# Piperine effectively protects primary cultured atrial myocytes from oxidative damage in the infant rabbit model

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Abstract. Piperine is an important active component of the Chinese herb Large leaf moss. The aim of this study was to investigate the effects of piperine on oxidative stress. An oxidative stress model was developed in rabbit atrial cells treated with low concentrations of hydrogen peroxide  $(H_2O_2)$ . A primary cell culture of the atrial cells was established and the cells were randomly divided into three groups: A piperine group, an H<sub>2</sub>O<sub>2</sub> group and a control group. The results demonstrated that the cell viability and superoxide dismutase activity in the piperine group were significantly higher than in the  $H_2O_2$  group (P<0.05), and the expression levels of malondialdehyde and glutathione were significantly reduced in the piperine group compared with the  $H_2O_2$  group (P<0.05). The intracellular free calcium concentration and the expression level of mitochondrial mRNA in the piperine group were also significantly lower than in the H<sub>2</sub>O<sub>2</sub> group (P<0.05). In conclusion, piperine was important in protecting the primary rabbit atrial cells from oxidative stress.

# Introduction

Large leaf moss, the dried or fresh grass of bryaceae, such as *Rhodobryum roseum* (Hedw.) Limpr. [Mnium roseum Hedw.], *R. giganteum* (Schwaegr) par. and *R. giganteum* par. (1-3), is a traditional herbal remedy widely used in the Yunnan province of China. Large leaf moss is commonly used to treat diseases, including heart palpitations, chest tightness and neurasthenia.

Myocardial ischemic injury is associated with the generation of reactive oxygen species (ROS); it has been demonstrated that the production of ROS increased and antioxidant enzyme activity decreased during myocardial ischemia (4). Oxidative stress resulting from oxygen free radical production may exceed the capacity of endogenous free radical scavenging mechanisms, resulting in lipid peroxidation and DNA damage in mitochondria (5,6). Data have indicated that cellular oxidative stress is important in the pathogenesis of atrial fibrillation (7). Results from another study have indicated that trying to prevent and avoid oxidative damage to atrial cells may prevent and relieve the development of atrial fibrillation to a certain extent (8).

In a study of monomeric compounds in Large leaf moss, piperine and pepper acid methyl ester protected cardiomyocytes from the injury induced by hydrogen peroxide ( $H_2O_2$ ), and piperine effectively protected ventricular myocytes from the injury induced by oxygen radicals (1). In the present study, an oxidative injury model with low concentrations of  $H_2O_2$  was used to analyze the key steps in the oxidative stress response and further investigate the protective effect of piperine on primary atrial cells in the oxidative injury model.

## Materials and methods

*Reagents*. The superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione (GSH) kits were from Jiancheng Bioengineering Corporation (Nanjing, China), and piperine and MTT were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), 3%  $H_2O_2$  and fetal bovine serum were purchased from Gibco-BRL (Carlsbad, CA, USA). The penicillin-streptomycin mixture and 0.25% trypsin-EDTA were supplied by Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The primer probes and SYBR Green were purchased from Shanghai ShineGene Molecular Biotech, Inc. (Shanghai, China). Fluorochrome Fluo-2/acetoxymethyl ester (Fura-2 AM) was obtained from Anaspec (Fremont, CA, USA).

*Animals*. Neonatal New Zealand rabbits (age, 3-5 days; weight, 150-200 g) were obtained from the Experimental Animal Center of Military Medical Sciences (Beijing, China) and housed in specific pathogen-free conditions. The protocols were approved by the Institutional Animal Care and Use Committee of General Hospital of People's Liberation Army. All procedures were in accordance with the Declaration of Helsinki of the World Medical Association.

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Animal grouping and primary culture of atrial muscle cells. A total of 18 animals were randomly divided into three groups: Piperine, H<sub>2</sub>O<sub>2</sub> and control groups. Briefly, following sacrifice by injection of ~10 ml air into the ear vein, the neonatal rabbit was fixed in a supine position and the chest skin was disinfected. The chest wall was cut and removed along the right edge of the sternum. The heart was rapidly removed and washed in D-Hanks fluid (Baihao, Tianjin, China) and the blood was removed. The left and right atria were washed with serum-free medium under sterile conditions and cut into small ~1-mm diameter sections with ophthalmic scissors. A volume of 5 ml 0.08% trypsin was added to the tissue sections, which were incubated in a 37°C water bath for 5 min. Digestion was then terminated by adding serum to the medium. A single cell suspension culture was prepared and the concentration of cells was adjusted to  $1 \times 10^{5}$ /ml. To inhibit the growth of fibroblast cells, 1 ml of 0.1 mmol/l bromodeoxyuridine (Sigma-Aldrich) was added. The cultures were incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

The primary atrial myocytes in the piperine group were pretreated with  $7x10^{-6}$  mol/l piperine for 1 h, then  $100 \ \mu$ mol/l H<sub>2</sub>O<sub>2</sub> was added and the cells were incubated for 2 h. The H<sub>2</sub>O<sub>2</sub> and control groups were treated with  $100 \ \mu$ mol/l H<sub>2</sub>O<sub>2</sub> or phosphate-buffered saline (PBS) for 2 h, respectively.

Detection of cell viability. The cell concentrations were adjusted to  $1x10^6$  cells/ml in DMEM supplemented with 10% fetal calf serum. Samples of the suspensions (100  $\mu$ m) were dispensed into 96-well round-bottom culture plates (Costar, Cambridge, MA, USA) and incubated for 72 h at 37°C in a 5% CO<sub>2</sub> humid incubator. Cell proliferation was then determined with the MTT assay (9).

Detection of oxidative stress markers SOD, MDA and GSH. Supernatant (50  $\mu$ l) was extracted from the myocardial cell cultures. SOD expression levels were determined with the xanthine oxidase method, MDA expression levels were measured by thiobarbituric acid assay and GSH expression levels were determined by a colorimetric method. All assays were performed according to the manufacturer's instructions of the respective kits.

Measurement of intracellular Ca<sup>2+</sup>. Intracellular Ca<sup>2+</sup> levels were determined with Ca<sup>2+</sup>-sensitive Fluo-2/acetooxymethyl ester by a Becton Dickinson FACS Calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) or a Perkin-Elmer LS 55 fluorescence spectrophotometer (Perkin-Elmer, Waltham, MA, USA) (10,11). The cells were incubated with 3  $\mu$ M Fluo-2/AM at 37°C for 30 min in the dark following treatment with salidroside and H<sub>2</sub>O<sub>2</sub> as described. The cells were then gently rinsed three times with D-Hanks solution and the fluorescence was analyzed by flow cytometry. Fluo-2/AM bound to cytoplasmic free calcium generates a strong fluorescence at an excitation wavelength of 330-350 nm, which is reduced at the 380 nm excitation wavelength. Thus, the fluorescence ratio of 340 to 380 nm was used to detect the intracellular calcium ion concentrations.

Quantitative analysis of mitochondrial mRNA in atrial myocytes. TotalRNA was isolated with an RNA pure kit (Bioteke,

Beijing, China) and retrotranscribed with MLV-reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) with random primers. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed by real-time PCR ABI Prism 7500 (Applied Biosystems China, Beijing, China), using 40 cycles of 95°C for 12 sec and 60°C for 1 min with SYBR Green. The primers were designed using Primer Premier, version 5 (www.premierbiosoft.com and OLIGO Primer Analysis Software, version 6 (oligo.net). The sequences of the primers were 5'-AGGATGGTAGCAAGGAGGAAG-3' and 5'-ACCCTGGAGCGATGTGGA-3'. The length of p22-phox (*Oryctolagus cuniculus*) was 137 bp.

Statistical analysis. Data were analyzed using SPSS software (SPSS Inc., Chicago, IL, USA). All data are expressed as the mean  $\pm$  standard deviation or standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Primary culture of atrial myocytes and morphological observation.* After 72 h culture, cells with a variety of shapes were visible under an inverted microscope, although the cell morphology was dominated by spindle, triangular and irregular shapes (Fig. 1A). Hemotoxylin and eosin staining of the cells is presented in Fig. 1B. Certain adherent cardiomyocytes formed multicellular beating cell clusters within 24 h.

Cell viability of primary atrial myocytes increases following piperine treatment. In order to determine the role of piperine on the cell viability of primary atrial myocytes, an MTT assay was performed. The results demonstrate that the cell viability of the primary atrial myocytes significantly increased following treatment with piperine compared with the cells only treated with  $H_2O_2$ . Furthermore, the cell viability increased in a time-dependent manner, as analyzed by MTT (Fig. 2A). The results detected by MTT were consistent with those observed with FACS analysis (Fig. 2B).

Detection of oxidative stress markers SOD, MDA and GSH. The levels of oxidative stress markers were detected in different groups. As shown in Fig. 3 and Table I, the mean SOD production in the piperine group was significantly higher than that in the  $H_2O_2$  group (P<0.05) and the expression levels of MDA and GSH in the culture supernatant of primary myocardial cells in the piperine group were significantly lower than those in the  $H_2O_2$  group (P<0.01). Untreated cells served as negative controls.

Detection of the intracellular calcium concentration. Impaired oxidative phosphorylation may result in lowered ATP synthase function and reduce the ability of the mitochondria to synthesize ATP. In addition, oxidative stress may reduce the activity of ATP-dependent ion pumps in the cell membrane and intracellular calcium levels may become elevated. Thus, the intracellular calcium levels in the different groups were examined. As shown in Fig. 4, the intracellular calcium concentration was significantly lower in the group treated with piperine than in those treated with  $H_2O_2$  alone (P<0.01).

Table I. Effects of piperine on oxidative stress markers SOD, MDA and GSH.

Group	SOD (U/l)	MDA (U/l)	GSH (µM/ml)
Control	1706.666±13.278	0.333±0.101	0.189±0.047
$H_2O_2$	202.423±65.498	12.593±0.201	3.869±0.192
Piperine	407.272±59.598ª	4.965±0.335ª	2.485±0.117 <sup>a</sup>

<sup>a</sup>P<0.05 compared with the H<sub>2</sub>O<sub>2</sub> group. SOD, superoxide dismutase; MDA, malondialdehyde; GSH, glutathione. H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.



Figure 1. Images of cultured primary atrial myocytes. (A) Primary rabbit atrial myocytes were cultured for 48 h and observed under an inverted microscope (magnification, x200). (B) Hematoxylin and eosin staining of the atrial myocytes (magnification, x200).



Figure 2. Cell vitality of primary atrial myocytes increased following treatment with piperine. (A) MTT assay. Primary atrial myocytes were cultured for 24 and 48 h. Data are shown as the mean  $\pm$  standard deviation of at least three independent experiments on different individuals. \*P<0.05 and \*\*P<0.01 compared with the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) group. (B) Fluorescence-activated cell sorting (FACS) analysis by Annexin V and propidium iodide (PI). Primary atrial myocytes were cultured for 48 h and cell apoptosis was detected by FACS.



Figure 3. Production of oxidative stress-related markers. Primary atrial cells were cultured and were randomly assigned to three groups: Piperine, hydrogen peroxide  $(H_2O_2)$  and controls. Untreated cells served as a negative control. Data are presented as the mean  $\pm$  standard deviation. \*P<0.05 and \*\*P<0.01.



Figure 4. Histogram of intracellular calcium concentration. Activity and variation of intracellular calcium in different groups was detected by fluorescence-activated cell sorting. Data are presented as the mean  $\pm$  standard deviation. \*\*P<0.01 compared with the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) group.



Figure 5. Quantitative polymerase chain reaction in atrial myocytes. (A) Amplification curves of mitochondrial mRNA indicates the specificity of the primer. (B) Histograms of the relative quantitation of mitochondrial mRNA in different groups. Data are presented as the mean  $\pm$  standard deviation. \*P<0.05 and \*\*P<0.01 compared with the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) group.

*RT-qPCR in atrial myocytes.* Quantitative analysis of mitochondrial mRNA expression levels in atrial myocytes was performed. The results demonstrated that the mitochondrial mRNA expression levels were significantly reduced in the groups treated with piperine compared with those treated with  $H_2O_2$  alone (P<0.01) (Fig. 5). Piperine significantly reduced the mitochondrial mRNA levels in a dose-dependent manner following  $H_2O_2$  stimulation as analyzed by RT-qPCR.

# Discussion

Oxidative injury/oxidative stress refers to cell damage resulting from excessive oxidation, when the body produces excessive ROS and/or the antioxidant capacity is reduced (12). The reactive oxygen species (ROS) and secondary ROS, such as peroxynitrite, are mainly generated in the mitochondrial respiratory chain reaction and by monoamine oxidase (13,14). ROS are involved in metabolism and signal transduction, which are important in normal physiology. Under normal circumstances, the body removes reactive oxygen free radicals to maintain ROS production and clearance in a dynamic equilibrium. Certain antioxidants, such as vitamin E, vitamin C, trace elements (including selenium and zinc), SOD and catalase are utilized for ROS clearance (15,16).

SOD is an active protease containing metallic elements, which is present in the cytosol and mitochondria of eukaryotic and prokaryotic cells (17,18). SOD is an important *in vivo* antioxidant enzyme, which catalyzes the disproportionation reaction of superoxide anion to  $H_2O_2$  and oxygen. To a certain extent, SOD activity may reflect the degree of oxidative damage to tissue cells and the body's ability to scavenge free radicals.

GSH is the main source of the sulfhydryl group in the majority of living cells and is important in maintaining the thiol redox state of the sulfhydryl group in proteins (19). In addition, GSH is a key antioxidant in animal cells. Several studies have found that a reduction in GSH levels is an early signal of apoptosis (20,21). In addition to directly scavenging oxygen free radicals, GSH also participates in the myeloperoxidase-derived oxidase reaction to reduce the generation of ROS.

In the present study, SOD activity was significantly increased in the group treated with piperine compared with the group treated with  $H_2O_2$  (P<0.05). Furthermore, the GSH levels were reduced in the piperine group compared with the  $H_2O_2$  group (P<0.05). Thus, piperine may increase the activity of SOD and enhance the activity of antioxidant enzymes to reduce oxidative stress damage to cells.

MDA is a lipid peroxide, which is produced by peroxidation of polyunsaturated fatty acids and induced cell damage (22). In oxidative injury reactions, ROS and macromolecules in the biofilm, including the side chains of polyunsaturated fatty acids and nucleic acids, react to form lipidperoxidation products by lipid peroxidation. The decomposition products of lipid peroxidation may result in cell damage, therefore MDA levels may reflect the extent of lipid peroxidation in the body (23). Simultaneously, MDA levels may also indicate the degree of oxidative damage to cells. The results suggest that piperine effectively reduces oxygen radicals and decreases lipid peroxidation by reducing the attack of polyunsaturated fatty acids by oxygen free radicals (24).

Mitochondria are intracellular organelles where oxidative phosphorylation and ATP synthesis are performed. Mitochondria are semi-autonomous organelles that contain their own genetic machinery and their own genome. Mitochondrial DNA (mtDNA) has a closed circular double-strand structure. Mitochondria are important producers of oxygen free radicals, and mtDNA is vulnerable to oxidative damage and susceptible to mutations as it lacks histone protection and does not have effective repair mechanisms (25) in an environment with high levels of ROS. In addition, when oxidative injury occurs, lipid peroxidation occurs. ROS and polyunsaturated fatty acids react to produce lipid peroxides. The fluidity and permeability of the membrane may change, eventually resulting in changes in cell structure and function. When cells are damaged by  $H_2O_2$ , mitochondrial mtDNA levels undergo a compensatory increase. Piperine can enhance antioxidant enzyme activity, reduce the generation of oxygen free radicals and the oxidative damage of lipid peroxidation to the mitochondrial membrane. Thus, the function of mitochondrial oxidative phosphorylation is protected during the process of oxidative damage.

When oxidative damage was observed to occur, coordination activity between mitochondria-regulating genes and mtDNA was revealed to be impaired, and the oxidative phosphorylation and mitochondrial ATP synthesis abilities were damaged (26). The mtDNA compensatorily increased resulting in the upregulation of mitochondrial mRNA levels (27,28). Additionally, accumulated ROS exert a direct effect on mitochondria, which reduces the membrane potential. When the mitochondrial membrane potential reaches below a threshold value, cell apoptosis is induced (29). In the present study, mitochondrial mtDNA in primary cells was quantitatively analyzed, and the results demonstrated that mRNA expression levels were significantly reduced in the piperine group and the normal group, compared with the  $H_2O_2$  group (P<0.05). However, no significant differences were identified between the piperine group and the control group (P>0.05). The results revealed that when cells were damaged by  $H_2O_2$ , mitochondrial copies of mtDNA increased due to functional compensation. Piperine was found to enhance the activity of antioxidant enzymes, inhibit the production of oxygen free radicals, reduce the lipid peroxidation damage on the mitochondrial membrane and partially protect from mitochondrial oxidative phosphorylation.

Ca<sup>2+</sup> is a key second messenger in regulating signaling pathways, which is also involved in the activation of enzymes that decompose a variety of proteins, phospholipids and nucleic acids (30). In normal circumstances, Ca<sup>2+</sup> enters cells through intracellular calcium channels, proteins located and embedded in the cell membrane. Calcium homeostasis is co-manipulated by Ca<sup>2+</sup>-translocating enzymes in the plasma membrane and intracellular calcium pool. However, when cells are damaged by oxidative injury, a large quantity of oxygen free radicals are generated (31,32). Lipid peroxidation is induced by oxygen radicals and membrane polyunsaturated fatty acids, which may disrupt the structure of cell membranes. In addition, ATP synthesis is impaired by oxygen free radicals due to mitochondrial damage. In the presence of ROS, activities of the ATP-dependent Na<sup>+</sup> and Ca<sup>2+</sup> pumps in the cell membrane were found to be reduced, the concentration of intracellular Na+ was increased, the rate of Na<sup>+</sup>-Ca<sup>2+</sup> exchanges was enhanced, and  $Ca^{2+}$  influx was increased (33). In the present study, the concentration of intracellular calcium ions in the H<sub>2</sub>O<sub>2</sub> group was significantly higher than in the piperine or the normal groups (P<0.01), which demonstrated that the membrane structures of primary atrial cells were damaged by oxidative injury, and that the accumulation of uncleared oxygen free radicals results in abnormalities of Na<sup>+</sup>-Ca<sup>2+</sup> exchange in the cell membrane. Thus, intracellular calcium became elevated.

 $H_2O_2$ -induced oxidative damage may increase the production of oxygen free radicals, reduce the activity of antioxidant enzymes, and promote the peroxidation of polyunsaturated lipid acids and oxidative phosphorylation in mitochondria in primary atrial cells. In the present study, the results demonstrated that piperine extract from Large leaf moss, effectively reduced intracellular accumulation of ROS, decreased damage to cells resulting from lipid peroxidation, protected against mitochondrial oxidative phosphorylation and reduced oxidative injury to mitochondria. Piperine effectively enhanced the activity of antioxidant enzymes, reduced oxygen free radicals, enhanced oxygen radical scavenging and reduced calcium overload damage to cells. Piperine also reduced the effect of oxidative injury on cell viability. In conclusion, piperine exerted a protective function to relieve the oxidative damage to primary atrial myocytes, and has the potential to be used in future to create therapies for oxidative stress injuries.

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