Levosimendan Postconditioning Attenuates Cardiomyocyte Apoptosis after Myocardial Infarction

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1. Introduction

Acute or chronic myocardial infarction is caused by myocardial cell ischemia following coronary artery obstruction, resulting in secondary myocardial cell necrosis [1]. Globally, myocardial infarction is associated with high death rates and significantly high treatment expenses [2, 3]. Myocardial infarction is a serious type of coronary heart disease, which is caused by acute or chronic myocardial ischemia after coronary artery system lumen or obstruction due to various reasons. Furthermore, myocardial necrosis occurs, which can lead to heart rupture, ventricular septal perforation, papillary muscle rupture, cardiogenic shock, heart failure, and even death. Therefore, the diagnosis of MI, device and drug treatment, and prevention as well as mechanism research on how to improve the function of cardiomyocyte are becoming the focus of research. It is a great significance for improving the heart function and survival rate of patients with MI [1, 4].

Levosimendan, a novel calcium sensitizer, has various pharmacological cardiovascular benefits, including positive inotropic with energy-saving cardiotonic effects, anti-inflammatory effects, and antiapoptotic effects [5, 6]. During open-heart surgery, levosimendan has been successfully used to treat preoperative and perioperative cardiac failure [7, 8]. Levosimendan preconditioning prevented myocardial ischemic damage and inhibited cardiomyocyte apoptosis in ischemia-reperfusion (I/R) models [9–13]. Clinically, in addition to ischemia-reperfusion injury, a significantly high number of patients present with myocardial infarction (MI)
due to prolonged acute coronary artery occlusion. MI is associated with cardiomyocyte apoptosis, which is characterized by an irreversible loss of functioning cardiomyocytes, a reduction in coronary flow reserves, and cardiac dysfunctions [14]. In normal treatment, applications of levosimendan before acute myocardial infarction may be restricted [15]. Ischemic postconditioning has been shown to significantly reduce infarct sizes while exerting cardioprotective effects [16–19]. Therefore, MI patients may benefit from alternative therapeutic strategies including postconditioning to rescue ischemic myocardium and improve their surgical or survival outcomes. Recent studies have proposed levosimendan postconditioning treatment for MI. However, it has not been established whether levosimendan exerts protective effects against cardiomyocyte apoptosis following MI.

Levosimendan has been used well in clinical practice. To elucidate on the therapeutic effects of levosimendan postconditioning following MI, we investigated its antiapoptotic effects in in vitro anoxia-reoxygenation (A/R) models. Then, we established in vivo acute myocardial infarction (AMI) models to observe the effect of levosimendan on cardiomyocyte after MI. Thus, we will clarify a point to determine whether this treatment has protective effects against cardiomyocyte apoptosis following MI. This effect has a positive significance for clarifying the relevant mechanism of cardiomyocyte apoptosis.

2. Materials and Methods

2.1. Establishment of In Vitro H9c2 Cell Anoxia-Reoxygenation (A/R) Models. H9c2 cells were cultured in high-glucose Dulbecco’s modified Eagle medium (DMEM) and presaturated for 1h with 95% N2/5% CO2. To ensure (PO2) ≤ 4.0 kPa, O2 partial pressure was measured using a blood gas analyzer. Additionally, a reoxygenation medium was prepared and presaturated for 1h with 95% O2/5% CO2 to maintain PO2 = 20.9 kPa.

The H9c2 cells (Shanghai Institute for Biological Sciences) were cultured in the anoxic media for 3h and centrifuged. Then, anoxic H9c2 cells were placed in reoxygenation media for 2h after which the cell suspension was centrifuged to obtain the cells. The anoxic/reoxygenated H9c2 cells were divided into two groups: A/R and the A/R + levosimendan (A/R + Levo) groups. Cells in the A/R group were cultured in normal media, while those in the A/R + Levo group were cultured in normal media supplemented with 0.3 μmol/L levosimendan (QILU Pharmaceutical, China). Additionally, control group cells without A/R were cultured in normal media. After 24h, H9c2 cells from each group were digested, centrifuged, harvested, and used in subsequent experiments.

2.2. Flow Cytometry. An FITC Annexin V Apoptosis Detection Kit (BD, USA) was used to assay apoptotic H9c2 cells. In each fluorescence-activated cell sorting (FACS) tube, 100 μL of cell suspension (1.0 × 10⁶ cells), 100 μL of staining buffer, and 5 μL of annexin V and propidium iodide were added, after which the samples were incubated as instructed by the manufacturer. Apoptotic cells were evaluated by a FACSCanto II flow cytometer with the FACSDiva 6.1.2 software (BD, USA) used for data analysis.

2.3. RT-qPCR. Total RNA was extracted from H9c2 cells using the TRIzol reagent (Invitrogen, USA), while the All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopeia, USA) was used for first-strand cDNA synthesis as instructed by the manufacturers. FastStart Universal SYBR Green Master (Roche, Germany) and the PikoReal 96-well system (Thermo Fisher, USA) were used for RT-qPCR analyses, which were performed in three independent experiments. Primers were designed using the Primer 5 software (Premier Biosoft International, Canada), with 5-CTTCGTCG-CTACCGTCGC-3 and 5-TCCCCCGTTCACCCCATC-3 for Bcl2, 5-GGAACGAACGGACCTGTGGA-3 and 5-CGCGTGGCTGATAGTAAAGCA-3 for Caspase 3, and 5-CCGCAGGATTTGGAGATGTA-3 for Bax (all primers were synthesized by Sangon Biotech, China). The amount of each RNA sample was normalized to that of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the internal control.

2.4. Western Blotting. Caspase-3, Bax, and Bcl-2 were extracted from H9c2 cells and their expressions analyzed. After H9c2 cell lysis, protein concentrations were determined using a BCA protein assay kit (Beyotime, China). Protein samples (40 μg) were loaded onto polyacrylamide gels, electrophoresed, and transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked for 1h using 5% nonfat dry milk. The blots were probed with Bax (1:5000 dilution, Abcam, UK), Caspase-3 (1:2000 dilution, Abcam, UK), and Bcl-2 (1:1000 dilution, Abcam, UK) antibodies. Goat-anti-rabbit IgG (1:5000 dilution, Millipore, USA) and goat anti-mouse IgG (1:5000 dilution, Millipore, USA) were used as secondary antibodies. The membranes were incubated 3–5 seconds with the enhanced chemiluminescence (ECL) detection reagent (Millipore, USA). The β-actin antibody (1:1000 dilution, Sigma-Aldrich, USA) was used as the internal standard. Protein bands were analyzed using the ImageJ software, with their concentrations being estimated relative to those of β-actin.

2.5. In Vivo Assays. Male Sprague-Dawley (SD) rats (160–200 g) were purchased from Kunming Medical University (Kunming, China). This study was approved by the Ethical Committee of Kunming Medical University and all animal procedures were performed in accordance with the National Institute of Health (NIH Publication No. 85–23, revised 2011) Guide for the Care and Use of Laboratory Animals. Rats were randomized into three groups: sham-operated group (sham group), MI group, and levosimendan postconditioning group (MI + Levo group).

Rats were anesthetized with intraperitoneally administered sodium pentobarbital (30 mg/kg), intubated, and ventilated during operation. The heart was exposed via left
lateral thoracotomy and pericardectomy. Then, using a 6.0 Prolene suture, the left anterior descending branch of the coronary artery was identified and gently ligated. Sham group rats were subjected to the same procedures, apart from ligation of the left anterior descending branch of the coronary artery. Successful MI was confirmed by observing myocardium color change from red to pale. Immediately following MI, rats in the MI + Levo group were intravenously infused with levosimendan (1.2 μg/kg/min for 10 min followed by 0.2 μg/kg/min for 1 h), while rats in the sham and MI groups were administered with equal amounts of 5% glucose solution.

2.6. Echocardiography. Transthoracic echocardiographic tests were performed on 7-day-old anesthetized rats using a GE VIVID E9 system equipped with a 10.0 MHz probe. Left ventricle ejection fraction (LVEF), left ventricle end-diastolic diameter (LVEDd), and fractional shortening (FS) were obtained.

2.7. Tetrazolium Chloride (TTC) Staining. Following MI, all rats were sacrificed on the 7th day. Hearts were rapidly removed, washed, and placed in ice for 30 min at −20°C. Myocardial tissues from the left ventricle were sliced into 2 mm sections, which were then placed in 24-well plates. TTC was added to the wells, and the sections were incubated at 37°C for 30 min, followed by rinsing. Infarct areas in the left ventricle were marked and quantified using the Image-Pro Plus 6.0 software. The percentage of infarct area (%) = (Sn/Se) × 100%, where Sn is the infarct area (white) and Se is the entire cross-sectional area in the same layer.

2.8. Terminal Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining. Myocardial tissue samples were harvested from the left ventricle and fixed overnight in neutral buffered formalin. Then, they were dehydrated, cleared, paraffin-embedded, and sliced into 5 μm sections for apoptosis analysis. Cardiomyocyte apoptosis was detected using the TUNEL assay kit (Roche, Germany). Nuclei of apoptotic cells were stained brown, while the nuclei of normal cells were stained blue. Moreover, morphological characteristics of apoptotic cells, including karyopyknosis, nuclear fragmentation, and karyolysis, were evaluated. Ten randomly selected high-powered fields (200× magnification) were examined in each section. Apoptotic rates (%) = (the number of apoptotic cells/total number of cells) * 100.

2.9. RT-qPCR. Total RNA was extracted from frozen myocardial tissue samples using the TRizol reagent (Invitrogen, USA), as instructed by the manufacturer. The rest of the procedures were similar to those used in RT-qPCR of H9c2 cells.

2.10. Western Blotting. Protein levels of Caspase-3, Bax, and Bcl-2 were evaluated using total protein extracted from myocardial tissue samples. The rest of the procedures were similar to those of western blot analysis as performed for H9c2 cells.

2.11. Immunohistochemical Staining. Paraffin-embedded sections were dewaxed and rehydrated, after which antigen retrieval was performed. To inactivate endogenous enzymes, sections were incubated with 3% H2O2 in deionized water at 37°C for 10 min. Then, they were incubated at 4°C overnight in the presence of anti-Bax (1:100 dilution, Abcam, UK), anti-Caspase-3 (1:100 dilution, Abcam, UK), and anti-Bcl-2 (1:100 dilution, Abcam, UK) primary antibodies. A biotin-labeled secondary antibody (ZsBio, China) was added, followed by incubation at 37°C for 40 min. Then, sections were stained with diaminobenzidine (DAB) for 5 min, counterstained with hematoxylin for 5 min, dehydrated, hyalinized, and sealed with neutral balsam. To detect brown cytoplasmic granules, sections were observed by light microscopy (Zeiss, Germany) connected to an Advanced 310 Image Processing system (Motic, Canada), to obtain the images.

2.12. Statistical Analysis. The SPSS 13.0 statistical software (IBM, USA) was used for all statistical analyses. Data are presented as mean ± SD. Between-group differences were assessed by the paired Student’s t-test. p ≤ 0.05 was considered statistically significant.

3. Results

Levosimendan postconditioning inhibited H9c2 cell apoptosis in A/R models by elevating Bcl-2 and suppressing Caspase-3 as well as Bax expressions at the mRNA and protein levels.

Flow cytometry was performed to investigate apoptosis levels in each group of H9c2 A/R cell models (Figures 1(a) and 1(b) and Table 1). The A/R + Levo group exhibited a significantly low rate of apoptotic cells, compared to the A/R group (A/R + Levo 12.14 ± 0.94 versus A/R 21.41 ± 0.51, p < 0.05). To establish the apoptotic factors involved in the effects of levsimendan postconditioning in A/R H9c2 cell models, RT-qPCR (Figure 1(c) and Table 2) and western blotting (Figures 1(d) and 1(e) and Table 3) were performed to determine mRNA and protein levels of Caspase-3, Bax, and Bcl-2. Compared to the A/R group, the A/R + Levo group exhibited significantly suppressed Caspase-3 (A/R + Levo 1.17 ± 0.09 versus A/R 2.06 ± 0.13, p < 0.05) and Bax (A/R + Levo 1.91 ± 0.03 versus A/R 5.67 ± 0.34, p < 0.05) expressions at the mRNA levels, while Bcl-2 mRNA levels were significantly elevated (A/R + Levo 0.76 ± 0.04 versus A/R 0.32 ± 0.006, p < 0.05) (Figure 1(c) and Table 2, p < 0.05). Additionally, as shown in Figures 1(d) and 1(e), Bcl-2 protein levels were significantly upregulated (A/R + Levo 0.76 ± 0.06 versus A/R 0.63 ± 0.03, p < 0.05), while Caspase-3 (A/R + Levo 0.12 ± 0.02 versus A/R 0.26 ± 0.04, p < 0.05) and Bax (A/R + Levo 0.40 ± 0.06 versus A/R 0.81 ± 0.06, p < 0.05) protein levels were markedly downregulated in the A/R + Levo group, compared to the A/R group (Figures 1(d) and 1(e) and Table 3, p < 0.05). Compared to the control group, in the A/R + Levo group,
Table 1: Apoptosis rates of H9c2 cells in each group 24 h after A/R induction.

|          | Control     | A/R         | A/R + Levo  |
|----------|-------------|-------------|-------------|
| Apoptosis (%) | 7.58 ± 0.92 | 21.41 ± 0.51 | 12.14 ± 0.94 |

Data are presented as mean ± SD for n = 5. A/R: anoxia-reoxygenation; Levo: levosimendan. *p < 0.05, A/R + Levo group versus A/R group; #p < 0.05, A/R + Levo group versus control group; ∆p < 0.05, A/R group versus control group.

Table 2: mRNA expressions of Caspase-3, Bax, and Bcl-2 in H9c2 cells of each group 24 h after A/R induction.

|          | Control     | A/R         | A/R + Levo  |
|----------|-------------|-------------|-------------|
| Caspase-3 | 1           | 2.06 ± 0.13  | 1.17 ± 0.09  |
| Bcl-2     | 1           | 0.32 ± 0.006 | 0.76 ± 0.004 |
| Bax       | 1           | 5.67 ± 0.34  | 1.91 ± 0.03  |

Data are presented as mean ± SD for n = 5. A/R: anoxia-reoxygenation; Levo: levosimendan. *p < 0.05, A/R + Levo group versus A/R group; #p < 0.05, A/R + Levo group versus control group; ∆p < 0.05, A/R group versus control group.
Bcl-2 protein levels were suppressed (A/R + Levo 0.76 ± 0.06 versus control 0.98 ± 0.12, p < 0.05), while Caspase-3 (A/R + Levo 0.12 ± 0.02 versus control 0.13 ± 0.04, p < 0.05) and Bax (A/R + Levo 0.40 ± 0.06 versus control 0.34 ± 0.04, p < 0.05) protein levels were elevated. However, differences in expression levels between the control and A/R groups were not significant (Figure 1(e) and Table 3, p > 0.05).

3.1. Levosimendan Postconditioning Improved Cardiac Functions in MI Models. Echocardiographic measurements revealed that, on the 7th day after MI, rats receiving levosimendan postconditioning exhibited improved cardiac functions, compared to the MI group. Additionally, they exhibited significantly elevated LVEF and FS (Table 4, p < 0.05). As indicated by the significantly reduced LVEF as well as FS and elevated LVEDd, cardiac functions of the MI group were markedly decreased, compared to the sham group (Table 4, p < 0.05).

Livosimendan postconditioning attenuated myocardial injury and protected the myocardium against MI-induced cardiomyocyte apoptosis by elevating mRNA and protein levels of Bcl-2 while suppressing those of Caspase-3 and Bax. Of the thirty rats, MI was successfully established in 21 rats (70% success rate). The remaining 9 rats died due to anesthetic overdose, cardiac rupture, or other causes.

As shown in Figure 2(a), TTC staining revealed reduced areas of left ventricle infarctions in MI + Levo and MI groups (Figure 2(a)). The MI + Levo group had a lower percentage of infarct area, compared to the MI group (MI + Levo 18.03 ± 0.47 versus MI 20.58 ± 1.14, p < 0.05, Figure 2(b) and Table 5). TUNEL staining was performed to assess cardiomyocyte apoptosis following MI (Figures 2(a) and 2(c)). Compared to the control group, the MI and MI + Levo groups exhibited significantly more TUNEL-positive cell nuclei (brown) (Figure 2(a)). Interestingly, following MI, the MI + Levo group exhibited a lower percentage of apoptotic cardiomyocytes (brown), compared to the MI group (MI + Levo 0.15 ± 0.01 versus MI 0.21 ± 0.02, p < 0.05, Figure 2(c) and Table 6). RT-qPCR analysis revealed elevated Bcl-2 mRNA levels (MI + Levo 0.50 ± 0.08 versus MI 0.23 ± 0.07, p < 0.05) as well as significantly suppressed Caspase-3 (MI + Levo 2.84 ± 0.63 versus MI 5.73 ± 0.45, p < 0.05) and Bax (MI + Levo 7.91 ± 0.03 versus MI 15.67 ± 0.08, p < 0.05) mRNA levels in the MI + Levo group, compared to the MI group following MI (Figure 2(d) and Table 7). Western blotting analysis of myocardial tissues from each group revealed that, compared to the MI group, MI + Levo group had significantly suppressed Caspase-3 (MI + Levo 2.80 ± 0.02 versus MI 0.46 ± 0.04, p < 0.05) and Bax (MI + Levo 0.76 ± 0.06 versus MI 1.13 ± 0.16, p < 0.05) protein levels as well as elevated Bcl-2 protein levels (MI + Levo 0.87 ± 0.08 versus MI 0.69 ± 0.05, p < 0.05) (Figures 2(e) and 2(f) and Table 8). The immunohistochemical staining results for Caspase-3, Bax, and Bcl-2 were consistent with RT-qPCR and western blot analysis results (Figure 2(a)).

4. Discussion
Levosimendan preconditioning has been shown to protect cardiomyocytes against apoptosis [9–13]. Moreover, it exerted comparable antiapoptotic effects in organs such as the liver, lungs, and kidneys [20–24]. Even though recent studies have reported that pharmacological post-conditioning is a potential method for preventing cardiac injury [25, 26], studies on levsimendan postconditioning are few, compared to those on levsimendan preconditioning. Generally, levsimendan is used in cases of appropriate clinical indications, such as definitive MI [5, 7, 8]. Based on findings from basic research and clinical practice, a hypothesis regarding the influence of levsimendan postconditioning on MI-induced myocardial apoptosis has been proposed. However, mechanisms of this levsimendan–conferred cardioprotection remain unclear. In this study, an in vitro A/R model, which is a suitable model for cell oxygen deprivation, was used [27, 28]. In A/R cell models, levsimendan postconditioning reduced the number of apoptotic H9c2 cells, compared to the control group. It is providing stronger and more direct evidence of levsimendan protective effects against reperfusion injury in clinical application.

It has been reported that one of the apoptotic pathways is tightly regulated by proteins from the B-cell lymphoma-2 (Bcl-2) family, such as Bcl-2, which have the ability to prolong cell survival by inhibiting apoptosis [29]. On the other hand, Bax, a member of the Bcl-2 family, promotes cell apoptosis [30]. Caspase-3, a member of the cysteine protease family, is a key apoptotic mediator and has the ability to cause cell death, as evidenced by the presence of cell lysates [31]. In A/R models in this study, levsimendan postconditioning enhanced Bcl-2 mRNA and protein levels while suppressing those of Caspase-3 and Bax. These findings could explain why levsimendan postconditioning attenuated H9c2 cell apoptosis. Moreover, levsimendan

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**Table 3:** Protein levels of Caspase-3, Bax, and Bcl-2 in H9c2 cells of each group 24 h after A/R induction.

|         | Control | A/R | A/R + Levo |
|---------|---------|-----|------------|
| Caspase-3/β-actin | 0.13 ± 0.04 | 0.26 ± 0.04* | 0.12 ± 0.02* |
| Bax/β-actin | 0.34 ± 0.04 | 0.81 ± 0.06* | 0.40 ± 0.06* |
| Bcl-2/β-actin | 0.98 ± 0.12 | 0.63 ± 0.03* | 0.76 ± 0.06* |

Data are presented as mean ± SD for n = 5. A/R: anoxia-reoxygenation; Levo: levosimendan. *p < 0.05, A/R + Levo group versus A/R group; ∆p < 0.05, A/R + Levo group versus control group; †p < 0.05, A/R group versus control group.

**Table 4:** Parameters of cardiac functions in rats from each group 7 days after MI model establishment.

|         | Sham | MI | MI + Levo |
|---------|------|----|-----------|
| LVEF (%) | 76.99 ± 3.00 | 49.85 ± 2.65* | 57.25 ± 3.80** |
| FS (%) | 50.11 ± 3.24 | 42.15 ± 2.16* | 46.43 ± 2.93** |
| LVEDd (mm) | 4.90 ± 0.19 | 7.69 ± 0.21* | 6.68 ± 0.19** |

Data are presented as mean ± SD for n = 7. LVEF: left ventricular ejection fraction; FS: fractional shortening; LVEDd: left ventricular end-diastolic diameter; MI: myocardial infarction; Levo: levosimendan; *p < 0.05, MI + Levo group versus MI group; †p < 0.05, MI + Levo group versus control group; ‡p < 0.05, MI group versus control group. |
Figure 2: (a, b) The left ventricular infarct area was detected by TTC staining in each group 7 days after MI model establishment (n = 3). (a, c) Representative TUNEL staining analysis of cardiomyocyte apoptosis in rats from each group 7 days after MI model establishment (n = 4). Normal cell nuclei are stained blue, while apoptotic cell nuclei are stained brown (magnification: × 200). (a) Immunohistochemical staining of Caspase-3, Bax, and Bcl-2 in rats from each group 7 days after MI model establishment (n = 4). Normal and apoptotic cell nuclei are stained blue and brown, respectively (magnification: × 100). (d) Caspase-3, Bax, and Bcl-2 mRNA levels in rats of each group 7 days after MI model establishment, as determined by qRT-PCR analysis (n = 4). (e, f) Representative western blot images and quantitative analyses of Caspase-3, Bax, and Bcl-2 protein levels in rats from each group 7 days after MI model establishment (n = 4). MI: myocardial infarction; Levo: levosimendan; # p < 0.05, MI+Levo group versus MI group; Δp < 0.05, MI+Levo group versus control group; * p < 0.05, MI group versus control group.
postconditioning exerted protective effects on H9c2 A/R cells.

Based on findings from in vitro experiments, we hypothesized that levosimendan can suppress myocardial apoptosis in vivo following MI. In MI rat models, compared to control treatment, levosimendan postconditioning after MI significantly reduced the number of apoptotic cardiomyocytes. Notably, it upregulated mRNA and protein levels of Bcl-2 while suppressing those of Caspase-3 and Bax in MI rat models, compared to control group. Histologically, levosimendan postconditioning was associated with a reduced left ventricular infarct area, compared to control treatment. Additionally, it partially improved cardiac functions following MI on the 7th day. Therefore, a reduction in the number of apoptotic cardiomyocytes and significant downregulation of proapoptotic protein levels following levosimendan postconditioning are beneficial factors in reducing the left ventricular infarct area and partially improving cardiac functions. Although this finding suggested that the therapeutic effect observed in our study was insufficient to completely reverse the damage caused by MI, our findings indicated a protective effect of levosimendan postconditioning against myocardial apoptosis. In other words, while some of the MI-induced apoptosis or necrosis in cardiomyocytes was irreversible, our findings revealed that levosimendan postconditioning can attenuate cardiomyocyte apoptosis and improve cardiac functions.

Since the number of viable cardiomyocytes is critical for cardiac function improvements, levosimendan postconditioning, which exerts cardioprotective effects by inhibiting cardiomyocyte apoptosis following MI, may improve patient prognoses, thereby providing alternative strategies for interventions or surgical treatment.

5. Conclusions

Levosimendan inhibited apoptosis in H9c2 A/R cell models. Besides, in vivo, levosimendan postconditioning of MI rat models conferred cardioprotection by inhibiting cardiomyocyte apoptosis and improving cardiac functions.

Data Availability

The simulation experiment data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Ying Xie and Zhengjiang Xing are the co-first authors.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

Ying Xie and Zhengjiang Xing contributed equally to this work.

Acknowledgments

This work was supported in part by the Funds of Advanced Health Technical Project of Yunnan Province (no. H-2018028), Natural Science Foundation of Science Department of Yunnan Province (nos. 202102AA310003-12 and 2018FE001-276), and Health Science and Technology Project of Kunming (nos. 2018-SW-06 and 2020-04-02-001).

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