RETRACTED ARTICLE: Bilobalide protects H9c2 cell from oxygen-glucose-deprivation-caused damage through upregulation of miR-27a

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ABSTRACT
Background: Myocardial ischemia is a troublesome disease. Bilobalide possesses multiple biological functions. We researched the consequents of bilobalide in OGD-irritated H9c2 cells.
Methods: OGD-stimulated H9c2 cells were treated by bilobalide, and/or transfected with miR-27a inhibitor or negative control. Use CCK-8 and flow cytometry to test cell activity and apoptosis, respectively. Luciferase activity experiment was to test targeting link between miR-27a and Tmub1. Levels of cell-cycle and apoptosis relative proteins and phosphorylation of PI3K/AKT and Wnt/β-catenin related proteins were detected through western blot.
Results: OGD stimulation reduced cell activity and negatively regulated the expression of CDK4, CDK6 and CyclinD1. Cell apoptosis was increased and its related proteins were affected by OGD. Bilobalide administration reversed all the results above caused by OGD. OGD negatively regulated miR-27a while bilobalide upregulated miR-27a. miR-27a’s target gene was Tmub1. The protection consequents of bilobalide were suppressed when cells were transfected with a miR-27a inhibitor that cell activity was reduced and apoptosis was raised. Attenuation in the phosphorylation level of PI3K, AKT and β-catenin by OGD was reversed by bilobalide, whereas there were opposite results after transfected with miR-27a inhibitor.
Conclusion: Bilobalide relieved OGD-caused H9c2 cell damage, raising cell activity and attenuating apoptosis via upregulating miR-27a and activating of PI3K/AKT and Wnt/β-catenin signal pathway.

HIGHLIGHTS
1. Bilobalide alleviates OGD-induced H9c2 cell injury.
2. Bilobalide upregulates miR-27a expression in OGD-stimulated H9c2 cells.
3. Bilobalide alleviates cell injury by upregulation of miR-27a.
4. Bilobalide actuates PI3K/AKT and Wnt/β-catenin pathways.

Introduction
Myocardial ischemia (MI) is a troublesome illness caused by ischemic in occlusion site of myocardium distal. In other words, occlusion of a coronary artery caused loss of blood flow to the myocardium seriously and abruptly [1]. The sudden exact progression makes the blood perfusion can only achieve 10% or less as compared to normal, which triggers great damage to mitochondria, then finally leads to cardiomyocyte death [2]. Mitochondria are the workshop of oxidative metabolism in cardiomyocytes. Therefore, the damage to mitochondria finally causes the pathological condition in which oxygen supply to the myocardium is reduced and the energy metabolism of the myocardium is abnormal [3,4]. Nowadays, even though antiplatelet drugs and anticoagulant therapy are used for MI and improve the survival for MI patients, the outcome is still very low [5]. Therefore, new medicine or more effective treatment methods are urgently needed.

Bilobalide, a component extracted from Ginkgo biloba extract, possess various kinds of properties. For example, bilobalide revealed neuroprotective effects in brain ischemic injury [6]; Bilobalide showed antioxidant potential in alleviating hypoxia-induced injury in 3T3-L1 adipocytes [7]; Bilobalide inhibited autophagy and promoted angiogenesis in cerebral ischemia-reperfusion [8]. Importantly, bilobalide demonstrated functions in the protection of the mitochondrial respiratory activity [9,10]. Moreover, Ginkgo biloba L. extract revealed cardioprotective effects on MI in rats [11]. Considering the crucial roles of mitochondrial in MI and bilobalide was also from Ginkgo biloba L. extract, we, therefore, inferred that bilobalide might have functions in MI.

MicroRNAs (microRNAs) are a class of RNA that does not exert protein-encoding function, regulating diverse biological processes by targeting mRNAs [12]. Myocardial ischemia is often got along with the aberrant expression of miRNAs, such as miR-15a/b [13], miR-103/107 [14], miR-126 [15].
MiR-27a was involved in various diseases and regulated several important biological processes [16]. Interestingly, researches from Rodrigues et al. found that bilobalide led to a significant upregulation of miR-27a in Caco-2 cells while no obvious difference was observed for miR-27a in MCF-7 cells, which suggests that the effects of bilobalide on miR-27a expression was associated with cell lines. We employed a rat cardiomyocytes cell line H9c2 to research how bilobalide influences the expression of miR-27a.

We employed oxygen-glucose-deprivation (OGD) to mimic the damage of myocardial ischemia, and experiments were performed to research the consequents and mechanisms of bilobalide in OGD-caused H9c2 cell damage to supply a new vision.

Material and methods

Cell model establish

The rat cardiomyocytes cell line H9c2 was obtained from Procell life science and technology (Wuhan, China). The complete growth medium for H9c2 cells were Dulbecco’s modified Eagle medium (DMEM, Procell, PM150210), 10% FBS (Procell, 164210) and 1% Penicillin/Streptomycin (Procell, PB180120). Keep cells in the proper surrounding (37 °C and 5% CO2). Medium is changed every 2 to 3 days.

OGD treatment

For glucose-deprivation, cultured cells were changed into a glucose-free DMEM medium with or without bilobalide treatments. Then oxygen deprivation was fulfilled through placing the cultured cells into the atmosphere with 5% CO2 and 95% N2 (v/v) and maintained for diverse period intervals (0, 3, 6, 12 and 24 h). All the other conditions and atmosphere were set the same as the normal culture requirement. The normal medium of glucose under normoxia was considered as a control.

Bilobalide (Cat. No.: 79593) was bought from Sigma-Aldrich (St Louis, MO) dissolved in DMSO and then was diluted into different consistencies (0.1, 1, 5 and 10 μM). H9c2 cells were treated by bilobalide for 24 h.

Cell viability assay

Cell Counting Kit-8 (CCK-8, Yeasen, Shanghai, China) was to detect cell activity. Treat H9c2 cells with hypoxia, or bilobalide or transfection with miR-27a inhibitor were seeded in a 96-well plate at the denseness at 2 × 10^4 cells/well. Set the surrounding in a proper condition (37 °C and 5% CO2). Then add 10 ml CCK-8 solution and cells were incubated for 1 h. Finally, absorption values were got at 450 nm through a Microplate Reader (Bio-Rad, Hercules, CA).

Cell apoptosis assay

PI and FITC-conjugated Annexin V staining (BD Pharmingen, San Diego, CA) was used to test cell apoptosis. First, the cell number of every sample was 1 × 10^6/ml. The suspended cells were gathered into tubes and centrifuged at 111.8 g for 5 min to discard the supernatant. The left cells were cleaned by PBS and then centrifuged again. Then cells were resuspended through 100 μl 1 × binding buffer and were kept for 10–15 min in the room without light. Centrifuge and clean cells again. Finally, flow cytometry (Beckman Coulter, Fullerton, CA) was used to analyze cell apoptosis. 515 and 560 wavelengths were used for FITC and PI fluorescence, respectively. Regular cells (FITC −/PI −), necrotic cells (FITC −/PI +) and apoptotic cells (FITC +/PI −) were got in our experiments.

miRNAs transfection

Adjust the number of every cell sample to 2 × 10^5 cells/well. Transfection was performed until cells reached 70–80% confluence [17]. MiR-27a inhibitor and negative control (NC) were synthesized through Life Technologies Corporation (Carlsbad, CA) and their final concentration was adjusted to 100 nM. Next, transfect H9c2 cells with miR-27a inhibitor and NC through Lipofectamine 2000 reagent (Invitrogen). The miR-27a inhibitor sequence was as follows: 5’-GCGGAACUCUGCCACUGUGAA-3’. The NC sequence was as follows: 5’-UCACAAACCUCUGAGAAGAGUGA-3’.

qRT-PCR

We used Trizol reagent (Invitrogen) to gather experimental RNA. Then, we performed the testing assay of miR-27a through Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (all from Applied Biosystems, Foster City, CA). U6 was worked as the inside control [18].

Western blot

RIPA lysis buffer (Cat. No.: R0010, Solarbio, Beijing, China) and protease inhibitors (Roche, Basel, Switzerland) were used to gather experimental protein. Then, use BCA™ Protein Assay Kit (Pierce, Appleton, WI) to quantitate the protein [18]. Third, protein samples were isolated through SDS-PAGE and subsequently, the gel was transferred to PVDF membrane. Fourth, a protein-free blocking solution (Sangon Biotech, Shanghai, China) was used to soak the membrane. Primary antibodies included: anti-cyclin-dependent kinase 4 (CDK4) antibody (ab199728), anti-CDK6 antibody (ab131469), anti-Cyclin D1 antibody (ab134175), anti-β actin antibody (ab8227), anti-p53 antibody (ab131442), anti-Bcl-2 antibody (ab32124), anti-Bax antibody (ab32503), anti-pro-Caspase-3 antibody (ab13847), anti-Caspase-3 antibody (ab49822), anti-phosphatidylinositol 3'-kinase (PI3K) (ab151549), anti-phospho-PI3K antibody (ab182651), anti-protein kinase B (AKT) antibody (ab8805), anti-phospho-AKT antibody (ab38449), anti-β Catenin antibody (ab32572) all from Abcam (Cambridge, UK). These primary antibodies were kept with the membrane, kept in a room for 10 min and maintained at 4 °C overnight. Fifth, rinse the membrane and incubate it...
with secondary antibody for 1 h indoors. Then 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA) was added to cover the surface of the membrane. Finally, Image Lab™ Software (Bio-Rad, Hercules, CA) was used to catch the bands.

**Dual luciferase activity assay**

Luciferase reporter that constructed the Tmub1 3’UTR carrying an assumed miR-27a binding site was ligated into the pMIR-Report Luciferase vector (Promega, Madison, WI). Use Lipofectamine 3000 (Life Technologies Corporation) to co-transfect miR-27a mimic with the Tmub1-wild-type (Tmub1-WT) or Tmub1-mutated-type (Tmub1-MUT) into H9c2 cells. Results were got through a dual-luciferase assay system (Promega) according to the manufacturer’s information.

**Statistical analysis**

Data were revealed as mean ± standard deviation (SD). Statistical analyses were done through Graphpad 6.0 statistical (GraphPad, San Diego, CA). The p-values were calculated through a one-way analysis of variance (ANOVA) and student’s t-test. p-Values less than .05 would be treated as a significant difference.

**Results**

**OGD caused H9c2 cell damage**

As shown in Figure 1(A), cell activity was notably reduced with the raising OGD therapy period (3, 6, 12 and 24 h) as compared with control (3 h, p < .05, 6 and 12 h, p < .01, 24 h, p < .001). OGD therapy period 6 h was selected for the following experiments. CDK4, CDK6 and CyclinD1 are all cell cycle-related proteins [19]. We got that the expression of CDK4 (p < .05), CDK6 and CyclinD1 (both p < .01) were all down-regulated by OGD stimulation (Figure 1(B,C)). Furthermore, it is shown in Figure 1(D), cell apoptosis was enhanced by OGD stimulation as a contrast with comparison (p < .001). In addition, levels of apoptosis relative factors (p53, Bax, Bcl-2, cleaved-Caspase-3) were tested through western blot. Results demonstrated that p53 and Bax were statistically positively regulated (both p < .01, Figure 1(E,F)), while Bcl-2 was negatively regulated (p < .01, Figure 1E,F). Meanwhile, cleaved-Caspase-3/pro-Caspase-3 was raised (p < .01, Figure 1(E,F)). These results validated the finding of OGD-induced cell apoptosis. Taken together, OGD caused cell damage via reducing cell proliferation and increased apoptosis.

**Bilobalide alleviated OGD-induced cell injury**

Results in Figure 2(A) showed that, under OGD treatment for 6 h, cell activity was notably raised when bilobalide was 0.1 and 1 µM (both p > .05). Besides, bilobalide administration markedly upregulated the expression of CDK4, CDK6 and Cyclin D1 in OGD-stimulated H9c2 cells (p < .05 or p < .01, Figure 2(B,C)). On the other hand, the group treatment with OGD and bilobalide notably reduced apoptosis contrasted with OGD set (p < .05, Figure 2(D)). Meanwhile, bilobalide administration also reversed the expression trend of apoptosis-related proteins presented by downregulation of p53 and Bax while Bcl-2 was upregulated (all p < .05, Figure 2(E,F)). Also, the rate of cleaved-Caspase-3/pro-Caspase-3 was decreased by the treatment of bilobalide (p < .01, Figure 2(E,F)). In a word, bilobalide alleviated OGD-induced H9c2 cell injury.

**Bilobalide positively regulated miR-27a**

miR-27a was found to be up-regulated in MI [20]. In addition, miR-27a was found to protect cells from OGD-caused damage in hippocampal neurons [21]. Therefore, we hypothesized that miR-27a was related to bilobalide in protecting cells from damage. Interestingly, bilobalide upregulated miR-27a (p < .05 or p < .01), which was downregulated by OGD (Figure 3). Hence, miR-27a participated in protection consequences of bilobalide in OGD-caused cell damage.

**Tmub1 was the target of miR-27a**

To further study the mechanism, we found that Tmub1 mRNA level was notably reduced by miR-27a mimic contrasted with mimic NC (p < .01, Figure 4(A)), whereas was notably enhanced by miR-27a inhibitor (p < .05, Figure 4(A)). Similarly, western blot experiment indicated that Tmub1 was down-regulated when cells were transfected with miR-27a mimic, whereas was up-regulated when miR-27a was inhibited (Figure 4(B)). These findings indicated that Tmub1 was down-regulated through miR-27a. Additionally, luciferase activity was notably reduced after Tmub1-WT transfection (p < .05), whereas was not affected when cells were transfected with Tmub1-MUT (Figure 4(C)). Our findings suggested that Tmub1 was the target of miR-27a.

**Bilobalide alleviated OGD-induced H9c2 cell injury through upregulation of miR-27a**

Figure 5(A) revealed high transfection efficiency in cells transfected with miR-27a inhibitor (p < .01, Figure 5(A)). Notably, downregulation of miR-27a caused contrary consequences as compared with NC in OGD-stimulated and bilobalide-treated H9C2 cells. It mainly presented by reducing cell activity (p < .05) and increasing apoptosis (p < .01, Figure 5(B,E)). Afterwards, cell cycle relative factors (CDK4, CDK6 and Cyclin D1) were downregulated, which indicated the reduction of cell proliferation (Figure 5(C,D)). Moreover, positive regulation of p53, Bax, and cleaved-Caspase-3 and negative regulation of Bcl-2 in Figure 4(F,G) confirmed the result that transfection with miR-27a inhibitor enhanced apoptosis. Taken together, bilobalide alleviated OGD-caused cell damage by positively regulating miR-27a.
Bilobalide activates PI3K/AKT and Wnt/β-catenin signal pathways through miR-27a overexpression

PI3K/AKT and Wnt/β-catenin signal pathways were reported to be closely related to MI [22,23]. We got that OGD decreased phosphorylation of PI3K, AKT and β-catenin (all \( p < .01 \), Figure 6(A,B)). Furthermore, bilobalide led to the opposite results revealed by increasing phosphorylation of PI3K, AKT and β-catenin (all \( p < .01 \), Figure 6(A,B)). Interestingly, groups transfected with miR-27a inhibitor indicated the downregulation of PI3K, AKT and β-catenin (all \( p < .01 \), Figure 6(A,B)). Thus, we inferred that bilobalide actuated PI3K/AKT and Wnt/β-catenin signal pathways via miR-27a overexpression.

Discussion

MI is a disastrous illness with serious morbidity and mortality in seniors [24]. The sudden ischemia leads to low oxygen supply and finally initiate cell death [25]. On the other side, increasing evidence demonstrated that bilobalide has significant effects on hypoxia-induced injury [26,27]. Our study employed OGD-stimulated H9c2 cells to build up MI cell...
mold in vitro and researched consequents of bilobalide in OGD-caused H9c2 cell damage. Consequences revealed that bilobalide relieved OGD-caused cell damage evidenced as raising cell activity and decreasing apoptosis. MiR-27a was positively regulated after bilobalide treatment in OGD-stimulated H9c2 cells. We thereafter found that protection consequents of bilobalide were performed through positively regulating miR-27a, and along with the actuation of PI3K/AKT and Wnt/β-catenin signal pathways.

The cardiomyocytes cells stimulated by OGD was often used to mimicked MI injury in vitro [28]. Our study employed OGD to induce H9c2 cell injury to build up in vitro MI cell mold. Consequences revealed that OGD decreased cell activity and raised apoptosis.

CDK4, CDK6 and CyclinD1 are key modulators for cell cycle [29]. CDK4/6 is important in the phase from G1-to-S transition and promotes cell proliferation [30]. Subsequently, CyclinD1 binds to and activates CDK4/6, the CyclinD1-CDK4/6 axis triggered the G1/S transition to enhance cell cycle [31]. In this study, CDK4/6 and CyclinD1 were both negatively regulated after OGD treatment (6 h), suggesting that OGD obstructed cell cycle or cell proliferation.

Figure 2. Bilobalide (Bilo) alleviated oxygen-glucose-deprivation (OGD)-induced cell damage. (A) Cell activity was tested by Cell Counting Kit-8. (B–C) Cell cycle-related proteins cyclin-dependent kinase (CDK) 4/6 and CyclinD1 were examined through western blot. (D) Cell apoptosis was measured via flow cytometry. (E–F) Expression of apoptosis relative factors was tested through western blot. Data were revealed as mean ± standard deviation (SD). *p < .05, **p < .01 and ***p < .001 were notable consequences.

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Moreover, apoptosis relative factors (p53, Bcl-2, Bax and Caspase-3) were detected via western blot. p53 is a regulator which might cause cell cycle stagnation or apoptosis [32]. This hinted that p53 might reach a threshold in causing apoptosis by OGD. Bcl-2, Bax and cleaved Caspase-3 are crucial in apoptosis [33,34]. In our study, the expression of p53, Bax and cleaved-Caspase-3 was positively regulated whereas the expression of Bcl-2 was negatively regulated after OGD treatment contrast with the comparison group. In a word, OGD successfully induced H9c2 cell injury.

Bilobalide is commonly used to protect cells to multiple stimulations in the previous literatures. For example, bilobalide revealed protective effects in hypoxia-induced endothelial cells injury [35], in activated oxygen species-caused apoptosis in PC12 cells [36], and in amyloid beta-peptide 25–35-caused PC12 cell [37]. Similarly, bilobalide was reported to protect BV2 microglia cells against OGD/reoxygenation injury [27]. Nevertheless, we used H9c2 cells to research the consequents of bilobalide in OGD-caused damage. Notably, bilobalide administration caused the reverse results in OGD-handled cells. Our consequence was consistent with the former study that bilobalide alleviated hypoxia-induced cell injury [7]. Additionally, we found that 10 μM of bilobalide protection effect is reduced. This finding was consistent with that in Lu et al.’s research [38]. It has been reported that endoplasmic reticulum (ER) is the position for folding and maturation of proteins [38]. Unfolded protein response (UPR) signal pathway elevates recovery of ER homeostasis and cell survival, whereas it can cause apoptosis under continued ER stress [39]. UPR pathway causes a transient inhibition in protein translation via phosphorylation of eIF2α [38]. This inhibition causes the induction of CHOP, which is a notable mediator of apoptosis under ER stress [40]. Therefore, a high concentration of bilobalide treatment may over-stimulate UPR signaling to cause the inhibition of cell viability. There may be a certain concentration range of bilobalide protective effects. This conjecture needs further research.

OGD stimulation often triggers the aberrant miRNAs expression, like miR-7 [41], miR-155 [42], miR-132 [43], miR-27a [21]. Interestingly, Cai et al. found that miR-27a overexpression attenuated OGD-induced injury in hippocampal neurons [21], which was consistent with our results. In our study, transfection with miR-27a inhibitor notably reduced cell activity and raised apoptosis which impaired the protective effects of bilobalide on OGD-caused cell damage. Of notably, cell-cycle and cell apoptosis relative factors validated the influence of downregulation of miR-27a on H9c2 cells. This consequence was consistent with the former literature report that overexpression of miR-27a decreased cell proliferation [44]. However, different finding was observed by Li et al. that miR-27a elevated hepatocellular carcinoma cell proliferation [45]. Hence, the expression of miR-27a might play various roles in
Figure 5. Bilobalide (Bilo) inhibited oxygen-glucose-deprivation (OGD)-caused cell damage through positively regulating miR-27a. (A) miR-27a inhibitor was transfected into H9C2 cells and the expression of miR-27a was tested by qRT-PCR. (B) Cell activity was tested through Cell Counting Kit-8. (C–D) Cell cycle-related proteins cyclin-dependent kinase (CDK) 4/6 and CyclinD1 was examined by western blot. (E) Cell apoptosis was measured through flow cytometry. (F–G) Expression of apoptosis relative factors was detected by western blot. Data were revealed as mean ± standard deviation (SD). *p < .05, **p < .01 and ***p < .001 were notable consequences.
various cells. We can only achieve that bilobalide defensed H9c2 cells against OGD-caused cell damage via upregulation of miR-27a. In addition, miRNAs play its roles through corresponding target genes, such as miR-155 and its target gene PDCD4 [46], and miR-27a is no exception. Lan et al. confirmed that the Tmub1 mRNA 3′-UTR was the target of miR-27a [47]. Consistently, we got that Tmub1 was the target gene of miR-27a and was negatively regulated by miR-27a. Further experimentation needs to be done to verify the role of this negative regulation in protective conquests by bilobalide.

PI3K/AKT and Wnt/β-catenin signal pathways participated in the progression of MI [22,23]. In our study, bilobalide administration actuated PI3K/AKT and Wnt/β-catenin signal pathways via upregulation of miR-27a. A similar conclusion was found from Shi et al. that bilobalide reduced apoptosis via actuation of PI3K/AKT pathway in SH-SY5Y cells [48] and bilobalide activated Wnt/β-catenin signal pathway in P19 embryonic carcinoma cells [49]. Taken together, it revealed that the protective effects of bilobalide on OGD-caused damage might through actuation of PI3K/AKT and Wnt/β-catenin signal pathways.

In conclusion, we got that bilobalide attenuated OGD-caused cell damage via positively regulating miR-27a presented as increasing cell activity, proliferation and reducing apoptosis in H9c2 cells. Further experiment validated that this process might be modulated by activation of PI3K/AKT and Wnt/β-catenin signal pathways. Our study might offer a novel insight into curing MI.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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