Panax notoginseng saponins protect rabbit bone marrow stromal cells from hydrogen peroxide-induced apoptosis

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Objective: To investigate the effects of Panax notoginseng saponins (PNSs) on hydrogen peroxide-induced apoptosis in rabbit bone marrow stromal cells (BMSCs).

Methods: BMSCs were isolated from 2-month-old New Zealand rabbits and cultured with different doses of PNSs to determine the most effective dose of PNSs by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and alkaline phosphatase (ALP) assay. The most effective dose of PNSs was used in subsequent experiments. Apoptosis of BMSCs was induced by hydrogen peroxide (100 μmol/L). BMSCs in PNSs group were also pretreated with PNSs before hydrogen peroxide exposure. Reactive oxygen species (ROS) levels were measured by using 2',7'-dichlorodihydrofluorescein diacetate. Apoptosis rate of BMSCs was observed by flow cytometry after staining with Annexin V-fluorescein isothiocyanate/propium iodide. The protein expression of Bax in BMSCs was analyzed by Western blotting. Activity of caspase-3 was measured by spectrofluorometry.

Results: The most effective dose of PNSs was 0.1 g/L. PNSs at dose of 0.1 g/L markedly reversed the augmentation of ROS level, decreased the apoptosis rate of, and the Bax expression and activity of caspase-3 in BMSCs treated with hydrogen peroxide (P<0.01).

Conclusion: PNSs can protect cultured rabbit BMSCs from hydrogen peroxide-induced apoptosis by decreasing oxidative stress, Bax expression and caspase-3 activity.

Keywords: Panax notoginseng, hydrogen peroxide, bone marrow stromal cells, apoptosis, rabbits

三七总皂苷对过氧化氢诱导的骨髓基质细胞凋亡的保护作用

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目的：观察三七总皂苷（Panax notoginseng saponins，PNS）对过氧化氢诱导的兔骨髓基质细胞（bone marrow stromal cell，BMSC）凋亡的影响。

方法：从 2 月龄新西兰兔获得原代 BMSC，给予不同浓度 PNS 处理后，通过检测 BMSC 增殖能力和碱性磷酸酶活性观察 BMSC 早期骨分化能力，筛选出 PNS 对 BMSC 作用的最佳浓度。采用过氧化氢 (100 μmol/L) 诱导 BMSC 凋亡。PNS 对过氧化氢诱导前的 BMSC 进行预处理后，应用 2',7'-二氯荧光素双乙酸盐法检测细胞活性氧水平，流式细胞术检测细胞凋亡率，免疫印迹法检测 BMSC 内 Bax 蛋白水平，荧光分光光度计检测 BMSC 内半胱氨酸天冬氨酸半胱氨酸特异性蛋白酶 3 (caspase-3) 活性。

结果：PNS 作用的最佳浓度是 0.1 g/L。与单纯过氧化氢处理组相比，0.1 g/L PNS 预处理能减少过氧化氢诱导后
The detailed mechanism by which steroid-induced osteonecrosis develops is not understood. It is important to understand the pathogenesis of steroid-induced osteonecrosis and to establish preventive methods to avoid this condition. Steroid-induced osteonecrosis is accompanied by widespread apoptosis of osteoblasts. Evidences have found a tight association between oxidative stress and apoptosis. Recently, oxidative stress and apoptosis have received increasing attention about the mechanism of steroid-induced osteonecrosis.

Bone marrow stromal cells (BMSCs) have been found to differentiate into a variety of cell types, including osteoblasts, chondrocytes and adipocytes, depending on the stimulatory microenvironment. BMSCs have been characterized in terms of their potential for differentiation into osteoblasts and are widely used as experimental models of bone remodeling, bone regeneration and bone diseases. It was reported that abnormalities in BMSCs might play a crucial role in the development of osteonecrosis.

*Panax notoginseng* is a highly valued and widely used traditional Chinese medicine. *Panax notoginseng* saponins (PNSs) were reported to be the active constituents responsible for the therapeutic action of this medicine. Various injection reagents of PNSs are commercially available and widely applied to the clinic in China. Researchers have focused on the anti-oxidative effect of *Panax notoginseng*, which acts as an anti-oxidative planta medica against many diseases. Therefore, the present study aimed to survey the effects of PNSs on hydrogen peroxide-induced apoptosis in cultured rabbit BMSCs.

1 Materials and methods

1.1 Chemicals and reagents  Fetal bovine serum (FBS), low-glucose Dulbecco’s modified Eagle’s medium (LG-DMEM), and Percoll were purchased from Gibco (USA). PNSs injection (Batch No. 08FL03, 250 mg (10 mL) each ampoule) was purchased from Kunming Pharmaceutical Corporation (Kunming, China), and its purity was more than 99% detected by high-performance liquid chromatography. Alkaline phosphatase (ALP) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

1.2 Determination of the most effective dose of PNSs

1.2.1 Determination of growth curve of cell proliferation  BMSCs were harvested and dispensed into 96-well culture plates at a concentration of 1×10⁶ cells/mL in LG-DMEM containing 10% FBS. Cells were treated with different doses of PNSs (0, 0.025, 0.05, 0.1 and 0.2 g/L). 20 µL MTT stock solution (5 g/L) was added to each well, and after incubation at 37 °C for 4 h, supernatants were removed and replaced by 150 µL dimethyl sulphoxide (DMSO). The plates were mounted on a micro-mixer for 5 min, and then they were placed in an Automated Microplate Reader (Bio-Rad 550) and the optical density (OD) of each well was measured at 570 nm. The assay was used for cultures every 24 h and the growth curve was constructed to assess cell proliferation.

1.2.2 Determination of ALP activity  BMSCs were subcultured into 96-well plates and treated
with different doses of PNSs (0, 0.025, 0.05, 0.1 and 0.2 g/L). Briefly, cells were washed three times with ice-cold Tris-buffered saline, pH 7.4, immediately harvested by scraping, and sonicated for 20 s at 4 °C. Total protein levels were determined by the biecinchonic acid assay and used to normalize ALP activity. ALP activity was determined by using the ALP assay kit according to the manufacturer’s instructions. The OD value of each well was measured at 410 nm by using Automated Microplate Reader. This assay was repeated for cultures at 48 h intervals (total seven days).

The most effective dose of PNSs was determined from the growth curves and ALP activity data and this dose was used in the following experiments.

1.4 Induction of oxidative stress and treatment

Oxidative stress was induced by H$_2$O$_2$ addition as described previously.$^{[15]}$ In brief, the cells were treated with 100 µmol/L H$_2$O$_2$ in LG-DMEM containing 10% FBS for 24 h. In control group, the cells were treated only with LG-DMEM containing 10% FBS. In PNSs group, the cells were pre-incubated for 4 h with PNSs before H$_2$O$_2$ exposure. The cells in each group were cultivated in different medium for 72 h.

1.5 Detection of cell apoptosis

The adherent cells were detached with 0.25% trypsin. The cells in each group (2×10$^6$ cells/group) were rinsed with ice-cold PBS and were centrifuged two times (1 000 r/min, 25 min), then re-suspended in reaction buffer. Annexin V stock solution was added to the cells and incubated for 30 min at 4 °C. The cells were further mixed with PI and immediately analyzed by a flow cytometer (BD Biosciences Clontech, USA) equipped with Cell Quest software. Apoptosis was routinely quantified by counting the number of cells stained with FITC-labeled Annexin V.

1.6 Determination of reactive oxygen species

Reactive oxygen species (ROS) levels in BMSCs were measured by using the DCFH-DA. Following PNSs and H$_2$O$_2$ treatment, the cells were incubated with 20 mmol/L DCFH-DA for 20 min. After centrifugation for removing DCFH-DA, the cells were washed twice with natural saline. DCFH fluorescence was detected by fluorescence-activated cell sorting (FACS) analyses by using Automated Microplate Reader (Bio-Rad, USA) at excitation wavelengths of 485 nm and 538 nm emission.

1.7 Bax expression observed by Western blot analysis

Stimulated cells (1×10$^7$) were washed with cold PBS and subsequently lysed in a lysis buffer. Insoluble material was removed by microcentrifugation at 13 000 r/min for 15 min at 4 °C. Cell lysates were analyzed byodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membrane. After blocked with Tris-buffered saline, membranes were incubated with different antibodies. For chemiluminescence detection, membranes were incubated in the dark with enhanced chemiluminescence substrate for horseradish peroxidase. The luminescent signal was recorded and quantified with the Syngene G box in a mini-darkroom enclosure. It was linked to a computer that controls the instrument and handles the data. The luminescent signal was detected by the camera with charge-coupled device and transmitted to the controller unit and the data were sent to the computer for analysis and documentation.

1.8 Caspase-3 activity assay in BMSCs

Caspase-3 activity was measured by using the caspase-3 activity assay kit. Standard curve was drawn by detecting the absorbance of standard samples with terminal concentrations at 0, 0.5, 1.0 and 1.5 g/L, respectively, at a wavelength of 490 nm. Then BMSCs were collected and lysed in caspase assay buffer completely. Supernatants were collected. The activity of the caspase-3 was detected by the enzyme-labeling meter with the wavelength of 405 nm. The activity of caspase-3 was represented with nkat.

1.9 Statistical analysis

SPSS software (Version 13.0, SPSS, USA) was used for all statistical comparisons. All experiments were performed for at least 3 times. All data were expressed as $\bar{x} \pm s$. Statistical analysis of data was performed by applying one-way analysis of variance. $P < 0.05$ was considered to be statistically significant.

2 Results

2.1 The most effective dose of PNSs on BMSCs

Growth curves of BMSCs treated with and without PNSs are shown in Figure 1A. The effects of PNSs with different doses on ALP activity of BMSCs are shown in Figure 1B. The results showed that PNSs had different effects on proliferation and osteoblastic differentiation in BMSCs. PNSs at the dose of 0.1 g/L caused the greatest increase of proliferation and ALP activity. According to the results of the growth curves and ALP activity, the dose of 0.1 g/L was determined as the most effective dose. Therefore, this dose was used to investigate the protective effect of PNSs on H$_2$O$_2$-induced apoptosis in BMSCs.

2.2 Effects of PNSs on ROS level of BMSCs

Intracellular ROS level of BMSCs were measured by using the DCFH-DA. Compared with the control group, the ROS level of BMSCs was increased significantly after being treated with
H$_2$O$_2$ ($P<0.01$). When the cells were pretreated by PNSs, the ROS level was decreased significantly ($P<0.01$). Significant alteration in ROS level was observed, indicating severe oxidative stress in the cells. Pre-incubation with PNSs could significantly alter ROS level of BMSCs induced by H$_2$O$_2$, showing the biological antioxidative effect of PNSs (Figure 2).

2.5 Effects of PNSs on activity of caspase-3 in BMSCs Activity of caspase-3 in BMSCs was measured by using caspase-3 activity assay kit. When BMSCs were treated with H$_2$O$_2$, a significant increase in activity of caspase-3 was observed ($P<0.01$). Pre-incubation of cells with PNSs markedly reduced the alteration in activity of caspase-3 induced by H$_2$O$_2$ ($P<0.01$). The results showed that PNSs had a protective effect on H$_2$O$_2$-induced apoptosis by decreasing the activity of caspase-3 in BMSCs (Figure 5).

2.3 Effects of PNSs on BMSC apoptosis The cells were analyzed with flow cytometry after staining with Annexin V-FITC/PI. Cells treated by H$_2$O$_2$ displayed much higher apoptosis rate (38.68%) than that of the control group (4.66%) ($P<0.01$). After pretreatment with 0.1 g/L PNSs, the apoptotic rate of BMSCs was markedly decreased to 19.24% ($P<0.01$). The results showed the protective effects of PNSs against H$_2$O$_2$-induced apoptosis in cultured rabbit BMSCs (Figure 3).

2.4 Effects of PNSs on protein expression of Bax in BMSCs The protein expression of Bax in BMSCs was analyzed by Western blotting (Figure 4a). H$_2$O$_2$ treatment markedly increased the protein level of Bax as compared with the control ($P<0.01$). Pretreatment with PNSs markedly reversed H$_2$O$_2$-induced augmentation of Bax expression ($P<0.01$). The results showed that alteration in the protein expression of Bax was involved in the anti-apoptotic effect of PNSs (Figure 4b).
oxidative planta medica against many diseasest\textsuperscript{[15,12]}]. So we aimed at the role of PNSs in H\textsubscript{2}O\textsubscript{2}-induced apoptosis of BMSCs for preventive treatment of osteonecrosis. And understanding the mechanism of the effects of PNSs on BMSCs would be helpful for the study of Panax notoginseng.

MTT is known to interact with components of the mitochondrial respiratory chain and its reduction has often been used as a convenient and precise means of assessing cell proliferation. ALP activity is one of the most frequently used parameters for evaluating cell osteogenic differentiation and for identifying osteogenic properties. The PNSs at dose of 0.1 g/L could cause the greatest increase of proliferation and ALP activity without any obvious cytotoxicity. Depending on the results of growth curves and ALP activity, the most effective dose of PNSs (0.1 g/L) was firstly determined and used to investigate its effect on H\textsubscript{2}O\textsubscript{2}-induced apoptosis of BMSCs.

ROSs induce a number of molecular alterations in cellular components, leading to changes in cell morphology, viability and function. High levels of ROSs perturb the normal redox balance and shift cells into a state of oxidative stress\textsuperscript{[16]}. Oxidative stress is believed to contribute to etiology of various diseases\textsuperscript{[17]}. In this study, ROS level was selected as an index of oxidative stress, and it was determined whether PNS pretreatment could protect apoptosis of the cells from H\textsubscript{2}O\textsubscript{2}-induced oxidative stress. The results showed that ROS level altered significantly in different groups, indicating that PNS was a real antioxidant.

Moreover, the apoptotic rate was observed by using flow cytometry after Annexin V/PI staining. The apoptotic rate induced by H\textsubscript{2}O\textsubscript{2} had the same significant alteration as ROS level in different groups. This study confirmed a tight association between oxidative stress and apoptosis\textsuperscript{[18,19]}. It first showed that PNSs had a protective effect on H\textsubscript{2}O\textsubscript{2}-induced apoptosis in rabbit BMSCs by decreasing oxidative stress. So it was necessary to investigate the possible mechanism of antiapoptotic effects of PNSs.

It has been documented that the progress of apoptosis is regulated by the Bcl-2 family. Bax, the pro-apoptotic gene of the family, is expressed abundantly and selectively, and promotes apoptosis. In addition, Bax, a related homologue of Bcl-2, forms heterodimers with Bcl-2, effectively antagonizing Bcl-2 function and promoting apoptosis\textsuperscript{[20]}. In this study, treatment with H\textsubscript{2}O\textsubscript{2} increased the Bax protein expression level. It indicated that oxidative stress increased Bax expression and induced
cell apoptosis. However, PNSs decreased the Bax expression. It showed that PNSs could alter the change by decreasing oxidative stress.

Caspase-3, one member of the caspase family, is in the downstream of Bax and particularly believed to be most commonly involved in the execution of apoptosis induced by many stimuli. It has been proved that caspase-3 induces apoptosis through different ways including cleaving DNA repair molecules, degrading the anti-apoptosis proteins, cleaving extracellular matrix proteins, skeleton proteins and other related molecules. Our data showed that low-dose H2O2 increased ROS level, activated caspases and induced apoptosis at last as described previously. However, pretreatment with PNSs decreased caspase-3 activity and apoptotic rate. All these indicated that PNSs decreased H2O2-induced apoptosis by down-regulating activity of the caspase-3.

Taken together, the present study has demonstrated that pretreatment with PNSs had a protective effect on H2O2-induced apoptosis in cultured rabbit BMSCs by acting as a biological antioxidant. The mechanistic study has also helped show that PNSs protected BMSCs against H2O2-induced apoptosis by decreasing oxidative stress, Bax expression and caspase-3 activity. Thus, PNSs could be developed to a novel preventive agent against steroid-induced osteonecrosis with further experiments.

4 Acknowledgment

This study was supported by National Natural Science Foundation of China (No. 30600624).

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全国中西医结合防治变态反应疾病中青年学术论坛征文通知

为了加强变态反应领域中青年医师的学术交流，促进变态反应学的发展，由中国中西医结合学会变态反应专业委员会主办、北京皮肤病医院承办的“全国中西医结合防治变态反应疾病中青年学术论坛”定于2010年8月7～8日在北京召开。

本次会议将邀请约20位变态反应领域（包括变态反应科、皮肤科、呼吸科、耳鼻咽喉科、儿科、免疫科及针灸科等）的国内外知名学者及最新科研和临床研究进展以及热点问题进行主题讲座和学术交流。变态反应专业委员会还将在会议期间增补15名青年委员。欢迎各相关学科同仁踊跃投稿并出席会议，现将征文事项通知如下。

1 征文范围 变态反应性疾病临床诊断、防治、流行病学及其基础研究，涉及中西医结合反应科、皮肤科、呼吸科、耳鼻咽喉科、儿科、免疫科及针灸科等相关领域。

2 征文要求 （1）未在国内公开发行刊物上发表的论文（勿提交综述类文章）。 （2）非结构式摘要1份，约1000字，编排顺序为题目、单位、邮政编码、姓名、摘要正文。全文限制在4000字以内。 （3）请务必附通讯地址，联系电话（单位、住宅电话及手机）、E-mail，以便及时联系。

3 投稿方式 请将论文以Word格式发送至qnlh2010@yahoo.com.cn。邮件标题请注明“中青年论坛投稿”字样。

4 截稿日期 2010年5月15日。

5 其他事项 会议代表可获得国家中医药继续教育学分，会议录用的论文可获得中国中西医结合学会论文证书。会议的具体报到时间和地点将另行通知。

中国中西医结合学会
2010年1月18日