Salvianolic Acid B Protects Against Myocardial Ischemic Injury Through Inhibiting Apoptosis and Promoting Autophagy

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Research

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Abstract

Aim of the study: Salvianolic acid B (Sal B) as a natural compound extracted from Salvia miltiorrhiza, has been extensively used to protect cardiomyocytes from myocardial ischemia. Although Sal B has shown evident effects on cardiovascular diseases, the detailed mechanism is still unclear as yet. Herein, we intended to explore the protective effects of Sal B on myocardial ischemic injury and the underlying mechanism.

Methods and Results: Western blotting, immunofluorescence assay, flow cytometry and lentiviral transfection were performed. The mice with myocardial ischemic injury were intravenously given 10 mg/kg Sal B once daily for seven days, and then H9c2 cells were treated with Sal B (20, 40, 80 μmol/L). Sal B treatment protected cardiomyocytes from myocardial ischemia through relieving apoptosis. Transmission electron microscopy and fluorescence microscopy exhibited that Sal B significantly increased autophagic lysosomes and vacuoles in H9c2 cells. Administration with Sal B significantly up-regulated the expressions of autophagy-related factors such as LC3, Atg5 and Beclin 1 in H9c2 cells and myocardial tissues. The beneficial autophagic changes induced by Sal B were abrogated through pharmacological inhibition.

Conclusions: This study provides a molecular mechanism by which Sal B potently inhibits apoptosis and oxidative stress upon myocardial ischemia by activating the AMPK-autophagy pathway. Sal B is a potential agent for treating myocardial ischemia.

1. Introduction

Ischemic heart disease has seriously affected patient quality of life worldwide and become the leading cause of death (Zhang et al., 2016). Although the mechanism by which myocardial ischemia leads to heart lesions remains elusive, cardiomyocytes of the ischemic heart undergo considerable metabolic changes, such as mitochondrial dysfunction, excess oxidative stress and cell death (Chen et al., 2016; Chen et al., 2014). Thus, therapeutic interventions focusing on relieving mitochondrial disorder, oxidative stress and cell death may efficiently promote the prevention and treatment of ischemic heart disease.

Autophagy can eliminate damaged organelles under starvation conditions, and maintain the energy and viability of cardiomyocytes (Delbridge et al., 2015; Ge et al., 2011; Han et al., 2012; Kang and Avery, 2008). This process widely exists in eukaryotic cells and helps to maintain homeostasis by recycling aging or damaged organelles. Under stresses such as nutritional deficiencies and hypoxia, autophagy is enhanced via various pathways to meet normal metabolic requirements and to maintain cell survival (Liu et al., 2016). During myocardial ischemia, autophagy is augmented in cardiomyocytes, which produces ATP to alleviate energy crisis and eliminates intracellular ubiquitinated proteins to maintain homeostasis. However, activation of autophagy excessively in the late stage of myocardial ischemia may aggravate heart lesions.
Danshen, a traditional Chinese herb, has been widely used to clinically treat coronary artery disease, angina pectoris and myocardial infarction for thousands of years (He et al., 2008; Jiang et al., 2010). One of the major water-soluble components in Danshen is Salvianolic acid B (Sal B). Emerging evidence has verified that Sal B had the effects of not only sedative, antioxidant, hepatoprotective and antifibrogenic (Lin et al., 2006) but also improved coronary microcirculation and cerebral blood flow (Tang et al., 2002).

Although Sal B can prevent cardiomyocytes from myocardial ischemia, the detailed mechanism is still unclear hitherto. Herein, we intended to assess the protective effects of Sal B on myocardial ischemic injury and the underlying mechanism. To investigate whether Sal B protected against myocardial ischemia in vitro and in vivo and explore the molecular mechanism is the primary aim of this study.

2. Material And Methods

2.1 Chemicals and Reagents

Sal B was obtained from Nanjing Hongqiao Medical Technology Research Institute (No. 151218, China; purity > 98.0%). The DMEM was purchased from Thermo Fisher Scientific Inc. (Waltham, USA). Fetal bovine serum (FBS) and glucose-free DMEM (GFM) were obtained from Gibco (Grand Island, NY, USA). Annexin V-FITC/PI apoptosis detection kit, Hoechst 33258 and autophagy detection kit were obtained from Millipore (Billerica, MA, USA). The protease inhibitor, Lyso-Tracker Red, BCA, phosphatase inhibitor and mitochondrial membrane potential (Δψm) detection kits were purchased from Beyotime Biotechnology Co., Ltd. (Haimen, China). Anti-LC3A/B antibody was obtained from Cell Signaling Technology Inc. (Boston, MA, USA). Anti-Bax, anti-Beclin 1, anti-caspase-3, anti-Apg5 and anti-ULK1 antibodies were obtained from Abcam Inc. (Cambridge, UK). Atg5 siRNA virus suspension was obtained from Nanjing Dirui Biotechnology Co., Ltd. (China). 3-Methyladenine (3-MA) was purchased from Selleck Chemicals LLC (Houston, CA, USA). DMSO was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Cell culture and sample preparation

The H9c2 cells were obtained from Shanghai Bioleaf Biotech Co., Ltd. (China). The cells were cultured in DMEM supplemented with 10% FBS, 100 µg/ml streptomycin as well as 100 unit/ml penicillin at 37 °C with a humidified atmosphere of 5% CO2. The glucose-starved cells were cultured in high-glucose DMEM for 24 h and incubated in serum-free DMEM and GFM for 3, 6, 12, 24 and 48 h, respectively. The control group was incubated in high-glucose DMEM with 10% FBS. Sal B was dissolved by saline into 100 µmol/L and diluted with GFM to 20, 40 and 80 µmol/L, respectively. The solution of 3-MA was diluted with GFM into 100 µmol/L (Lin et al., 2017).

2.3 Animals

The male ICR mice of ten weeks old were purchased from Suzhou Zhaoyan New Drug Research Center Co., Ltd. (China). With unlimited supply to water and food, all of the mice were maintained in a 12 h/12 h light/dark cycle with controlled temp at (23 ± 1)°C and humidity of (40 ± 5)% All the animals were closely
monitored to ensure that none lived through stress or discomfort. After acclimating 1 week, the mice were divided into 6 groups (n = 10) randomly, i.e. control, isoproterenol (ISO), Sal B (10 mg/kg/day), siNTC + ISO, siAtg5 RNA + ISO and siAtg5 + ISO + Sal B. The drug treatment groups were administered intravenously for 7 days. On the fifth day, all mice except for the control group were administered by intraperitoneal injection with ISO (3 mg/kg/day) for two consecutive days. On the third day after ISO injection, electrocardiogram (ECG) was recorded for 20 min without interruption. After the experiment, blood samples were taken from the eyelids and centrifugated to obtain the plasma which was stored at −80 °C until further analysis. Heart tissues were dissected, rinsed, weighed and stored at -80 °C prior to use, and small amounts of them were stored in 10% formalin for histological analysis. All animal experimental procedures were conducted accordance with the animal ethics of Nanjing University of Chinese Medicine. Every possible effort was made to minimize the suffering of animals and reduce the number of animals used.

2.4 SiRNA treatment

Injected SiAtg5 at 5⋅10^8 pfu/g in 0.1 mL of PBS into the mouse tail vein. The control group was injected with an inactive scrambled siRNA construct in a similar manner. RT-PCR were performed to confirm the inhibition of Atg5 transcription level.

Si-Control and si-Atg5 duplexes contained 3 target sequences. H9c2 cells were seeded in plates of 6-well (200,000/well) and incubated with DMEM containing 10% FBS overnight under normal conditions. After that, before transfection, the cells were cultured in serum-free medium for 2 h and washed twice with cold PBS. According to the manufacturer’s instructions, the pre-designed Atg5-specific siRNAs were transfected into H9c2 cells using transfection reagent. The cells were incubated for 4–6 h, and the medium was replaced with complete growth medium subsequently. After 24 h of transfection, the cells were collected.

2.5 Cell apoptosis analysis

Apoptosis was detected by Hoechst 33258 fluorescence staining according to a previous literature (Gao et al., 2015).

H9c2 cells were seeded in the plate of 6 wells (10,000/well). The cells were washed twice with iced PBS and resuspended in buffer at a concentration of 10^6/ml after treatment with Sal B. Then the cells were mixed with 10 μl of 3 mM PI as well as 10 μl of FITC-conjugated annexin-V reagent, and analyzed by flow cytometry after incubation at room temperature for 15 min. Flow cytometry was conducted by using BD Accuri™ C6 analyzer (Franklin Lakes, USA) with 15 mW argon ion laser (488 nm). Annexin-V staining was determined in the FL1 channel, and PI staining was detected in the FL2 channel. Appropriate quadrants were set, and the percentages of cells negative for stains (viable cells), positive for annexin-V (apoptotic cells) and positive for PI (dead cells) were acquired.

2.6 Western blotting
Western blotting was performed to detect protein expression levels with parameters reported in our previous study (Lin et al., 2016)

2.7 Mitochondrial membrane potential

JC-1 mitochondrial membrane potential assay kit was used to determine the levels of Δψm in H9c2 cells as previously described (Zhang et al., 2013). Observed cells under a fluorescence microscope (Olympus, Tokyo, Japan) and quantified with Image-Pro Plus 6.0 software (Lin et al., 2017). All measurements were performed in triplicate.

2.8 LC3 immunofluorescence

Autophagy was tested by lysosomal and fluorescence staining with parameters reported in our previous study (Lin et al., 2017).

2.9 Monodansylcadaverin staining and autophagic flux detection

Autophagic flux was determined by MDC staining with parameters reported in our previous study (Lin et al., 2017).

2.10 Statistical analysis

All data were collected and analyzed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) and represented as mean ± standard deviation. The results were subjected to Tukey’s post-test for one-way analysis of variance, and Student’s t test were used for comparison between groups. P < 0.05 was considered statistically significant.

3. Results

3.1 Establishment of the model of myocardial ischemia by glucose deprivation

The model of myocardial ischemia was established by culturing H9c2 cells with serum-free DMEM and GFM for 3, 6, 12, 24 and 48 h, respectively (Dan et al., 2016; Wanka et al., 2016). Then the cells were stained by Hoechst 33258 and observed by fluorescence microscopy. The apoptotic cells emitting strong blue fluorescence significantly increased after incubation with GFM for 6 h compared with the control group, and decreased after 12 and 24 h of GFM incubation (Fig. 1A and C).

Flow cytometry showed that the proportions of H9c2 cells undergoing early apoptosis and late apoptosis induced by glucose starvation were significantly different (Fig. 1B, D and E).

In addition, compared with the control group, the Bcl-2/Bax ratio of the group receiving 6 h of glucose deprivation was significantly decreased, and the expression level of cleaved caspase 3 (c-caspase 3) was
3.2 Sal B inhibited GFM-induced H9c2 cell apoptosis and oxidative stress injury

To study whether Sal B protected H9c2 cells under nutrient deficiency conditions, cell apoptosis as well as oxidative stress injury were tested after treatment with Sal B at 20, 40 and 80 μg/mL for 24 h. Apoptosis is typified by cell shrinkage, nuclear fragmentation, chromatin condensation and low Δψm (Hengartner, 2000). Compared with the model group, Sal B observably attenuated DNA fragmentation and condensation and increased Δψm (Fig. 2A-E) in a dose-dependent manner. In addition, compared with the control group, glucose deprivation significantly reduced the Bcl-2/Bax ratio and increased the c-caspase 3 expression in the model group while Sal B treatment significantly elevated the Bcl-2/Bax ratio and decreased the c-caspase 3 expression and ROS levels dose-dependently (Fig. 2F-H), suggesting that Sal B effectively inhibited GFM-induced cell apoptosis and oxidative stress injury.

3.3 Sal B promoted autophagy in glucose-deprived cells

To explore the mechanism by which Sal B protected H9c2 cells from apoptosis and oxidative damage, the bilayer membrane autophagosomal structure (autophagosomes) and LC3 levels were examined by transmission electron microscopy and immunofluorescence staining. As shown in Fig. 3A and D, the fluorescence intensity of LC-3 and amount of autophagosomes markedly increase in Sal B-treated cells compared with those of the model group, indicating that Sal B enhanced autophagy in glucose-starved H9c2 cells. Moreover, the glucose-deprived H9c2 cells were stained with MDC, which is a specific marker for autophagosomes and autophagolysosomes located in the cytoplasm of the autophagic vacuole membrane structures (Shi et al., 2016). Compared with the model group, MDC dots markedly increased in Sal B-treated cells, so Sal B increased autophagic vacuoles in glucose-starved H9c2 cells (Fig. 3E and F).

To clarify the molecular mechanism for the moderating effect of Sal B on autophagy, the expression levels of LC3-II/I, Atg5, Beclin 1 and P62 were detected by Western blotting. Beclin 1 allows the nucleation of autophagic vesicle (Furuya et al., 2005). LC3-II is another common autophagosome marker (Shi et al., 2016). In this study, the conversion levels of LC3-II/I in glucose-deprived H9c2 cells significantly up-regulated with rising dose of Sal B treatment for 24 h (Fig. 3C). Besides, Sal B markedly enhanced the expression levels Atg5 of and Beclin 1, and decreased that of P62 in a dose-dependent manner compared with those of the model group (Fig. 3G). Taken together, Sal B promoted the autophagy of the glucose-starved H9c2 cells.

3.4 Sal B protected glucose-starved H9c2 cells by inducing autophagy

To investigate whether Sal B protected H9c2 cells mainly by activating autophagy, an autophagy inhibitor 3-MA was used. Compared with the Sal B group, the DNA condensation and fragmentation of the Sal B + 3-MA group were evidently enhanced, and markedly decreased Δψm (Fig. 4A and B). Additionally, the ROS release in Sal B- and 3-MA-treated cells significantly exceeded that in the Sal B group (Fig. 4C). Treating
glucose-starved H9c2 cells with 3-MA significantly inhibited Sal B-induced conversion of LC3-I to LC3-II, as evidenced by immunofluorescence staining (Fig. 4D). Furthermore, Western blotting showed that after 3-MA treatment, the expressions of apoptotic proteins Bax and c-caspase 3 significantly increased in glucose-starved H9c2 cells (Fig. 4E), and those of antiapoptotic protein Bcl-2 and autophagy proteins LC3-II/I decreased (Fig. 4F). In addition, the expressions of Beclin 1, Atg5 induced by Sal B were abolished in 3-MA-treated H9c2 cells (Fig. 4G).

After transfection, compared with sham group (DMSO), Atg5 gene expression significantly in the Atg5 siRNA group (Fig. 5A). Western blotting and immunostaining presented that autophagy-associated protein expression was inhibited in the Atg5 siRNA group compared with those in sham and Sal B groups (Fig. 5B and C).

In order to clarify the relationship between autophagy and apoptosis, the expression levels of apoptotic proteins were detected. The expression levels of c-caspase 3 and Bax in the Atg5 siRNA group evidently surpassed those of the sham group. However, Sal B-induced expressions of c-caspase 3 and Bax were reversed by Atg5 siRNA treatment (Fig. 5D). Collectively, Sal B inhibited the oxidative stress damage of glucose-starved H9c2 cells and apoptosis by inducing autophagy.

3.5 Cardioprotective effect of Sal B on myocardial ischemia in mice

As mentioned above, Sal B was in close relationship in the autophagy and apoptosis of H9c2 cells induced by glucose deprivation. To clarify the role of Sal B-induced autophagy in regulating angiogenesis, the mouse model of myocardial ischemia was first established by intraperitoneally injecting ISO. Meanwhile, Atg5 was knocked down by transfection with Atg5 adenovirus.

After ISO administration, ECG showed that the J-point of the model group was significantly elevated, which was recovered to normal with Sal B treatment (Table 1). The optical microscope of the myocardial tissue section of the control mice showed normal myofibril structure with stripes, branching appearance and continuity with adjacent myofibrils. The tissues of the untreated model mice treated with ISO showed obvious cardiomyocyte swelling, degeneration, disappearance of transverse stripes and a large number of infiltrating inflammatory cells. Tissues from Sal B-pretreated mice presented a normal, well-preserved myocardial cell histology (Fig. 6A). Furthermore, Sal B-induced expressions of Bcl-2, Bax and c-caspase 3 proteins were significantly blocked in Atg5 siRNA-treated mice (Fig. 6C). Thus, Sal B significantly protected against myocardial ischemic injury.

Moreover, the LC3 level and the expressions of autophagy-related proteins were measured by immunofluorescence staining, immunohistochemical staining and Western blotting. As shown in Fig. 6B, D and E, Sal B significantly facilitates autophagy in myocardial tissue. However, Sal B-induced changes of autophagy were blocked in the myocardial tissue of Atg5 siRNA-treated mice. In combination with the results of in vitro experiments, Sal B significantly suppressed myocardial ischemic injury and exerted cardioprotective effects by promoting the autophagy of cardiomyocytes.
3.6 Sal B induced AMPK activation and up-regulated autophagic pathways during myocardial ischemia

As the master energy sensor, AMPK can be selectively activated by the binding to aminoimidazole-4-carboxamide ribonucleotide (AICAR) or by glucose deprivation (Krieg et al., 2018). During ischemia, AMPK is activated by low ATP level in cardiomyocytes (Ma et al., 2015), then activating autophagy through AMPK-mTORC1 signaling. Moreover, novel pathways via which AMPK activated autophagy have recently been reported. AMPK can directly phosphorylate and activate ULK1 to initiate autophagy (Alers et al., 2012). Sal B was herein found to stimulate the phosphorylation of AMPK which was significantly attenuated by compound C (Fig. 7A). Additionally, phosphorylated AMPK promoted the formation of bilayer membrane autophagosomal structure by up-regulating ULK1 to recruit Atg13-Atg101-Atg7/Fip100 complexes in the cytosol for binding (Fig. 7B), which further facilitated the formation of autophagy lysosomes and completed the self-degradation and energy reuse of damaged organelles (Fig. 7C-E). Moreover, Sal B activated the AMPK pathway and up-regulated the expressions of autophagy-related proteins in vivo (Fig. 7F). In short, Sal B and autophagy synergistically combated myocardial ischemia through AMPK phosphorylation.

4. Discussion

The incidence rate of cardiovascular disease is soaring worldwide, accompanied by high morbidity from ischemic heart disease. Coronary atherosclerosis (Gimelli et al., 2017), inflammation (Bai et al., 2018), hypotension (Wu et al., 2015) and coronary artery spasm (Kuczkowski, 2006) have been involved in the development of ischemic heart disease. Myocardial ischemia can be treated by drugs, interventional therapy and surgery alone or in combination. In any case, the patients often suffer severe and persistent pain or even death, thereby requiring individualized medication risk-benefit profiles. Therefore, this study provided an innovative strategy for treating myocardial ischemia by oral administration of natural bioactive Sal B.

As one of the most abundant and bioactive salvianolic acids in Danshen (Chen et al., 2006), Sal B has been approved by the Chinese State Food and Drug Administration as a cardioprotective agent for coronary artery disease (State Drug Permit Doc: Z20050249). This compound can protect against injury of the skin, heart and brain caused by ischemia-reperfusion (Du et al., 2000), improve cerebral blood flow as well as inhibit platelet aggregation (Yang et al., 2010). Qiao and Xu reported that Sal B protected cardiomyocytes by inhibiting apoptosis and reducing the formation of peroxynitrite (Qiao and Xu, 2016). However, the antiapoptotic and antioxidative mechanisms underlying the protective role of Sal B in cardiomyocytes remain largely unknown.

The mouse model of myocardial ischemia and glucose starvation-induced cardiomyocytes, which can be established stably within relatively short duration, were used in this study to assess the cardioprotective effects of Sal B in vivo and in vitro, respectively. Sal B significantly attenuated DNA fragmentation and condensation and increased Δψm. Moreover, Western blotting showed that the expression levels of
apoptotic markers (e.g. Bcl-2 and Bax) changed remarkably in Sal B-treated cells and myocardial tissues. Given that the levels of ROS in Sal B-treated cells significantly decreased, Sal B protected cardiomyocytes by suppressing apoptosis and oxidative stress damage.

Autophagy is an evolutionarily conserved catabolic process, in which the inner membrane structure of the cell wraps protein complexes and organelles to degrade and renew these cytoplasmic components (Boya et al., 2013). Thus, it acts as a housekeeper to remove damage organelles (such as broken mitochondria), remove misfolded proteins, eliminate intracellular pathogens and recovered cellular components, and plays an important role in cell homeostasis (Xilouri et al., 2016; Yu et al., 2018). In the case of myocardial ischemic injury, cell energy depletion, apoptosis and oxidative stress may occur, and finally damaged organelles can be eliminated by autophagy (Wu et al., 2019). Normally, autophagy and apoptosis occur simultaneously during starvation, and the interaction between autophagy and apoptosis pathways dominantly controls the initiation of programmed cell death (Boya et al., 2005; Liang et al., 1999). Autophagy can protect cardiomyocytes from oxidative stress and apoptosis, and maintain the energy homeostasis and viability of cell starvation models (Ge et al., 2011; Yan et al., 2005).

To unravel the mechanism by which Sal B protected H9c2 cells from apoptosis and oxidative stress, the levels of LC3 were detected by fluorescence microscopy. Sal B increased autophagic lysosomes, LC3 level, autophagic flux and autophagic vacuoles in H9c2 cells, as indicated by transmission electron microscopy and fluorescence microscopy. Besides, Sal B administration increased LC3-II/I, Beclin 1 and Atg5 expression levels in H9c2 cells and myocardial tissues of ISO-treated mice, suggesting that this compound facilitated autophagy in cardiomyocytes upon myocardial ischemia. Moreover, Sal B-induced beneficial autophagic changes in H9c2 cells were abrogated through pharmacological inhibition and Atg5 siRNA. Finally, the antiapoptotic and antioxidative effects of Sal B on H9c2 cells were blocked by using autophagy inhibitor 3-MA or Atg5 siRNA. Therefore, Sal B prevented apoptosis in the case of myocardial ischemia probably by promoting autophagy. Autophagy is regulated by numerous complex signaling pathways, including AMPK, mTOR and Bcl-2/Beclin 2 (Yang and Klionsky, 2010). To adapt to energy metabolism, AMPK may inhibit mTOR, which induces the activation of autophagy (Gao et al., 2019).

AMPK predominantly regulated cell metabolism and exerted a key role in cardiovascular disease. Activation of AMPK in the heart is an important component of the adaptive response to cell stress that occurs during myocardial ischemia (Qi and Young, 2015). Under glucose starvation conditions, activated AMPK can inhibit mTORC1 to weaken Ser 757 phosphorylation, leading to Ulk1–AMPK interaction. The activation of Ulk1 kinase eventually induces autophagy (Kim et al.). AMPK is an “endogenous survival mechanism" (Qi and Young, 2015), and activation of AMPK pivotally protecting against myocardial ischemic injury (Russell et al.).

We herein found that Sal B suppressed the apoptosis of cardiomyocytes during ischemic injury. In addition, Sal B exerted antiapoptotic effects mainly by promoting autophagy in cardiomyocytes through activation of the AMPK/mTOR pathway. This compound significantly enhanced the level of p-AMPK and
decreased that of p-mTOR. Treatment with compound C decreased the level of autophagy marker LC3-II and increased that of P62 compared with the control group. In contrast, autophagy was facilitated by using AMPK inducer AICAR. Hence, AMPK protects cells by regulating autophagy.

Collectively, the biochemical, molecular and histological results proved that Sal B protected against myocardial ischemia by promoting autophagy and inhibiting apoptosis. As Sal B show no toxic effect at the tested doses and can be found in nature widely, it is a novel cardioprotective agent potentially applicable to the treatment of acute myocardial infarction.

**Conclusion**

From the results of the cell and animal experiments, we demonstrated that Sal B could protect cardiomyocytes from apoptosis by promoting autophagy upon myocardial ischemia. In addition, we verify the efficacy of Sal B in promoting autophagy is mediated by up-regulating AMPK phosphorylation and down-regulating mTOR signaling. Here we provide detailed evidence that a model of myocardial ischemia was replicated at the cellular level by glucose deprivation at different time points, which elucidated the aging relationship between autophagy and apoptosis in the process of ischemia and these properties of Sal B can be further explored to develop eligible anti-MI agents with therapeutic promises.

**Abbreviations**

Sal B: Salvianolic acid B; IHD:Ischemic Heart Disease; FBS:Fetal bovine serum; Δψm:mitochondrial membrane potential; 3-MA:3-Methyladenine; ISO:isoproterenol; ECG:electrocardiogram; GFM:Glucose-Free DMEM; AICAR:aminoimidazole-4-carboxamide ribonucleotide;

**Declarations**

Acknowledgments

Not applicable

Authors’ contributions

Chao Lin and Huimin Bian carried out all the experiments, analyzed data and wrote the manuscript, as well as designed the study, supervised the experiments and validated the data. Qi Chen, Yongming Li, Linxiu Peng, Xiao Wu and Junyi Wang helped with doing experiments and analysing data as well as performed experiments.

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Consent for publication

All authors consent to the publication of this manuscript. Neither the article nor portions of it have been previously published elsewhere.

Competing interests

The authors declare no conflict of interest.

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**Tables**

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.