temperature in the dark, a FACSCalibur flow cytometer (BD Biosciences, USA) equipped with Cell Quest software was used to collect and analyze the data, and platelets labeled with CD61 were used to determine the percentage of CD62p-positive platelets.

Detection of ROS production

Intracellular ROS measurements were performed by measuring the fluorescence intensity of 2, 7-dichlorofluorescein (DCF). The platelets in each group was incubated with the fluorescent probe 2, 7-dichlorodihydrofluorescindiacetate (DCFH-DA) (1:1 000) at 37°C for 30 min in the dark, and then washed three times. The fluorescence signals produced were analyzed using a FACSVese flow cytometer (BD, Germany). Fluorescence was measured with a fluorescence microscope (Olympus IX81, Japan) at 488 nm (excitation) and 525 nm (emission) wavelengths, and photos were captured.

Determination of the Nitric oxide (NO) content and superoxide anion (O_2^-) level in platelets

The NO detection kit (nitric acid deoxidize enzyme method) was provided by Nanjing Jiancheng Bioengineering Institutes. Platelets were centrifuged at 3500 g for 15 min, and aliquots of 100 μl of supernatant were mixed with an equal volume of Griess reagent (containing 1% sulphanilamide and 0.1% naphthlethylenediamine in 5% phosphoric acid). Platelets were first incubated at room temperature for 10 min and then their absorbance (550 nm) was measured using a micro-plate reader (Titertek multiskan MCC). Solutions of sodium nitrite were used as standards. The nitrite concentrations in platelets were calculated by comparing their OD values with standard solutions. The results are presented as NO release corrected for platelet counts in picomoles per 10^8 platelets. The specific procedures were performed according to the manufacturer's instructions. Lucigenin-enhanced chemiluminescence (CL) was used to determine the platelet $O2^-$ level. Platelets were first equilibrated in Krebs solution at 37°C and then equilibrated in Krebs-HEPES buffer at 37°C for 30 min. Platelets were then placed in 0.5 ml of Krebs-HEPES buffer containing 250 mmol/L lucigenin at 37°C in a Picolite luminometer (Packard), and luminescence counts were measured every minute for 15 min. Background counts were determined for 15 min before adding the platelets; these values were then subtracted from the luminescence counts, and the results are presented as O_2^- concentrations in picomoles per 10^8 platelets.

Observation of autophagosome formation by transmission electron microscopy

The suspended platelets in each group were pelleted twice for fixation with a 2.5% glutaraldehyde solution and a 1% osmium tetroxide solution. Finally, the platelets were dehydrated with an ethanol solution, embedded and cut into ultrathin sections with a thickness of 100 nm. After staining with a saturated uranyl acetate solution and a lead acetate solution, the platelets were observed and photographed under a transmission electron microscope.

Detection of LC3 expression by immunofluorescence staining

After grouping, platelets were fixed with 4% paraformaldehyde for 20 min, washed three times and sealed with sealing solution. After the sealing solution was removed, the platelets were incubated with a primary antibody against LC3 (1:50) at 4°C overnight, followed by washes with PBS. Next, a fluorescent phycoerythrin (PE)-labeled secondary antibody was added to platelets and incubated for 1 h at room temperature in the dark; platelets were then washed with PBS for 3 times. After adding 40-6-diamidino-

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2-phenylindole (DAPI) and incubating the platelets with the dye at room temperature for 10 min, platelets were sealed with anti-fluorescence quenching liquid, observed and photographed under a fluorescence microscope.

Western blotting

According to instructions of the BCA (bicinchoninic acid) protein assay (Beyotime Biotechnology Co., Shanghai, China), cell proteins were extracted to determine the protein concentration. Loading buffer was added to the extracted proteins and boiled at 95°C for 10 min. Thirty micrograms of protein sample were loaded into each well of a 10% polyacrylamide gel (Wuhan Boster Biological Technology Ltd., Wuhan, Hubei, China) and separated by electrophoresis. Then, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and incubated with 5% bovine serum albumin (BSA) at room temperature for 1 h. Next, the PVDF membrane was incubated with primary antibodies against, p-PI3K, PI3K, phosphorylated (p)-AKT, AKT, p-mTOR, mTOR, LC3, Beclin1, p62 and GADPH (Cell Signaling Technology, Inc.) at 1:1000 dilutions overnight at 4°C. After three washes with Tris-buffered saline containing Tween (TBST) for 5 min each, the signals on the PVDF membrane were developed by incubating the membrane with chemiluminescence reagents; GADPH was used as the internal reference. The gray value of each target band was analyzed using ImageJ software.

Statistical analysis

All data were analyzed with SPSS 21.0 software (SPSS Inc., Chicago, IL, USA), and values are reported as means ± standard deviation ($\overline{x}$ ± s). All experiments were repeated three times to obtain the mean value and standard deviation. The data from multiple groups were compared using one-way ANOVA, whereas pairwise comparisons between two groups were performed using t-tests. A P value of < 0.05 was considered statistically significant.

Results

Platelet aggregation and adhesion in each group

Scanning electron micrographs revealed that ox-LDL induced the formation of platelet microaggregates (Fig. 1A). Platelets in the Rapamycin group formed large, stable aggregates. However, NAC, 3-MA and NAC + 3-MA clearly prevented aggregate formation. Compared with the Control group, the ox-LDL and Rapamycin groups exhibited an evident increase in platelet aggregation and adhesion (all $P < 0.05$). Moreover, platelet aggregation and adhesion were substantially increased in the Rapamycin group (both $P < 0.05$), but were dramatically decreased in the NAC, 3-MA and NAC + 3-MA groups compared to the ox-LDL group (all $P < 0.05$). Significant differences in platelet aggregation and adhesion were not observed among the Rapamycin + 3-MA, NAC + Rapamycin and ox-LDL groups (all $P > 0.05$). Meanwhile, the Rapamycin + 3-MA and NAC + Rapamycin groups showed significant reductions in platelet aggregation and adhesion compared with the Rapamycin group (all $P < 0.05$, Fig. 1B and C). Based on these findings, ox-LDL-treated platelets aggregated, whereas the ROS scavenger NAC and the autophagy inhibitor 3-MA reversed ox-LDL-induced platelet aggregation.

Expression of platelet activation markers in each group

As shown in Fig. 2, a greater percentage of platelets in the ox-LDL group and rapamycin group expressed CD62p than the Control group and ox-LDL group, respectively (both $P < 0.05$). In contrast, a critical decrease in the percentage of platelets expressing CD62p was observed in the NAC group, 3-MA group and NAC + 3-MA group (all $P < 0.05$). No significant differences were observed among the ox-LDL, Rapamycin + 3-MA, and NAC + Rapamycin groups (all $P > 0.05$). However, the percentages of CD62p-positive platelets were noticeably decreased in the Rapamycin + 3-MA group and NAC + Rapamycin group compared with the Rapamycin group (both $P < 0.05$).

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Fig. 1. Comparison of platelet aggregation and adhesion in each group. Notes: A, Scanning electron micrographs reveal that ox-LDL induces the formation of platelet microaggregates (scale bar: 1 μm). B, Platelet aggregation was monitored in a Lumi-Aggregometer, and the quantification of platelet aggregation is shown. C, Quantification of platelet adhesion to a collagen-coated surface under high shear rates (1000 s^{-1}). All data are presented as means ± standard deviations. Different letters refer to significant differences, while the same letters do not indicate significant differences among groups.
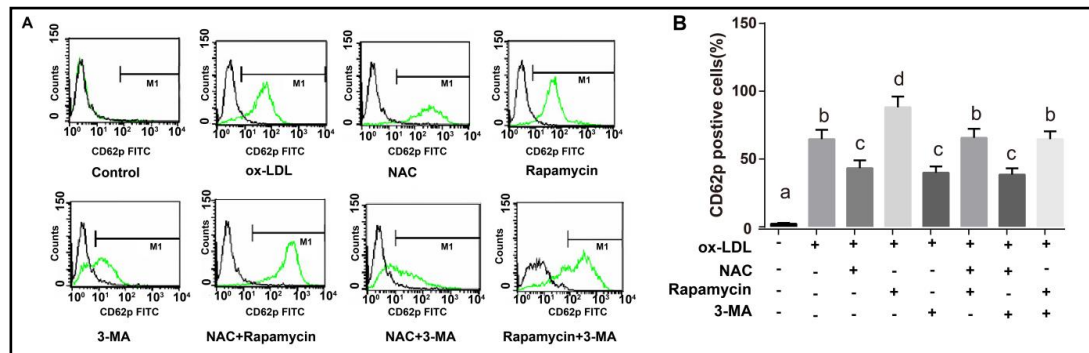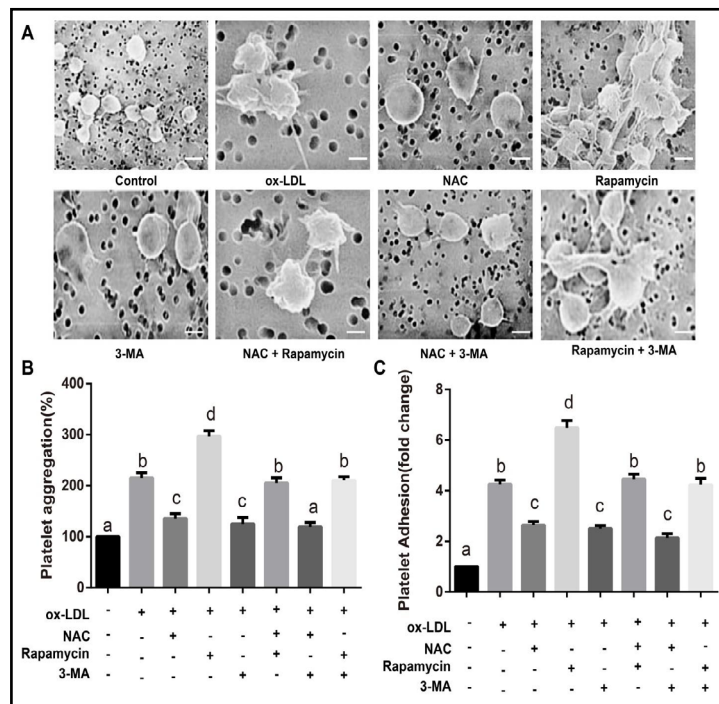

Fig. 2. Comparison of the expression of a platelet activation marker (CD62p) in each group. Notes: A, Platelets in the ox-LDL, NAC, Rapamycin, 3-MA, NAC + Rapamycin, NAC + 3-MA and Rapamycin + 3-MA groups were stimulated with 50 μg/ml ox-LDL; platelets were collected, stained with CD62p monoclonal antibodies, and subjected to flow cytometry. B, Histogram showing the percentages of CD62p-positive platelets in each group. All data are presented as means ± standard deviations. Different letters refer to significant differences, while the same letters do not indicate significant differences among groups.

Comparison of ROS contents in each group of platelets

Fluorescence microscopy was used to observe the fluorescence intensity in each group. Compared with Control group, the fluorescence intensity was increased and the ROS level was substantially increased in the ox-LDL group (both $P < 0.05$). Compared to the ox-LDL group, the ROS level was significantly increased in the Rapamycin group ($P < 0.05$), but was decreased in the NAC group, 3-MA group and NAC + 3-MA group (all $P < 0.05$). Both the Rapamycin + 3-MA group and NAC + Rapamycin group exhibited a noticeable reduction in the ROS level compared with the Rapamycin group ($P < 0.05$). No significant changes were observed between the rapamycin + 3-MA, NAC + Rapamycin, and ox-LDL groups (all $P > 0.05$, Fig. 3).
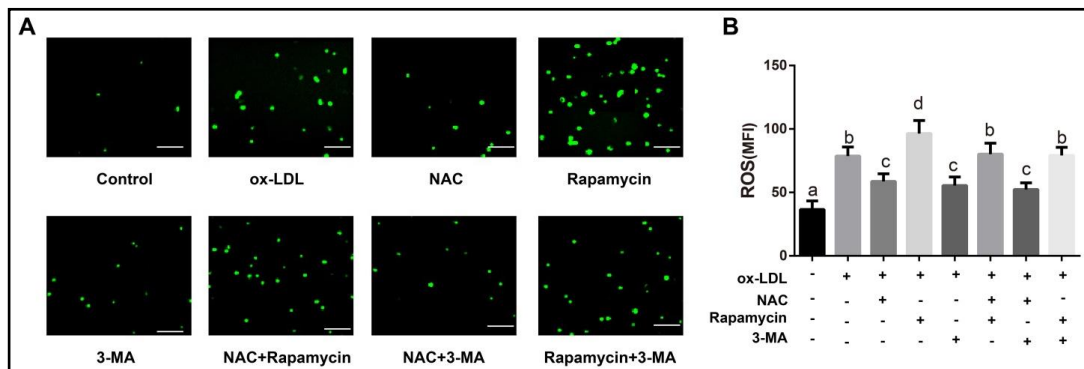
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Fig. 3. Intracellular ROS generation in platelets was measured using DCFH-DA staining. Notes: A, Representative fluorescence microscopy images of ROS staining (scale bar: 50 μm). Platelets in the ox-LDL, NAC, Rapamycin, 3-MA, NAC + Rapamycin, NAC + 3-MA and Rapamycin + 3-MA groups were stimulated with 50 μg/ml ox-LDL. B, Comparison of ROS contents in platelets from each group. All data are presented as means ± standard deviations. Different letters refer to significant differences, while the same letters do not indicate significant differences among groups.
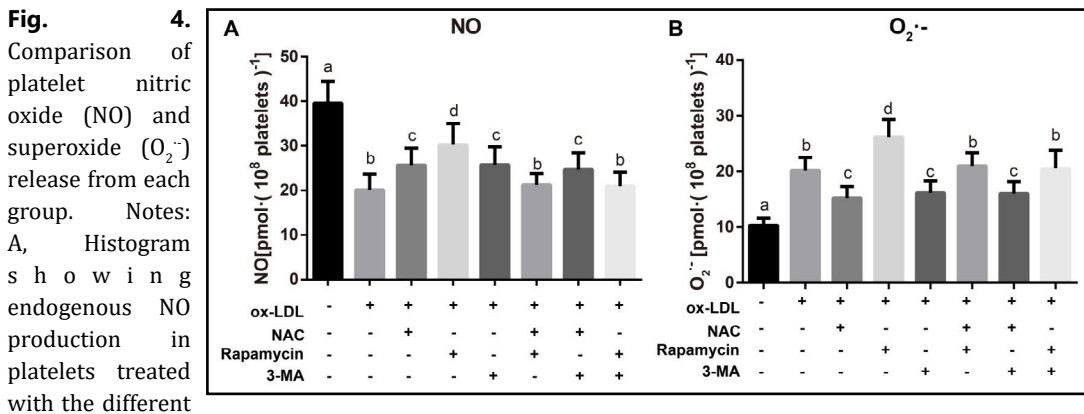

Fig. 4. Comparison of platelet nitric oxide (NO) and superoxide ($O_2^{\cdot-}$) release from each group. Notes: A, Histogram showing endogenous NO production in platelets treated with the different compounds. B, Histograms show the quantification of $O_2^{\cdot-}$ production in platelets in response to the different treatments. All data are presented as means ± standard deviations. Different letters refer to significant differences, while the same letters do not indicate significant differences among groups.

Comparison of NO and $O_2^{\cdot-}$ contents in each group of platelets

Decreased NO contents and increased $O_2^{\cdot-}$ contents were detected in the ox-LDL group and Rapamycin group compared to the Control group and ox-LDL group, respectively, as shown in Fig. 4 (all $P < 0.05$). Compared with the ox-LDL group, the NAC group, 3-MA group and NAC + 3-MA group exhibited a substantial increase in the NO content and a noticeable decrease in the $O_2^{\cdot-}$ content, findings that were opposite to those observed in the Rapamycin group (all $P < 0.05$). Additionally, the NO content was higher but the $O_2^{\cdot-}$ content was lower in both the Rapamycin + 3-MA group and NAC + Rapamycin group than in Rapamycin group (both $P < 0.05$).

Observation of autophagosomes and expression of the autophagy-related protein LC3

As shown in Fig. 5A, the Control group exhibited the normal, long, strip-shaped mitochondria with a clean ridge structure, but no autophagosomes, while autophagosomes were present in the ox-LDL group under the transmission electron microscope. Furthermore, a large number of autophagosomes with distinct double membranes or myelin figures was present and accumulated in the Rapamycin group. Compared with the ox-LDL group, the number of autophagosomes was significantly decreased in the NAC group, 3-MA group and NAC + 3-MA group (both $P < 0.05$). Immunofluorescence staining was used to observe the
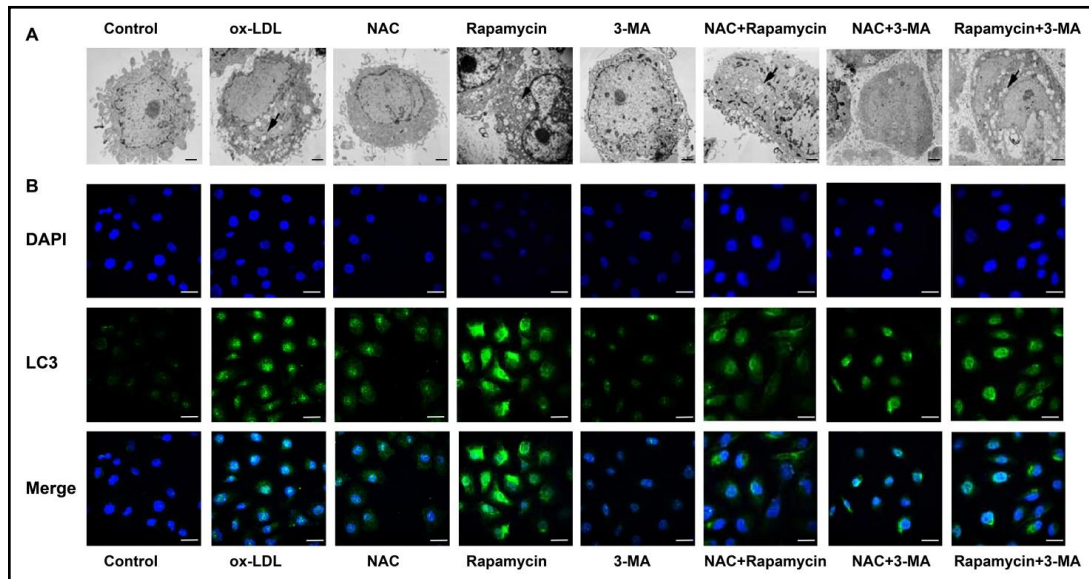
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Fig. 5. Observation of autophagosomes and distribution of the autophagy-related protein LC3 . Notes: A, Morphological alterations in platelets occurring in response to the various treatments were detected by transmission electron microscopy (the black arrow indicates autophagosomes, scale bar: 2 μm). B, Platelets treated with the different compounds were stained with an anti-LC3 antibody and DAPI and observed under a fluorescence microscope (scale bar: 20 μm).

Fig. 6. Western blot analysis of the levels of autophagy-related proteins in each group of platelets. Notes: Levels of the LC3-I, LC3-II, Beclin1, and p62 proteins were analyzed by Western blotting, and quantification of the LC3 II/LC3-I ratio, Beclin 1, and p62 levels are shown. All data are presented as means ± standard deviations. Different letters refer to significant differences, while the same letters do not indicate significant differences among groups.
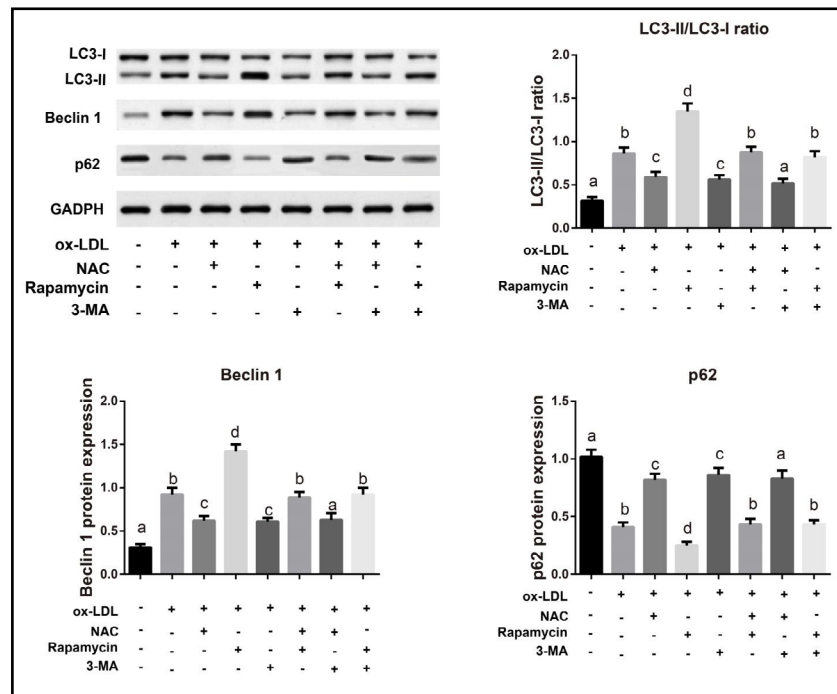
distribution of LC3. No LC3 green fluorescence was detected in the Control group, while the ox-LDL group displayed an increase in LC3 expression, which was distributed around the nucleus in the cytoplasm and formed various scattered green fluorescent dots. However, LC3 expression was significantly increased in the Rapamycin group, but was reduced in the NAC group, 3-MA group and NAC + 3-MA group compared with the ox-LDL group (all $P < 0.05$). In contrast to the Rapamycin group, LC3 expression was dramatically decreased in the NAC + Rapamycin group and Rapamycin + 3-MA group (both $P < 0.05$, Fig. 5B).
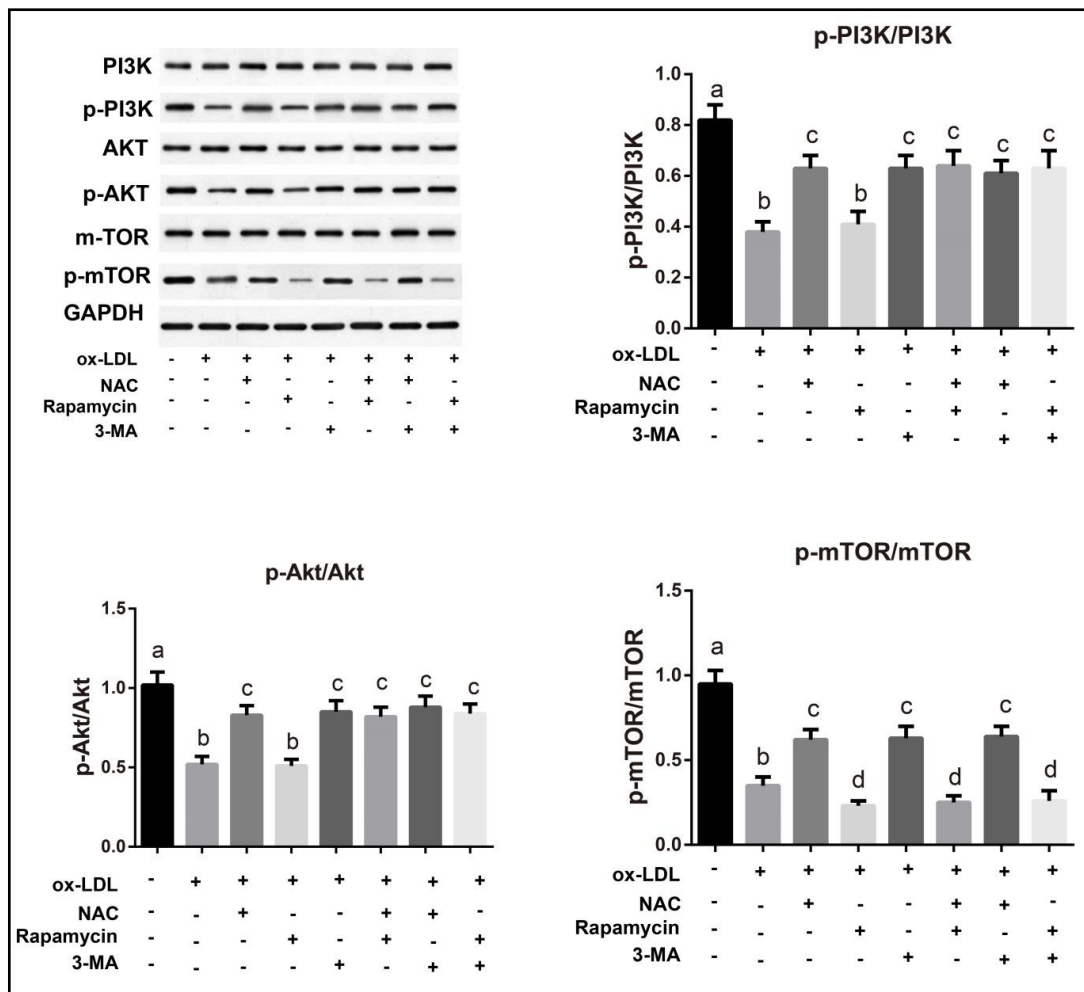
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Fig. 7. Western blot analysis of the levels of PI3K/AKT/mTOR pathway-related proteins in each group of platelets. Notes: Levels of the PI3K, p-PI3K, AKT, p-AKT, mTOR, and p-mTOR proteins in the different groups were analyzed by Western blotting, and the quantification of the PI3K/p-PI3K ratio, p-AKT/AKT ratio and p-mTOR/mTOR ratio is shown. All data are presented as means ± standard deviations. Different letters refer to significant differences, while the same letters do not indicate significant differences among groups.

Levels of autophagy-related proteins in each group of platelets

A significant up-regulation of LC3II/LC3I and Beclin1 and a substantial down-regulation of p62 were observed in the ox-LDL group and Rapamycin group compared with the Control group and ox-LDL group, respectively (all $P < 0.05$). Additionally, LC3II/LC3I and Beclin1 expression were down-regulated in the NAC group, 3-MA group and NAC + 3-MA group, but p62 expression was up-regulated. These changes differed from the changes observed in the Rapamycin group compared with the ox-LDL group (all $P < 0.05$). Moreover, lower levels of LC3II/LC3I and Beclin1, and higher levels of p62 were observed in the NAC + rapamycin group and Rapamycin + 3-MA group than Rapamycin group (all $P < 0.05$, Fig. 6).

Levels of PI3K/AKT/mTOR pathway-related proteins in each group of platelets

Western blotting was used to detect the levels of PI3K/AKT/mTOR pathway-related proteins in each group. As shown in Fig. 7, evident decreases in the levels of p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR were observed in the ox-LDL group and Rapamycin group compared with the Control group (all $P < 0.05$). Additionally, much higher levels of p-PI3K/PI3K and p-AKT/AKT were observed in the NAC, 3-MA, NAC + 3-MA, NAC + Rapamycin and

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Rapamycin + 3-MA groups than in the ox-LDL group (all $P < 0.05$). In addition, in contrast to the ox-LDL group, p-mTOR/mTOR levels were significantly increased in the NAC, 3-MA, and NAC + 3-MA groups, but were dramatically decreased in the Rapamycin, NAC + Rapamycin, and rapamycin + 3-MA groups (all $P < 0.05$).

Discussion

To our knowledge, platelet activation and aggregation are recognized as complex and key processes involved in thrombus formation after the rupture of an atherosclerotic plaque. Meanwhile, ox-LDL is not only important for cardiovascular diseases but is also an independent inducer of platelet activation. Specifically, ox-LDL increases platelet activity and promotes platelet adhesion, aggregation and release by directly interacting with flowing platelets in circulating blood [29, 30]. Notably, alterations in platelet membrane glycoproteins are specific molecular markers of activated platelets. One of these markers is CD62p (also called P-selectin), a glycoprotein that was initially identified on activated platelet membranes by Blann *et al.* [31], and was only selectively distributed on α particles in resting platelets, but was not expressed or was expressed at persistently low levels on the surface of normal platelets [32]. However, CD62p was redistributed in activated platelets, thereby mediating the adhesion and interaction of platelets with granulocytes and endothelial cells to facilitate platelet-monocyte aggregation and accelerate intravascular thrombosis [33, 34]. In the present study, ox-LDL induced platelet activation *in vitro*, significantly increased platelet aggregation and adhesion, and dramatically increased the percentage of platelets expressing the activation marker CD62p, suggesting that ox-LDL promotes platelet adhesion, aggregation, release and the deformation of particles, contributing to the occurrence of coronary heart disease and thrombus [35]. Additionally, ROS and O_2^- levels were noticeably increased but the NO content was substantially decreased in ox-LDL-treated platelets. A close relation between ROS and platelets has been reported, and ROS (including O_2^-, OH^- or H_2O_2) function as second messengers and are produced by resting or thrombin-activated platelets [36]. Once platelets are stimulated with certain agonists, the nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidases are specifically activated [37], further generating ROS and O_2^- [38]. In addition, the enzyme nitric oxide synthase (NOS) determines NO formation through the oxidation of L-arginine in platelets [39]. Ox-LDL-mediated eNOS uncoupling inhibits NO generation [40]. Moreover, the uncoupling of eNOS leads to higher O_2^- and lower NO production under oxidative stress conditions [41], indicating that an ox-LDL treatment may decrease NO production and increase O_2^- by inhibiting eNOS activation. More importantly, Rapamycin-treated platelets exhibited higher O_2^- levels and lower NO production than cells treated with ox-LDL alone in our study. Notably, rapamycin-mediated decreases in the levels of the eNOS mRNA and protein have been documented in several studies. For example, the Rapamycin-induced reduction in eNOS production in endothelial progenitor cells occurred in a concentration-/time-dependent manner in a previous study by Chen TG *et al.* [42]. According to David C. Reineke *et al.* [43], treatment of endothelial cells with Rapamycin significantly reduced eNOS release and activity in a concentration-dependent manner by inhibiting the PKB/Akt pathway, which activate mTOR, ultimately leading to the decreased production of NO [44, 45] and further suggesting that rapamycin may decrease NO production and stimulate O_2^- production owing to reduce eNOS levels.

Autophagosome formation, increased levels of LC3II/LC3I and Beclin1, and decreased levels of p62 were also simultaneously observed in ox-LDL-treated platelets, implying that activated platelets underwent autophagy, consistent with the results from a previous study [15]. Generally, ROS production may induce autophagy to overcome oxidative stress [46, 47] by specifically inactivating Atg4 to cause the accumulation of LC3II and increase the formation of autophagosomes [48]. On the other hand, ROS contribute to the ubiquitination of the degraded substances, and p62 interacts with LC3 (a member of a novel ubiquitin-like protein family) to promote its degradation by the autophagosome-lysosome system [49].

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Moreover, ox-LDL induces autophagy, as evidenced by increased LC3 and Beclin-1 expression [50]. More importantly, ROS induce autophagy by modulating the activity of related signaling pathways. For example, in the study by Mi *et al.*, Momordin Ic induced autophagy by inhibiting the ROS-mediated activation of the PI3K/AKT pathway, and apoptosis was also suppressed by the autophagy inhibitor 3-MA [51]. Additionally, ROS overproduction led to the activation of autophagy by suppressing the PI3K/AKT/mTOR signaling pathway, which plays a critical role in regulating autophagy [52]. Consistent with these results, our findings revealed the restricted activity of PI3K/AKT/mTOR in activated platelets induced by ox-LDL, indicating that platelet activation increased platelet oxidative stress and ROS levels, thereby promoting platelet autophagy possibly via the inhibition of PI3K/AKT/mTOR signaling.

We applied the ROS scavenger NAC, autophagy inhibitor 3-MA and/or mTOR inhibitor Rapamycin to activated platelets to clarify the regulatory mechanism underlying platelet autophagy. Both NAC and 3-MA significantly inhibited activated platelet aggregation and adhesion, along with decreasing ROS levels and the expression of LC3II/LC3I and Beclin1, elevating p62 levels, and activating the PI3K/AKT/mTOR signaling pathway. In contrast, Rapamycin reversed these processes to inhibit the phosphorylation of mTOR and facilitate the activation and autophagy of platelets. Traditionally, PI3K, a class I PI3K in the PI3K/AKT/ mTOR signaling pathway, activates the serine/threonine kinase AKT, and then phosphorylates mTOR via a series of regulatory steps, ultimately inhibiting autophagy [53]. Akt, a member of the family of serine/threonine protein kinases, is activated in a PI3K-dependent manner in response to various stimuli [54], while the phosphorylated class I PI3K enzymes and protein kinase B (Akt/PKB) also activate mTOR [55]. As one of the downstream signaling targets of PI3K/Akt, mTOR was suggested to regulate the signaling proteins responsible for protein synthesis, such as ribosomal p70S6 kinase [56],indicating that during platelet activation, Rapamycin induces the autophagy reaction that has been repressed by class I PI3K by inhibiting the phosphorylation of mTOR. In contrast, class III PI3K (Vps34) interacts with Beclin1 and Vps15 to form class III PI3KC, which initiates autophagy during the formation of the pre-autophagy bilayer membrane in the early stage of autophagy [57]. As discussed above, starvation or the mTOR inhibitor Rapamycin trigger platelet autophagy to induce platelet aggregation and adhesion [16]. Furthermore, a subsequent study confirmed that 3-MA suppresses autophagy by inhibiting class III PI3K [58]. The inhibitor of class III PI3K 3-MA blocks the plasma membrane translocation of important cytoplasmic subunits of NOX2 NADPH oxidase, further affecting the assembly and activation of NOX2 complex on the platelet membrane and subsequently reducing ROS production, ultimately affecting platelet activation and thrombosis [59]. Furthermore, the NOX2 NADPH was identified as one of the key sources of ROS, and a recent study has revealed a novel role for the NOX2 NADPH oxidase in the activation of autophagy [60]. As shown in the present study, platelets treated with 3-MA exhibited lower ROS and autophagy levels, suggesting that 3-MA blocks autophagy by inhibiting class III PI3K activity, which may block NOX2-derived ROS production. More importantly, this study confirmed the hypothesis that Rapamycin-induced autophagy is blocked by the ROS scavenger NAC and autophagy inhibitor 3-MA. In the study by Roy *et al.*, zinc oxide nanoparticles induced ROS formation in macrophages to inhibit the PI3K/AKT/ mTOR signaling pathway and subsequently promote cell autophagy and apoptosis, whereas NAC blocked ROS production, cell autophagy and apoptosis [61], further indicating that inhibition of the activity of the PI3K/AKT/mTOR pathway is partially regulated by ROS and subsequently promotes autophagy in activated platelets.

Conclusion

Taken together, as illustrated schematically in Fig. 8, the present study has revealed that ox-LDL-induced oxidative stress triggered platelet activation and increased ROS production, thereby suppressing the downstream activity of the PI3K/AKT/mTOR signaling pathway, promoting platelet autophagy, and consequently exacerbating platelet aggregation and
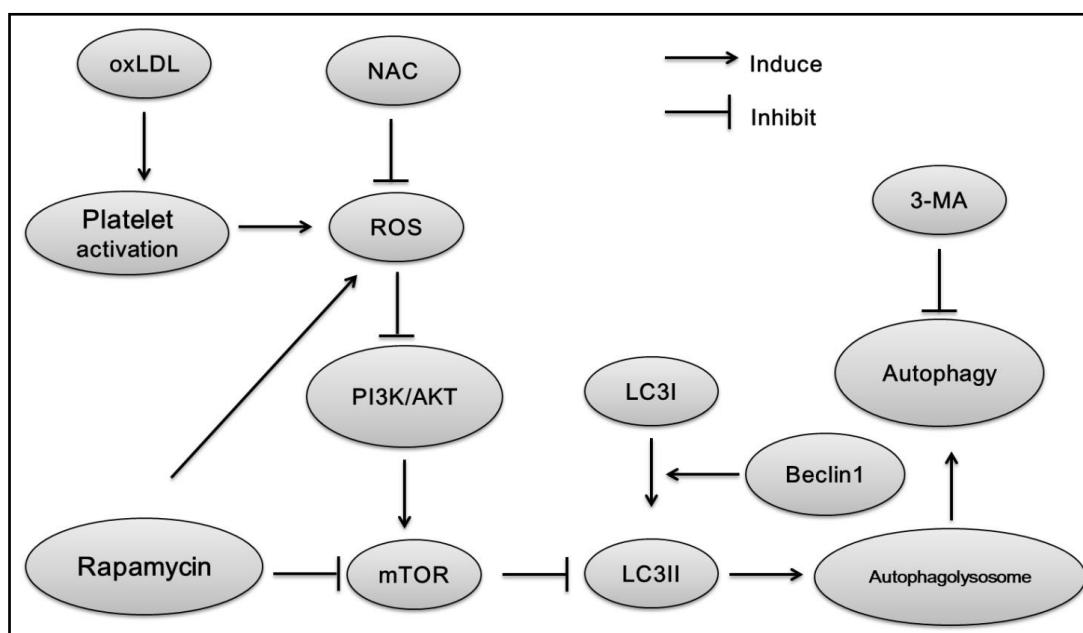
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Fig. 8. Schematic illustrating the proposed mechanism by which ROS induce autophagy to promote ox-LDL-induced platelet activation by inhibiting the PI3K/AKT/mTOR pathway.

adhesion. However, strategies that repress ROS activity may influence the platelet NO and O_2^- contents to reduce autophagy through the activation of the PI3K/AKT/mTOR pathway, thus blocking platelet activation to a certain extent and ultimately achieving the beneficial effects of inhibiting platelet aggregation.

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Disclosure Statement

The authors have no conflicts of interest.

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