Effects of Purified *Salvia miltiorrhiza* Extract on Cardiac Vascular Smooth Muscle Hypoxic Cells

Begum Husna¹, Todd On¹, and Yi Zhun Zhu¹,²,*

¹Department of Pharmacology, Faculty of Medicine, National University of Singapore, 119260, Singapore
²School of Pharmacy and Institute of Biomedical Sciences, Fudan University, Shanghai 200032, China

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Abstract. Recently, we have reported that purified *Salvia miltiorrhiza* extract (PSME) could prevent myocardial infarction in vivo and myocardial ischemia/reperfusion injury in isolated rat hearts (ex vivo). The aim of this project is to determine whether PSME exerts any cardio-protective effects in vitro. The vascular smooth muscle cell line was used and the effects of the drugs were determined after inducing hypoxia. Gene expression levels of the pro-apoptotic genes Asp53, Bax, and Fas were significantly down-regulated by 0.78-, 0.82-, and 0.87-fold, respectively, and Bcl-2 was up-regulated by 0.82-fold in the PSME-treated groups as compared to the hypoxic group (*P* < 0.05). Significant reduction in immunoreactivity of the protein products of these genes as well as least nuclear green fluorescence observed in TUNEL staining indicate the therapeutic potential of this drug. Furthermore, cardiac antioxidant enzymes assay confirmed this deduction as PSME had slight preserving effects on superoxide dismutase and catalase (0.25 ± 0.01 vs 0.488 ± 0.02 units/mg protein and 0.026 ± 0.012 vs 0.076 ± 0.01 µmol per min per mg protein, respectively; each *P* < 0.05). No significant results were obtained with glutathione S-transferase and GSH peroxidase antioxidant tests. Our results demonstrated that PSME exerts antioxidant effects in vitro, indicating the therapeutic potential of this drug.

Keywords: ischemic myocardium, purified *Salvia miltiorrhiza* extract, hypoxia

Introduction

Ischemic heart diseases, especially acute myocardial infarction (AMI), remain the leading cause of death in both developed and developing countries as seen over the past quarter century (1). Clinical study shows that most of the cases were caused by blockage or narrowing of the coronary artery, where vascular remodeling plays a critical role (2). Pathological studies show that the remodeling is mediated by ischemic apoptosis (3). Reactive oxygen species (ROS), which possess highly reactive and toxic properties, can be generated as a result of ischemia and exacerbate the degree of myocardial damage sustained by the ischemic myocardium (4, 5). In response to this, animals have developed a natural defensive system to cope with these unwanted and toxic species. Such defense mechanisms include enzymes like superoxide dismutase (SOD) and catalase (CAT). In pathological or disease conditions, such as myocardial infarction, diabetes, stroke, and others, the production of free radicals may override the scavenging effects of antioxidants leading to oxidative stress (5 – 7). Also, in recent years, oxidative stress has been proven to be a powerful inducer of programmed cell death (8).

*Salvia miltiorrhiza* (SM) is an important Chinese natural herb used in the treatment of many diseases, especially ischemic cardiovascular diseases (6, 7, 9, 10). Danshen, the dried root of SM, has been widely used for the treatment of cardiovascular and cerebrovascular diseases (11). It is considered to possess slightly cold and bitter properties and enters the heart, pericardium, and liver channels. Experiments using modern techniques showed that SM dilates coronary arteries, increases coronary blood flow, and scavenges free radicals in patients with ischemic diseases, thus reducing cellular damage from ischemia (12). Clinical trials have also indicated that SM is an effective medicine for
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angina pectoris, myocardial infarction and stroke (6, 7, 9, 10, 12, 13). To date, 20 major ingredients in SM have been identified (13). The purified SM used in the current study is an extract consisting of six active watersoluble ingredients from the 20 major ingredients of crude SM.

In recent years, researchers have found that many herbal drugs provide antioxidant effects on ischemic heart diseases (9, 14, 15). SM has demonstrated that it could scavenge the oxygen free radicals generated from ischemia-reperfusion injury in the myocardium as effectively as SOD using the low temperature electron spin resonance technique. Recently, we have reported that purified SM extract (PSME) could prevent myocardial infarction in vivo (12) and myocardial ischemia /reperfusion injury in isolated rat hearts (ex vivo) (16).

Despite all this, it is important to note that the underlying scientific principles of the action of herbal drugs is still unsatisfactory, resulting in their limited widespread use in patients worldwide.

Materials and Methods

Purified Salvia miltiorrhiza extract (PSME)

*Salvia miltiorrhiza* was originally collected from Si Chuan Province, China. PSME was supplied by CMM Corporation Pte, Ltd. (batch No. IV-S-900-2, 30-4-2003; Singapore), which was originally developed by Shanghai Material Medica Bioengineering Institute. It comprises 6 bioactive ingredients: salvianoic acid (*Danshensu*), rosmarinic acid, caffeic acid, protocatechualdehyde, and salvianolic acid A and B. The chemical structures of these compounds were shown in our recent report (17).

Cell culture materials

Dulbecco’s modified Eagle's medium (DMEM) [vascular smooth muscle cells (VSMC) medium] with 2.2% genetin and 10% fetal bovine serum were purchased from Sigma (St. Louis, MO, USA), and 0.25% trypsin-EDTA and trypan blue solution were from Gibco, Invitrogen (Singapore).

Maintenance of cell line

Rat VSMC cell line, SV40LT-SMC, which was derived from the aorta, was purchased from American Type Culture Collection (ATTC) (Manassas, VA, USA). The cells were cultured in VSMC and kept in 37°C humidified air with 5% CO₂ in the incubator.

Study design: treatment groups

The treatment groups used in this experiment are as shown in Table 1.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>1</td>
<td>Normoxic group: normal conditions (normal control)</td>
</tr>
<tr>
<td>2</td>
<td>Hypoxic group: hypoxia (hypoxic control)</td>
</tr>
<tr>
<td>3</td>
<td>Hypoxia + 0.1 mg/ml PSME group: drug-treatment</td>
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</table>

*Stimulated hypoxia-induced ischemic model*

Hypoxia was induced based on a technique described by Rakhit et al. (18). All the culture plates excluding the normoxic control were placed in anaerobic Gaspak pouches (BD Diagnostics System, NJ, USA), and the pouches were heat-sealed twice before incubation at 37°C for 8 h. Normoxic control cells were also incubated for the same length of time.

*Evaluation of cell viability*

Trypan blue is one of several stains used for the dye exclusion procedure for viable cell counting. The primary assessment of cell viability in this study is based on measurement of lactate dehydrogenase enzyme (LDH) leakage from the cells into the medium; an indicator of relative cell viability, using the TOX-7 LDH-based in vitro toxicology assay kit (Sigma). This method was described by Legrand et al. (19) and Decker et al. (20). The LDH assay was measured spectrophotometrically at 490 nm.

*Total RNA isolation and quantitation*

The total RNA of the samples was extracted by TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The absorbance at wavelengths of 260 nm (A260) and 280 nm (A280) were read using an Ultraviolet Spectrophotometer. Calculations of stock total RNA concentrations was done based on the extinction coefficient of 1 unit (O.D) A260 reading = 40 µg/ml RNA.

*Reverse transcriptase-polymerase chain reaction (RT-PCR)*

A 1-µg sample of total RNA was used for RT-PCR by using the Qiagen OneStep RT-PCR Kit (Hilden, Germany). The PCR was carried out in a total volume of 12.5 µl. Glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) was considered as an internal standard gene.

Primers sequences used for each gene are shown in Table 2. PCR was run at 25 cycles for GAPDH, 30 cycles for p53, 33 cycles for Bax, 31 cycles for Fas, and 33 cycles for Bel-2 in a GeneAmp® PCR System 9700 (Applied Biosystem, Singapore). Reverse transcription reaction was performed at 50°C for 30 min. Samples were then heated at 95°C for another 15 min for the
initial PCR activation step. Three-step PCR of denaturing, annealing, and extension proceeded at 94°C for 30 s (denaturation); at 55°C (GAPDH), 58°C (Asp53), 51°C (Bax), 59°C (Fas), 55°C (Bcl-2) for 30 s (annealing); and 72°C for 1.00 min (extension). A final extension was carried out for 15 min at 72°C.

**Agarose gel electrophoresis**

Gel electrophoresis was performed using 1.5% agarose gel, imaged by a MultiGenius Bioimaging system (Cambridge, UK). Blue/orange loading dye (Promega, Singapore) was mixed and loaded with the samples. Intercalation of EtBr with DNA allows visualization of the DNA bands under UV.

Intensity of each band was quantified by gel analysis software (Syngene, Cambridge, UK), and results were expressed relative to the corresponding intensity of the GAPDH bands from the same RNA sample (21).

**Immunohistochemical staining**

Immunohistochemical staining (Immunostaining) is a method to show the expression level regulation of the genes of interest. The ready-to-use UltraVision Detection System Anti-Polyvalent, HRP/DAB kit (Lab Vision Corporation, Singapore) was used for antibody staining as we previously described in Loh et al. (22). Polyclonal rabbit anti-p53 antibody (sc-6243), polyclonal rabbit anti-Bax antibody (sc-526), polyclonal rabbit anti-Fas antibody (sc-7886), and polyclonal rabbit anti-Bcl-2 antibody (sc-492) (all antibodies from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used for the primary antibody incubation. Hematoxylin staining was performed as counterstain. Slides were viewed and photographed using a Leica® fluorescence microscope.

**TUNEL (TdT-mediated dUTP nick-end labeling) assay**

A DeadEnd™ Fluorometric TUNEL System (G3250, Promega) kit was chosen for detection of apoptosis-induced nuclear DNA fragmentation via fluorescence microscopy. This assay was performed as we described previously (22). Slides were viewed and photographed using the Leica® fluorescence microscope using the standard fluorescein filter set to view the green fluorescence of fluorescein at 520 ± 20 nm while red DAPI was viewed at 460 nm.

**Antioxidant assay**

For the assays of SOD, CAT, GSH-peroxidase (GSH-Px), and glutathione S-transferase (GST) activities, cells was homogenized in 1 ml phosphate buffer (10 mmol/l, pH 7.5) by a Polytron homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min at 4°C; the supernatant was then divided into 2 parts for different enzyme assays. For SOD and CAT activity assays, the supernatant was centrifuged at 2,300 × g for a further 10 min; while the supernatant used for GSH-Px and GST activity assays was further centrifuged for 90 min at 100,000 × g at 4°C with an ultracentrifuge (Beckman L8-70; Beckman Instruments, Inc., Fullerton, CA, USA). After centrifugation, the pellets were discarded and the supernatants used for the assessment of enzyme activity. SOD activity was determined by a modified method of Marklund and Marklund as stated by Ji et al. (9). CA was assayed by the amended method of Aebi as stated by Ji et al. (9). For the assay of GSH-Px activity, glutathione peroxidase was determined by the amended method of Beutler as stated by Ji et al. (9). GST was assayed by the improved method of Habig and Pabst as stated by Ji et al. (9).

**Statistical analysis**

Data was represented as mean ± S.E.M. Statistical analysis was performed by the two-tailed Student’s t-test for difference between samples. A difference with \( P<0.05 \) was considered statistically significant.

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**Table 2. Primer sequences of GAPDH, Asp53, Bax, Fas, and Bcl-2 used in current study**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Function (base pairs)</th>
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<tbody>
<tr>
<td>GAPDH-Sense</td>
<td>5'-CATGGTCTACATGTCCAGT-3'</td>
<td>House keeping (253 bp)</td>
</tr>
<tr>
<td>GAPDH-Antisense</td>
<td>5'-GGCTAAGCAGTTGGTGCTGC-3'</td>
<td></td>
</tr>
<tr>
<td>Asp53-Sense</td>
<td>5'-GGACTAGCATTGTCTTGTACGC-3'</td>
<td>Apoptosis (270 bp)</td>
</tr>
<tr>
<td>Asp53-Antisense</td>
<td>5'-ATGTCCAGAGCTGAATGAGG-3'</td>
<td></td>
</tr>
<tr>
<td>Bax-Sense</td>
<td>5'-GCAAGGAGGATGCTGGGGGAGA-3'</td>
<td>Apoptosis (352 bp)</td>
</tr>
<tr>
<td>Bax-Antisense</td>
<td>5'-TCCAGACAAAGCAGCCAGTCAGC-3'</td>
<td></td>
</tr>
<tr>
<td>Fas-Sense</td>
<td>5'-ACCAGAAGACATCTGGTGCC-3'</td>
<td>Apoptosis (249 bp)</td>
</tr>
<tr>
<td>Fas-Antisense</td>
<td>5'-TCCCGTCTCATGATGCTTACC-3'</td>
<td></td>
</tr>
<tr>
<td>Bcl-2-Sense</td>
<td>5'-CGGGAGATCGATGAAGTA-3'</td>
<td>Anti-apoptosis (280 bp)</td>
</tr>
<tr>
<td>Bcl-2-Antisense</td>
<td>5'-CATATTGTTTGGGGCATGTCT-3'</td>
<td></td>
</tr>
</tbody>
</table>
**Results**

*Cell viability assay (LDH assay and trypan blue exclusion assay)*

As seen in Fig. 1, LDH release was observed to be significantly higher (2.83 ± 0.02 vs 1 ± 0.01) in the hypoxic control compared to the normoxic control, as expected ($P<0.001$). To further validate this, assessment of cell viability using trypan blue was also performed, and the results obtained (Fig. 2) are consistent (51.5 ± 6% viability in the PSME-treated group compared to 30 ± 7.2% in the hypoxia control group) with that shown in the LDH assay (1.6 ± 0.02 LDH release in the PSME-treated group compared to 2.83 ± 0.02 in the hypoxia control group) (Fig. 1). Normal control groups were used as the standard, where the cell count and LDH released to the medium from all other groups was calculated as fold against the standard.

Two concentrations of PSME, 0.1 and 1.0 mg/ml, were tried out to determine the better concentration to use. At 1.0 mg/ml, PSME-treatment had no significant effect on viable cell count, as compared with that of the hypoxia control group. Similar observations were made for the LDH assay where PSME-treated group showed no significant difference from the hypoxia control group. At 0.1 mg/ml concentration, PSME caused a significant increase in cell viability. Thus 0.1 mg/ml was then chosen for the rest of the experiments.

The statistics show the following points: Firstly, the hypoxic condition has a large effect on VSMC viability regardless of which treatment they were given (either hypoxia control or hypoxia with PSME-treatment). When comparing the hypoxic group with PSME-treated group, the results are extremely significant for both trypan blue and LDH assay ($P<0.001$).

*Gene-expression*

Relative Asp53, Bax, Fas, and Bcl-2 mRNA expression against the normal control standard were analyzed in each group as shown in Fig. 3, a – e. By considering the expression level of Asp53 across the different treatment groups, Asp53 expression in the normoxic group is comparatively weaker (Fig. 3: a and b) but has significantly increased in the hypoxic group (1.63-fold, Fig. 3b) ($P<0.001$). Meanwhile, PSME had significantly reduced Asp53 expression in hypoxic cells at 0.78-fold (Fig. 3b) ($P<0.05$).

As for expression of the pro-apoptotic protein Bax, its expression was also relatively weak in the normoxic group when considering the gene expression of Bax over the various treatment groups. Expression of Bax was significantly higher in the hypoxic group compared to that in the normoxic group (1.53-fold, Fig. 3c) ($P<0.001$). In addition, PSME had significantly down regulated the gene expression of Bax at 0.82-fold in the hypoxic group (Fig. 3c) ($P<0.001$).

As for expression of the pro-apoptotic protein Fas, it was found to be comparatively weaker in the normoxic group than in the PSME-treated group (0.87-fold, Fig. 3d). All groups were statistically significant when compared against the hypoxic control ($P<0.05$) and the PSME-treated group also showed significantly reduced Fas expression in comparison to the hypoxic control (Fig. 3d) ($P<0.001$).
For Bcl-2, its expression level was significantly reduced in the hypoxic groups compared to normoxic group (0.51-fold, Fig. 3e). PSME-treated group had significantly increased the Bcl-2 expression level in the hypoxic group (0.82-fold, Fig. 3e; \( P < 0.05 \)).

Bcl-2 is a classic anti-apoptotic protein while Bax is a classic pro-apoptotic protein. The Bcl-2/Bax ratio is very useful in determining the status of cells. According to Fig. 3f, the normoxic group and the PSME-treated group are neither pro- nor anti-apoptotic as they have a

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**Fig. 3.** RT-PCR gel electrophoresis pictures of the various pro and anti-apoptotic gene expression among various groups. 

- **a:** RT-PCR gel electrophoresis patterns of house-keeping gene GAPDH, which was used as an internal standard gene and of Asp53, Bax, Fas, and Bcl-2. Lane A: normoxic control group (NC), lane B: hypoxic control group (HC), lane C: hypoxia with 0.1 mg/ml PSME-treatment (HY + PSME).
- **b−e:** A comparison of the mRNA levels of Asp53 (b), Bax (c), Fas (d), and Bcl-2 (e) among each treatment group.
- **f:** Effects of the studied drugs on the state of cell via Bcl-2/Bax ratio. The hypoxic group has the lowest ratio, whereas the PSME-treated group has the same ratio as the normoxic group. *\( P < 0.05 \), normoxic control group vs hypoxic control group. *\( P < 0.05 \), PSME-treated group subjected to hypoxia vs hypoxic control group. ***\( P < 0.001 \), normoxic control group vs hypoxic control group. ***\( P < 0.001 \), PSME-treated group subjected to hypoxia vs hypoxic control group.
Fig. 4. Immunohistocytochemistry result. Light photomicrographs (60× magnification) of negative control in each treatment group (a), and Asp53 (b), Bax (c), Fas (d), and Bcl-2 (e) antibody staining of the rat vascular smooth muscle cells in each treatment group. i: normoxic control group, ii: hypoxia control, iii: hypoxia with 0.1 mg/ml PSME-treatment. The arrows are those positive staining with respective antibodies.

Magnification: —— = 20 μm

Fig. 5. Apoptotic staining in rat vascular smooth muscle cells after inducing hypoxia (60× magnification) for each treatment group. Apoptotic cells exhibited strong, nuclear green fluorescence (arrows). Treatment groups are as follows: a: normoxic (normal) control group, b: hypoxic (hypoxia) control, c: hypoxia with 0.1 mg/ml PSME-treatment.

Magnification: —— = 20 μm
ratio of 1, indicating equal amounts of Bcl-2 and Bax. Lastly, the hypoxic group is situated at a pro-apoptotic state having a ratio of 0.33.

**Immunohistochemical staining**

No positive staining of any protein product of targeted gene was observed in the negative control from each treatment group (Fig. 4a).

Very minimal Asp53 staining was observed in the normoxic group as shown in Fig. 4b-i. Strongest signal of Asp53 (arrow) was observed in hypoxic group (Fig. 4b-ii). Weaker positive staining of Asp53 (arrow) was observed in the group of hypoxia with PSME-treatment (Fig. 4b-iii).

No Bax staining was observed in the normoxic group (Fig. 4c-i). Same as the Asp53, the strongest signal of Bax (arrow) was in the hypoxic group (Fig. 4c-ii). Weak positive staining of Bax (arrow) was observed in the PSME-treated group subjected to hypoxia (Fig. 4c-iii).

No Fas staining was observed in the normoxic control. Strongest signal of Fas (arrow) was observed in hypoxic group (Fig. 4d-ii). Weak positive staining of Fas (arrow) was observed in the PSME-treated group subjected to hypoxia (Fig. 4d-iii). There was Bcl-2 positive staining (arrow) in the normoxic group (Fig. 4e-i). In addition, weak signal of Bcl-2 (arrow) was detected in the hypoxia group (Fig. 4e-ii). Positive Bcl-2 staining (arrow) was also observed in the PSME-treated group subjected to hypoxia (Fig. 4e-iii).

**TUNEL staining**

TUNEL staining was used to identify the apoptotic cells. Lowest level of apoptosis (green signal) was observed in the normoxic group (Fig. 5a). Strongest apoptosis marker (green signal, arrow) was observed in the hypoxic group (Fig. 5b). Less apoptosis was observed in the PSME-treated group (Fig. 5c). Overall, the PSME-treated groups showed a significant decrease in the level of apoptosis compared to the non-drug-treated hypoxic cells.

**Antioxidant tests**

Antioxidant tests showed significant differences in the SOD (0.25 ± 0.01 units/mg protein in the hypoxia control group vs 0.488 ± 0.02 units/mg protein in PSME-treated group) and CAT activity (0.026 ± 0.012 µmol per min per mg protein in hypoxia control group vs 0.076 ± 0.01 µmol per min per mg protein in PSME-treated group).
treated group) in all hypoxia groups regardless of which treatment they come from (either hypoxia control or hypoxia with PSME-treatment, *P*<0.05). Statistical significant differences were also observed when any hypoxia group was compared against the normoxic control group for both SOD (0.25 ± 0.01 units/mg protein in hypoxia control group vs 0.782 ± 0.02 units /mg protein in normoxic control group) and CAT activity (0.026 ± 0.012 µmol per min per mg protein in hypoxia control group vs 0.155 ± 0.01 µmol per min per mg protein in normoxic control group) (*P*<0.05). (Fig. 6: a and b, respectively).

However, no statistical significance was observed in any of the groups for both the GPx and GST antioxidant activities. (Fig. 6: c and d, respectively)

**Discussion**

In the present study, PSME was shown to display cardioprotective effects by lowering mortality rate, up-regulating expression of anti-apoptotic protein Bcl-2, and down-regulating expressions of pro-apoptotic proteins Asp53, Bax, and Fas after induction of hypoxia similar to previous studies (9, 12, 15, 17). PSME was also found to increase the antioxidant enzyme activities of SOD and CAT, which is consistent with the established results both in vitro and in vivo (7, 9, 12). For the cell viability assay (trypan blue exclusive assay and LDH assay) experiment, the right concentration of the drug had to be determined. Two starting concentrations were analyzed experimentally, i.e., 0.1 and 1.0 mg/ml. The 1.0 mg/ml PSME-treated group exhibited mass cell death and showed no significant difference with that of the hypoxia control in both cell viability tests. At 0.1 mg/ml concentration, PSME significantly increased cell viability, and thus this concentration was chosen for the rest of the experiments.

In the cell viability tests, the trypan blue exclusion test showed that there was a significant drop in the number of viable cells after induction of hypoxia. This is expected as the cells are undergoing stress and would have lower cell viability. PSME-treatment was able to significantly improve the cell viability, thus increasing the number of viable cells.

In the LDH assay, the results were consistent with the trypan blue cell viability results, with significant improvement in cell viability after treatment by PSME as observed in previous studies (15).

From the gene expression results, there was strong evidence for the over-expression of pro-apoptotic genes and down-regulation of anti-apoptotic genes after inducing hypoxia (similar to inducing myocardial infarction in an in vivo model). There was up-regulation of mRNA level of Asp53, Bax, and Fas and down-regulation of Bcl-2 mRNA expression. However, after drug-treatment, PSME was able to significantly reduce the expression of the pro-apoptotic genes of Asp53, Bax, and Fas. This would mean that PSME was able to reduce the amount of apoptosis after inducing hypoxia, similar to previous in vivo studies (9). Also, the expression of the anti-apoptotic protein Bcl-2 was up-regulated, although the expression was faint, hence increasing the chances of cell survival. These would result in higher cell viability and is consistent with the cell viability tests. This was similar to our other study on the animal model of stroke. Bcl-2 was observed to be up-regulated but not so strongly expressed due to its expression profile (22).

The down-regulation of Bax after inducing hypoxia is only marginally significant when compared against the normoxic group. This would mean that the level of Bax in the PSME-treated group is close to the level of Bax in the normoxic groups. This is a good sign as it indicates that the PSME is able to down-regulate the Bax expression in the hypoxic cells similar to the normoxic level, but still significant differences in levels are found.

As for Bcl-2, the level of Bcl-2 expression in the PSME-treated group is marginally significant. This could mean that PSME is very efficient because it can up-regulate the level of Bcl-2 in the hypoxic cells to essentially normoxic levels. This would not necessarily put the cells in an anti-apoptotic state as the state of the cells can only be determined after looking at the Bcl-2/Bax ratio.

The basis of survival by cells is dependent on the levels of the pro- and anti-apoptotic proteins expressed by their genes. Bax, a member of the Bcl-2 family, is able to heteromerize with the anti-apoptotic protein Bcl-2 to sequester its anti-apoptotic function, thereby initiating the intrinsic pathway of apoptosis. Thus, during PSME-treatment, when the level of Bcl-2 has been up-regulated, there will be more Bcl-2 available than what could be sequestered by the Bax. Hence, Bcl-2 will then be able to maintain the stability of the outer mitochondrial membrane and prevent the release of pro-apoptotic components from the mitochondria, which in turn would prevent cell death.

As known, Bcl-2 is a classic anti-apoptotic protein while Bax is a classic pro-apoptotic protein, so the Bcl-2/Bax ratio is very useful for determining the status of the cells (22). It is obviously shown in Fig. 3f that the PSME-treated group shows lower apoptosis potential, i.e., higher cell survival than the hypoxic control. However, PSME appears to bring the cells to the same state as the normoxic cells. It was observed that PSME-treated group has a Bcl-2/Bax ratio similar to that of the normoxic group. This could imply two things: either
PSME has lower capability in up-regulating the expression of anti-apoptotic protein Bcl-2 (or similarly down-regulating the expression of pro-apoptotic protein Bax) and hence leading to a state where both Bax and Bcl-2 were in equal activity (in this sense, the cell is neither in the pro- or anti-apoptotic state) or that the PSME is very efficient so that it was able to bring the cells to the normoxic state.

This indicates PSME has the ability to interfere with apoptosis, thereby potentially reducing the mortality rate of VSMC cells after inducing hypoxia. Thus, the increasing ratio of Bcl-2/Bax towards the anti-apoptotic situation further demonstrates that it has a promising therapeutic potential for ischemia.

In healthy individuals, Fas ligand (FasL) and Fas are mainly restricted to the immune tissues and to sites of immune privilege such as the eye and the testes where they can trigger apoptosis of invading immune cells (23). Fas expression was detected in the normoxic group and significantly higher in the hypoxic group in this experiment. The function of this death-inducing receptor in the heart however is not clear.

The gene expressions of most of the pro and anti-apoptotic genes detected in low levels in the normoxic group may be due to basal expression. However, the balance of the expression of these genes is greatly affected during hypoxia (either up-regulated or down-regulated) and PSME-treatment has shown to significantly help in restoring the balance of these gene expressions after induction of hypoxia.

As for the immunohistochemical staining, pro-apoptotic proteins Asp53, Bax, and Fas have been dramatically increased in the hypoxic group as compared to the normoxic group. The situation of increased pro-apoptotic proteins in the hypoxic group is consistent with the increased levels of pro-apoptotic gene expression in the hypoxic group. Furthermore, these pro-apoptotic proteins (especially Bax and Fas) could only be detected in the hypoxic group and not in the normoxic group, indicating up-regulation of these proteins during hypoxia as compared to undetectable levels of these proteins under normal conditions.

Although Bcl-2 could be detected in the normoxic group, its immunoreactivity is relatively weak. This result is consistent with the previous report by Ferrer et al. who showed that weak Bcl-x immunoreactivity was detected in the model of transient MCAO (16). However, stronger expression level could be detected in the PSME-treatment group. Concomitant expression of both distinct pro-apoptotic and pro-survival members of the Bcl-2 family during hypoxia offers little help towards the understanding of the role of the Bcl-2 family after hypoxia. Hence, in this experiment it can only be concluded that Bcl-2 plays an important role in the pathophysiological response after inducing hypoxia.

One of the most important biochemical hallmark of apoptosis in many cells is nuclear DNA fragmentation. The TUNEL system is designed for the detection of apoptotic cells via this hallmark. In this assay, fragmented DNA of apoptotic cells is catalytically incorporated with fluorescein-12-dUTP at the 3'-OH DNA ends using the terminal deoxynucleotidyl transferase. The fluorescein-12-dUTP-labeled DNA is then detected by fluorescence microscopy. The strongest nuclear green fluorescence was observed in the hypoxic group. This can be correlated with the up-regulation of Asp53, Bax, and Fas and the down-regulation of Bcl-2 as shown in gene expression as well as the highest immunoreactivity of pro-apoptotic proteins in hypoxia groups observed in the immunohistochemical staining. This indicates dramatic morphological changes and intense cell death taking place in the hypoxic group of cells. Less nuclear green fluorescence was observed in the PSME-treated group when compared to the hypoxic group. The ability of PSME to reduce the gene mRNA levels of pro-apoptotic genes as well as the lowered expression of pro-apoptotic proteins in immunostaining together with its reduced green fluorescence from the TUNEL assay all indicate the therapeutic potential of PSME after inducing hypoxia.

The results of the antioxidant studies demonstrate that PSME has antioxidant effects in vitro similar to previously published data (7, 9, 12). PSME appears to preserve the activities of SOD and CAT after induction of hypoxia. However, no significant results were obtained for both GPx and GST activities after inducing hypoxia.

In conclusion, this study shows that PSME was able to display cardioprotective effects on VSMC cells through the various experiments done on cell viability, gene expression, fluorescent TUNEL staining, immunohistochemical staining as well as antioxidant tests. Although PSME showed significant down-regulation of pro-apoptotic genes (Asp53, Bax, and Fas) and up-regulation of anti-apoptotic genes (Bcl-2) and hence is therapeutically promising, the underlying molecular mechanisms as well as the feasibility of long-term usage of this drug needs to be further elucidated. Future studies can also be done to isolate and identify the individual active compounds in this drug that contribute to their antioxidant properties. This might contribute to the acceptance of the complementary use of herbal medicines by Western doctors in treating cardiovascular disease in the future.
Acknowledgments

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