RETRACTED ARTICLE: Lentinan protects cardiomyocytes against hypoxia-induced injury by regulation of microRNA-22/Sirt1

Shaohui Zhang¹ and Yongliang Zhao²
¹Department of Cardiology, Affiliated Hospital of Jining Medical University, Jining, China; ²Department of Cardiac Surgery, Affiliated Hospital of Jining Medical University, Jining, China

ABSTRACT
Myocardial ischemia is a serious disease which threatens human’s life. Lentinan (LEN) possesses multiple biological properties: anticancer, antibacterial, antiviral and antioxidant effects. Our study investigated the effects of LEN on hypoxia-stimulated cardiomyocytes and the underlying mechanism. Primary neonatal rat ventricular cardiomyocytes (PNCM) were isolated from neonate rat pups. PNCM and H9c2 cells were stimulated by hypoxia and treated by LEN. Cell viability and apoptosis were detected by cell counting kit-8 and flow cytometry, respectively. Moreover, apoptotic factors were examined by western blot. Phosphatidylinositol 3’-kinase (PI3K)/protein kinase B (AKT) and β-catenin pathways related proteins were analyzed by western blot. Furthermore, the expression of microRNA-22 (miR-22) was detected by qRT-PCR. Altered expression of miR-22 and silenced information regulator 1 (Sirt1) was achieved by transfection. The relationship between miR-22 and Sirt1 was verified by luciferase assay. We found that LEN promoted cell viability and decreased apoptosis which led to the contrary results with what hypoxia induced. Moreover, LEN decreased the ratio of Bax to Bcl-2 and the level of cleaved caspase-3, as well as activated PI3K/AKT and β-catenin. LEN decreased the expression of miR-22 which was upregulated by hypoxia. miR-22 overexpression broke the promoting effects led by LEN. Moreover, Sirt1 was verified to be a target of miR-22. Silence of Sirt1 led to the opposite results with LEN. In conclusion, LEN relieved hypoxia-induced cellular injuries evidenced by increasing viability and decreasing apoptosis via down-regulation of miR-22, which was accompanied by activation of PI3K/AKT and β-catenin pathways.

Highlights
- Lentinan alleviates hypoxia-induced injuries of PNCM and H9c2 cells;
- microRNA-22 expression is decreased by lentinan;
- Lentinan reduces hypoxia-induced injury by microRNA-22 downregulation;
- Lentinan regulates PI3K/AKT and Wnt/β-catenin by regulation of microRNA-22/Sirt1.

Introduction
Myocardial ischemia is a pathological condition in which the blood perfusion of the heart is reduced, resulting in decreased oxygen supply to the heart and abnormal myocardial energy metabolism, which cannot support the normal work of the heart [1,2]. The main result caused by the unrelied ischemia is permanent damage to the myocardium which might bring great changes in other diseases [3]. On the other side, the damaged myocardium would be replaced by fibrous scar tissue which cannot contribute to myocardial contractile function and finally results in chronic heart failure [4]. For myocardial ischemia patients, if the coronary thrombosis was found, then reperfusion which means sending blood to the ischemic myocardium is the standard treatment [5]. Overwhelming evidences have proved that reperfusion is a good approach to limit infarct size, improve long-term myocardial function, change the healing pattern of the infarcted zone, and more importantly, decrease mortality [4]. Even though it can improve the outcomes of the patients, these treatments easily lead to chronic heart failure [4,6]. Therefore, new medicine and novel effective treatment for myocardial ischemia are needed.

Traditional Chinese medicine receives considerable attention due to its effective effects with low-side effects, including in the field of myocardial ischemia [7,8]. For example, Suxiao jiuxin pill as a traditional Chinese medicine reveals effects against myocardial ischemia in dogs [9]. Among all these identified medicine compound, lentinan (LEN) has been proved to be an interesting medicine used in various kinds of diseases because of its biological activities, such as anticancer, antibacterial, antiviral, and antioxidant effects [10–12]. From the above information, we inferred that there might be a potential of LEN in various diseases. We hypothesized that
LEN could also work on hypoxia-induced cell injury. In our study, we used hypoxia to stimulate cardiomyocytes to mimic the injury in myocardial ischemia to establish an in vitro cell model.

microRNAs (miRNAs) have been reported to participate in myocardial ischemia in exercise-induced cardiac growth, and protect against pathological cardiac remodeling by altering key signaling elements, which suggests that miRNAs can be regarded as the potential therapeutic targets [13,14]. For instance, microRNA-103/107 (miR-103/107) regulates programmed necrosis and myocardial ischemia/reperfusion (IR) injury through targeting fas-associated protein with death domain [15]. Increasing evidences demonstrated that miR-22 is closely connected with myocardial ischemia [16,17]. Then, we aimed to explore whether miR-22 was also cooperated with LEN to work in the hypoxia-treated cardiomyocytes. This is the first time to investigate the possible mechanism in which LEN protected cardiomyocytes against hypoxia-induced injury.

Material and methods

Cell isolation, culture and treatment

Primary neonatal rat ventricular cardiomyocytes (PNCM) were isolated from neonate rat pups (Sprague–Dawley rats) that were postpartum less than 2 days. The hearts were cut along the sternum and dissected through the chest wall. After washed in an isolated buffer, the obtained hearts were cut into 2 mm pieces used for incubation. After digestion seven times, the pellets were pooled and washed in 15% (v/v) fetal bovine serum (FBS) (Thermo Scientific, Waltham, MA, USA) in Dulbecco’s modified Eagle medium (DMEM) (American Type Culture Collection; ATCC, Rockville, MD, USA). PNCM cells were seeded onto 6-well plates at 2 × 10^6 cells per well and cultured in DMEM containing 15% (v/v) FBS. Rat H9c2 cardiomyocytes were purchased from ATCC. Collected cells were maintained in DMEM with 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Gaithersburg, MD, USA). Hypoxia injury was induced by incubating the cells in the incubator with 94% N2, 5% CO2 and 1% O2 for 24 h. A normoxia experiment was performed, whereby cells were incubated in 74% N2, 5% CO2 and 21% O2. Lentinan (Cat. NO. B24117), with an ultraviolet purity more than 98%, was provided by Shanghai Pharmaceutical Company (Shanghai, China). Then lentinan was diluted into different concentrations of 100–500 µg/ml to be incubated with cells for 24 h. A control experiment was carried out to value the effect of lentinan.

Cell viability assay

Cell viability was detected by using cell counting kit-8 (CCK-8) assay. In general, PNCM and H9c2 cells were seeded in a 96-well plate at the density of 1 × 10^3 cells/well. CCK-8 solution was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). The CCK-8 solution was administrated into the DMEM and after that incubated at 37°C in humidiﬁed 95% air and 5% CO2 for 1 h. Finally, the absorbance at 450 nm was determined by a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Apoptosis assay

Cell apoptosis was detected by the method of Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI). The apoptosis detection kit was obtained from Beijing Biosea Biotechnology (Beijing, China). The cells at the density of 1 × 10^5 cells/well were incubated in 6-well plate. Treated cells were washed two times with ice-cold phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA) and then centrifuged to re-suspend in binding buffer. Then 5 µl Annexin V-FITC was added and mixed gently and put in the dark and incubated for 15 min. In addition, added 5 µl PI to the samples. The apoptotic cell rate was measured with flow cytometer (Beckman Coulter, IN, USA).

Western blot

Radio Immunoprecipitation Assay (RIPA) lysis buffer (Solarbio, Beijing, China) with protease inhibitors (Thermo Scientific) was used for protein extraction. BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was used to measure total protein concentration compared to a protein standard. The western blot system was constructed using a Bio-Rad Bis-Tris Gel system. Primary antibodies were prepared in 5% blocking buffer and diluted following product instruction. These primary antibodies were incubated with membranes at 4°C overnight. Then, the primary antibodies were detected by secondary antibody (Abcam, Cambridge, UK) conjugated by horseradish peroxidase. Protein signals were captured, and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad).

qRT-PCR

Trizol reagent (Life Technologies, Carlsbad, CA, USA) was used for RNA isolation. The Taqman MicroRNA Reverse Transcription Kit (Thermo Scientific) was used for converting miRNA to cDNA. The Taqman Universal Master Mix II (Thermo Scientific) was used for PCR reaction. These two cooperated with TaqMan MicroRNA Assay (Thermo Scientific) for determining miR-22 in PNCM and H9c2 cells. U6 was the internal control for miR-22.

Cell transfection

miR-22 mimic, the negative control (NC mimic), si-NC and si-Sirt1 were synthesized by GenePharma Co. (Shanghai, China). Cells at the density of 2 × 10^5 cells/well were seeded and incubated until the cells arrived at 70–80% confluence, and then the cells were transfected with miR-22 mimic (NC mimic) and si-Sirt1 (si-NC) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).
Luciferase reporter assay

In brief, HEK 293 T/17 cells (ATCC) were co-transfected with miR-22 mimic and Sirt1 3′-untranslated regions (UTR), or mutant 3′-UTR, together with the controls. The DharmaFECT Duo transfection reagent (Thermo Scientific) was used for transduction, and the luciferase assays were performed with the Dual-Glo Luciferase assay (Promega, Madison, WI, USA).

Statistical analysis

Data are shown as mean ± standard deviation. Statistical analyses were performed using Graphpad 6.0 statistical software (GraphPad, San Diego, CA, USA). The p values were calculated using Student’s t-test and one-way analysis of variance (ANOVA) followed by Tukey’s homogenous variances post-hoc test. p Values <.05 would be treated as significant difference.

Results

LEN alleviated hypoxia-induced apoptosis process

Firstly, experiments were performed to detect which concentration was the best for the following experiments. Results showed that LEN at concentrations of 100–300 μg/mL could not significantly alter cell viability of PNCM and H9c2 cells, while the viability was apparently decreased by LEN at concentrations of 400 and 500 μg/mL (both p < .05) (Figure 1(A)), which indicated that LEN with the concentrations of 100–300 μg/mL were safe for PNCM and H9c2 cells. Then, the
cells were pre-incubated with 100–300 μg/mL LEN before hypoxia treatment. As shown in Figure 1(B), the significant increment in viability was found when LEN was at the concentration of 200 and 300 μM (both \( p < .05 \), Figure 1(B)). Considering that 300 μM was the greatest concentration of LEN which PNCM and H9c2 cells can tolerate and meanwhile exhibited improved viability in response to hypoxia. Therefore, LEN at the concentration of 300 μM was chosen in the following experiments. As expected, we found that hypoxia significantly increased cell apoptosis (\( p < .001 \), Figure 1(C)) compared with control. In addition, the ratio of Bax to Bcl-2 and abundance of cleaved caspase-3 were obviously upregulated by the treatment of hypoxia (both \( p < .001 \), Figure 1(D)). On the other side, we found that LEN pre-incubation led to the opposite results which was shown as decrease in apoptotic rate (both \( p < .01 \), Figure 1(D)), ratio of Bax to Bcl-2 and cleavage of caspase-3 (both \( p < .01 \), Figure 1(D)). Taken together, LEN alleviated hypoxia-induced apoptosis process in PNCM and H9c2 cells.

**LEN activated PI3K/AKT and wnt/β-catenin signal pathways**

PI3K/AKT and Wnt/β-catenin were involved in myocardial ischemia [18, 19]. In our study, we detected the effects of LEN on PI3K/AKT and Wnt/β-catenin pathways. Results showed that hypoxia decreased the phosphorylated levels of PI3K (\( p < .05 \), \( p < .01 \)) and AKT (\( p < .05 \)) compared with control, while PNCM and H9c2 cells pre-incubated by LEN prior to hypoxia treatment showed the reversal trend in the phosphorylation of PI3K (both \( p < 0.05 \)) and AKT (\( p < 0.05 \), \( p < 0.01 \)) (Figure 2(A)). Intriguingly, hypoxia increased the expression of β-catenin (\( p < .05 \)) and LEN further increased β-catenin expression (both \( p < .05 \), Figure 2(B)). From these results above, we inferred that LEN could activate PI3K/AKT and Wnt/β-catenin signal pathways.

**LEN induced miR-22 downregulation**

miR-22 was reported to be closely related to myocardial ischemia [16, 17], therefore, we explored the expression of miR-22 in hypoxia-treated H9c2 cells. Results showed that hypoxia significantly upregulated the expression of miR-22 (\( p < .01 \)) while LEN downregulated the expression of miR-22.
miR-22 overexpression negated the protective role of LEN against hypoxia-caused apoptosis

To identify whether the protective function of LEN was mediated by miR-22 silence, miR-22 mimic and its corresponding NC were transfected into PNCM and H9c2 cells. The upregulation of miR-22 by transfection with miR-22 mimic (both
p < .001, Figure 4(A)) indicated high transfection efficiency. Results showed that co-treated with miR-22 mimic and LEN decreased cell viability (both p < .05, Figure 4(B)) and enhanced apoptosis (p < .05 or p < .01, Figure 4(C)). Moreover, we also detected the expression of the apoptosis-associated proteins. Results showed that the ratio of Bax to Bcl-2 and the cleavage of caspase-3 were both upregulated by co-treatment with LEN and miR-22 mimic in hypoxia-stimulated cells (p < .05 or p < .01, Figure 4(D)). These findings suggested that transfection with miR-22 mimic impaired the promoting effects led by LEN, which further indicated that the promoting effects of LEN in PNCM and H9c2 cells were through downregulation of miR-22.

### LEN activated PI3K/AKT and wnt/β-catenin signal pathways via downregulation of miR-22

To reveal whether the expression of miR-22 was also involved in modulating signal pathways, further experiments were performed. Results demonstrated that overexpression of miR-22 decreased the phosphorylated level of PI3K and AKT (p < .05 or p < .01) (Figure 5(A)). Additionally, up-regulation of β-catenin by LEN was abolished by miR-22 mimic (p < .01) (Figure 5(B)). Collectively, miR-22 overexpression impaired the effects of LEN in activating PI3K/AKT and Wnt/β-catenin signal pathways.

In addition, due to Sirt1 was reported to be in the same pathway with miR-22 [20], and Sirt1 was also demonstrated as a highly effective therapeutic target for protection against IR injury [21]. Therefore, we further detected the expression of Sirt1 after miR-22 overexpression and its relationship with miR-22. Of note, we found that hypoxia downregulated Sirt1 expression compared with control (p < .01). Moreover, mi-22 upregulation significantly decreased the accumulated level of Sirt1 (p < .01, Figure 5(C)). In addition, luciferase activity assay showed that miR-22 mimic-transduced cells showed lower luciferase activity compared with NC in Sirt1 wild type (p < .01, Figure 5(D)), which suggested that Sirt1 was a target of miR-22.

### LEN blocked hypoxia-induced apoptosis of H9c2 cells by upregulation of Sirt1

To validate the role of Sirt1 in the effects of LEN in protecting H9c2 cells against hypoxia-induced apoptosis, si-Sirt1 was
transfected into H9c2 cells. The low expression of Sirt1 in the group of transfection with si-Sirt1 indicated the high transfection efficiency ($p < .01$, Figure 6(A)). Then, we found the number of apoptotic cells was not decreased by LEN in si-Sirt1-transduced cells in hypoxic circumstance ($p < .05$) (Figure 6(B)) with enhanced ratio of Bax to Bcl-2 and cleavage of caspase-3 (both $p < .01$) (Figure 6(C)). Moreover, we also detected Sirt1 silence blocked the activation of these two signal pathways (both $p < .01$) (Figure 6(D,E)). Taken together, we concluded that LEN could block apoptosis of H9c2 cells dependent on Sirt1 up-regulation.

**Discussion**

In our study, we investigated the effects of LEN on hypoxia-induced injuries of PNCM and H9c2 cells. Results showed that LEN has significant effects in maintaining viability and retarding apoptosis induced by hypoxia, as well as activating PI3K/AKT and β-catenin signal pathways. Further experiments indicated that LEN regulated the down-regulation of miR-22 which might be associated with its protective properties against hypoxia-elicited apoptosis.
PNCM and H9c2 cell lines are often used to establish the myocardial disease model in vitro [22,23]. In the current study, we observed that hypoxia decreased cell viability and increased cell apoptosis, which indicated that hypoxia induced the cellular injuries of PNCM and H9c2 cells. When administrated with LEN prior to hypoxia treatment, the viability of PNCM and H9c2 cells was enhanced by LEN in a dose-dependent manner while apoptosis was inhibited to some extent, which indicated that LEN inhibited hypoxia-induced cell injury in cardiomyocytes PNCM and H9c2 cells. The ratio of Bax to Bcl-2 and the cleavage of caspase-3 are two important apoptosis-related indications [24,25]. The decreased ratio of Bax to Bcl-2 and suppressive cleavage of caspase-3 by LEN in hypoxia-treated cell indicated that LEN inhibited cell apoptosis to some extent. Our results are consistent with the previous study that LEN protects pancreatic β cells from streptozotocin-induced injury [26].

Previous studies have reported that the amount of medicine or effective treatment approach was achieved through targeting PI3K/AKT and β-catenin pathways [18,27,28]. In the current study, we found that hypoxia inactivated PI3K/AKT while LEN activated this pathway, which suggested that the activation of PI3K/AKT was related with role of LEN in inhibiting cell growth. In addition, β-catenin expression was upregulated by hypoxia while was further upregulated by LEN. Of course, the alteration of β-catenin might not be directly ascribed to hypoxia treatment or LEN administration since AKT has been reported to phosphorylate β-catenin which increases its transcriptional activity [29]. Our study confirmed the point that PI3K/AKT and β-catenin were closely involved in the regulation system of LEN in hypoxia-treated cells. Our study was in line with the former studies that PI3K/AKT and β-catenin pathways were implicated in the protective process initiated by adipose-derived mesenchymal stem cells exosomes and natural compound rutin against cardiomyocytes injuries [30,31].

Next, we asked whether miRNAs were involved in the effects of LEN on hypoxia-stimulated H9c2 cells. miR-22 attracted our attention and we explored it in our study because of its close connection to the myocardial ischemia [16,32]. Interestingly, we found that miR-22 was significantly upregulated by hypoxia while decreased by LEN, which suggested that miR-22 might be involved in hypoxia-induced injuries as well as the protective role of LEN. This result was in line with the former study that upregulating miR-22 promoted H9c2 cell injury [17]. Further experiments were performed to identify the exact functions of miR-22 during the protection of LEN. As expected, we observed these interesting results that transfection with miR-22 mimic blocked the promoting effects of LEN in hypoxia-treated cell as evidenced by inhibiting viability and enhancing apoptosis. These results showed that the promoting effects of LEN on PNCM and H9c2 cells were through downregulation of miR-22. It is for the first time that LEN was reported to function through down-regulating miR-22.

In addition, we also investigated the underlying mechanism by which miR-22 achieved its functions. From the former report, we obtained the information that Sirt1 was a target of miR-22 and the functions of miR-22 were reached through targeting Sirt1 [33,34]. However, that research demonstrated the functions of miR-22 in regulating proliferation, motility, and invasion in human glioblastoma cells, then we asked what role of Sirt1 is during the process that miR-22 functioned in H9c2 cells. As predicted, Sirt1 was confirmed to be a target of miR-22 in H9c2 cells. Moreover, we also found that silence of Sirt1 promoted apoptosis which was accompanied by the bluntness of PI3K/AKT and β-catenin signal pathways. These results demonstrated that silence of Sirt1 revealed the similar effects with miR-22 mimic, suggesting that LEN regulated the apoptosis process might also be through modulating Sirt1. Taken all these together, the effects of LEN on apoptosis and signal pathways were through regulating miR-22 targeted by Sirt1.

Conclusions

In conclusion, we found that LEN has the potential effects in relieving hypoxia-induced apoptosis through down-regulating miR-22. PI3K/AKT and β-catenin were also involved in the biological process. It might provide some research foundation for further studies in the treatment of myocardial ischemia.

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Disclosure statement

Authors declare that there are no conflicts of interest.

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