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# The protective effect of magnesium lithospermate B against glucose-induced intracellular oxidative damage

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ABSTRACT

*Objectives:* To investigate the effects of magnesium lithospermate B (LAB) on intracellular reactive oxygen species (ROS) production induced by high dose of glucose or $H_2O_2$, we explored the influences of LAB on the expression of heme oxygenase-1 (HO-1) and nuclear factor E2-related factor-2 (Nrf2) in HEK293T cells after treatment with high dose of glucose.

*Materials and methods:* The total nuclear proteins in HEK293T cells were extracted with Cytoplasmic Protein Extraction Kit. The ROS level was determined by flow cytometry. The mRNA and protein expression of HO-1 and Nrf2 were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot.

*Results:* LAB reduced the ROS production in HEK293T cells cultured under oxidative stress. High dose of glucose enhanced the expression of HO-1 mRNA and HO-1 protein in a time-dependent manner. LAB enhanced the expression of HO-1 mRNA and HO-1 protein in a dose-dependent manner treated with high dose of glucose. The amount of Nrf2 translocation was enhanced after cells were pretreated with 50 μmol/L or 100 μmol/L LAB. Silencing of Nrf2 gene eliminated the enhanced expression of HO-1 protein induced by high dose of glucose plus LAB.

*Conclusions:* LAB plays an important role against glucose-induced intracellular oxidative damage. The enhanced expression of HO-1 mRNA and HO-1 protein caused by LAB is regulated via Nrf2 signal pathway.



## 1. Introduction

Nowadays, type 2 diabetes mellitus (T2DM) has become a major public health and economic problem in the world. It has been well known that prevalence of T2DM is increasing all over the world. High blood sugar induced oxidative stress is the common pathophysiological pathway in diabetic complications, especially in vascular disease [1,2]. Recent studies suggest that excessive reactive oxygen species (ROS) produced by mitochondrial electron transport chain after stimulation of high dose of glucose may be the first step for diabetes caused endothelial dysfunction [3]. Therefore, the treatment of oxidative stress prevention for patients with diabetes accompanied by vascular disease has become a new research hotspot.

Heme oxygenase-1 (HO-1) is the initial and rate-limiting enzyme of heme metabolism and heme can be decomposed to release free iron, carbon monoxide, and biliverdin, which can be quickly converted to bilirubin. There are three HO isozymes in human, which are HO-1, HO-2, and HO-3 [4]. HO-1, also known as heat shock protein 32 with molecular weight of 32 kDa, has low expression in most organs in human. However, the expression of HO-1 obviously increased after the body is induced by stimuli such as stress, hypoxia, ultraviolet radiation, heavy metals and a variety of disease states including diabetes, high cholesterol, smoking and

*Abbreviations:* LAB, magnesium lithospermate B; ROS, reactive oxygen species; HO-1, heme oxygenase-1; Nrf2, nuclear factor E2-related factor2; GSTs, glutathione *S*-transferases; CAT, hydrogen peroxidase; $H_2O_2$, hydrogen peroxide; SOD, superoxide dismutase; $O^{2-}$, superoxide anion; MDA, malondialdehyde; ARE, antioxidant response element; EpRE, electrophile response element; NF-κB, nuclear factor kappa B; Keapl, Kelch-like ECH-associated protein 1; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorodihydrofluorescein; CPK, creatine phosphokinase; AP-1, activator protein 1; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction; GSH-Px, glutathione peroxidase.

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other lesions [5]. The molecular structures of HO-2 and HO-3 are similar, but they are less powerful for the decomposition of hemoglobin [4–6]. Bilirubin is an endogenous antioxidant and an effective superoxide scavenger. It can inhibit the formation of oxidized low density lipoprotein and make a synergistic effect with other antioxidant defense system such as superoxide dismutase and vitamin E in human body. The antioxidant capacity is even more powerful than vitamins C and E [7]. Morita et al. [8] found that suitable level of endogenous bilirubin reduces the damage on body caused by oxidative stress. HO-1 is one of the most important endogenous protection system and plays an important role in the cell protection through anti-inflammation, anti-oxidation, inhibition of apoptosis, and improvement of microcirculation [9]. Induction of HO-1 gene is regulated primarily at the transcriptional level. A number of antioxidant response elements (ARE) or electrophilic response elements (EPRE) have been identified. ARE in the promoter regions contains binding sites for heat shock factor, NF-κB, and nuclear factor E2-related factor-2 (Nrf2) [10]. ARE is similar to the Maf recognition element and specifically combines with basic leucine zipper transcription factor including Jun, Fos and Nrf2 [11].

Nrf2 is basic leucine zipper (bZIP) family of white matter [12]. In non-active state, it is located in the cytoplasm and binds with cytoplasmic protein chaperone Keapl (Kelch-like ECH-associated protein 1). Under the stimulation of ROS or other nucleophilic agents, Nrf2 is activated and transferred into the nucleus after uncoupling with Keapl. Nrf2 regulates ARE-dependent gene expression of phase II detoxification enzymes and antioxidant genes including glutathione *S* transferase (GSTs), hydrogen peroxidase (CAT), superoxide dismutase (SOD), and HO-1[11]. Nakasolz et al. [12] reported that the expression of Nrf2 protein is both time and dose dependent, and reaches the peak value at 50 μmol/L at 6 h when neuroblastoma cells were treated with HO-1 classic inducer hemin. Recently, Chen's [13] study showed that LAB promotes Nrf2 translocation into the nucleus in aortic smooth muscle cells at the normal and glucose stress state. Many natural dietary antioxidants such as salvia magnesium acetate, curcumin, and bioflavonoids enhance the expression of HO-1.

Sal B is the most active component of Danshen water-soluble substances and has a very strong antioxidant activity due to a number of phenolic hydroxyl components. Previous studies showed that Sal B inhibits the production of superoxide anion ($O^{2-}$), prevent the formation of erythrocyte hemolysis induced by hydrogen peroxide ($H_2O_2$) and malondialdehyde (MDA) [14]. In addition, Sal B could delay the occurrence of renal failure in diabetic rats [15] and inhibited the activity of aldose reductase in vascular smooth muscle cell induced by high dose of glucose and endometrial hyperplasia, which result in a decreased damage of smooth muscle cell by high dose of glucose [16]. LAB, as the main form of Sal B, is the main component of salvianolate injection and its content is more than 80%. At present, LAB was widely used in the treatment of many diseases such as atherosclerosis, diabetes, and coronary heart disease in clinic. However, its mechanism for anti-oxidative damage is still unclear.

In this study, we aimed to assess the effect of LAB against high dose of glucose-induced intracellular oxidative damage and its action mechanism.

## 2. Materials and methods

### 2.1. Cell culture

HEK293T cells were maintained in Dulbecco's modified eagle's medium (DMEM, Invitrogen, CA, USA) containing 10% fetal bovine serum (Every Green, Hangzhou, China), 100 U/mL penicillin, and 100 μg/mL streptomycin before initiating differentiation.

### 2.2. Determination of ROS generation in treated cells

Differentiated HEK293T cells were grown in 6-well plates at $1 \times 10^5$–$2 \times 10^6$ cells per well. When the culture reached 90% confluence, cells were collected and divided into 5 experimental groups. The groups were set as follows: (1) normal control, (2) treated with 30 mmol/L glucose, (3) pretreated with 50 μmol/L LAB for 30 min, then 30 mmol/L glucose was added to cells, and cells were continuously cultured for an hour, (4) treated with 100 mmol/L $H_2O_2$ (5) pretreated with 50 μmol/L LAB for 30 min, then 100 mmol/L $H_2O_2$ was added to cells, and cells were continuously cultured for 15 min. After the cells of each group were treated, the cell culture medium was removed, 1 mL of 10 μmol/L DCFH-DA (diluted concentration) was added to cover the cells completely, and the cells were cultured for another 30 min. After rinsing with serum-free cell culture medium three times to remove free DCFH-DA, cells were centrifuged at 13,000*g* for 5 min and then resuspended in 0.5 mL PBS. The level of ROS in HEK293T cells was determined by flow cytometry with excitation wavelength at 488 nm and emission wavelength at 525 nm. Each experiment was repeated three times ($n = 3$).

### 2.3. RNA isolation and semi-quantitative RT-PCR on HO-1

Total RNA was extracted by using trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Total RNA concentration was determined spectrophotometrically at 260 nm. Genomic DNA from total RNA was removed by using DNaseI (Promega, Wisconsin, USA) before reverse transcription. Semi-quantitative RT-PCR was performed as described previously to determine the mRNA levels of β-actin and HO-1. The primer pairs used in the amplification of the HO-1 were: forward primer, 5′-TTGCCAGTGCCAC-CAAGTTC-3′; reverse primer, 5′-TCAGCAGCTCCTGCAACT-CTCC-3′. The primer pairs of β-actin were: forward primer, 5′-TGGCACCCAG-CACAATGAA-3′; reverse primer, 5′-CTAAGTCATAGTCCGCCTA-GAAGCA-3′. Polymerase chain reactions (PCR) were carried out using 2 μL cDNA, 12.5 μL SYBR ®Premix Ex TaqTM (Takara, Dalian, China), 0.5 μL Rox Reference Dye II (50) (Takara, Dalian, China), 0.4 μL of each primer (10 μM) (Takara, Dalian, China). The total reaction volume was brought up to 25 μL with $H_2O$. PCR was initiated by heating to 95 °C for 2 min, then amplified through 40 cycles: denaturing at 95 °C for 15 s, annealing at 59 °C for 30 s for HO-1.

### 2.4. Construction and transfection of pRNAT-U6.1/Neo-siNrf2 plasmid

The primer pairs used in the amplification of the Nrf2 shRNA were: forward primer, 5′-GATCCCGAGTATGAGCTGGAAAAACTTGA-TATCCGGTTTTTCCAGCTCATACTCTTTTTTCCAAA-3′: Reverse primer, 5′-AGCTTTTGGAAAAAAGAGTATGAGCTGGAAAAACCGGATATCAAG TTTTTCCAGCTCATACTCGG-3′ (Genescript, Guangzhou, China). pRNAT-U6.1/Neo vector (Genescript , NJ , USA) was double digested by *BamH* I and *Hind* III (Takara, Dalian, China). Double-stranded DNA and pRNAT-U6.1/Neo vector were connected at the ratio of 4:1. The constructed pRNAT-U6.1/Neo-siNrf2 plasmid was identified by PCR with forward primer: 5′-TACGATACAAGGCTGTTAGAG-3′ and reverse primer: 5′-TAGAAGGCACAGTCGAGG-3′. The pRNAT-U6.1/Neo-siNrf2 and non-specific control shRNA (Invitrogen, CA, USA) were transferred into HEK293T cells using lipofectamine 2000 (Invitrogen, CA, USA) by following the manufacturer's instruction.

### 2.5. Nuclear protein preparation and Western blot

Protein extraction reagent A of 200 μL was added to each 20 μL collected cell precipitation and then violently vortexed for 5 s. The mixture was incubated in ice bath for 15 min. Then, 10 μL protein extraction reagent B was added to the mixture,