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# Protective effect of *Potentilla anserine* polysaccharide (PAP) on hydrogen peroxide induced apoptosis in murine splenic lymphocytes

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ABSTRACT

The study was carried out to investigate protective effect of *Potentilla anserine* polysaccharide on the process of apoptosis induced by hydrogen peroxide ($H_2O_2$) in murine splenic lymphocytes *in vitro*. The results showed that apoptosis occured in murine splenic lymphocytes treated with $H_2O_2$ *in vitro* at a concentration of 200 μmol/L. Treatments with *Potentilla anserine* polysaccharide at concentrations of 50, 100, 200 or 400 μg/mL decreased the amount of apoptosis cells and the anti-apoptosis effect showed a dose dependent manner increase. The results showed that PAP reduced intracellular oxidative damages and dose-dependently inhibited apoptosis. This suggests that PAP is a potential anti-apoptotic agent.



## 1. Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide, super oxide anion and hydroxyl radical are generated in biological systems by aerobic metabolism and also by exogenous sources such as drugs, ultra violet light, ionizing radiation and pollution systems (Briviba & Sies, 1994). Over production of free radicals was ascertained to play multiple important roles in tissue damage and loss of function in a number of tissues and organs (Simic, Bergtold, & Karam, 1989; Zheng & Huang, 2001). The antioxidants could attenuate oxidative damage of a tissue indirectly by enhancing natural defenses of cell and/or directly by scavenging the free radical species. *Mango* peel extract exhibited protection against oxidative damage induced by $H_2O_2$ in normal rat erythrocytes (Ajila & Prasada, 2008). In recent years, more and more polysaccharides have been investigated and reported to exhibit a variety of biological activities, including anti-tumor (Jiao, Li, & Li, 2009; Wasser, 2002), signal transduction (Li, Lei, & Liang, 1999; Zhang, Xiang, & Cui, 1997), immunostimulation (Zhang & Huang, 2005), gene expression (Chen, Tan, & Chan, 2008a; Chen, Zhong, & Zeng, 2008b), anti-oxidation (Li et al., 2003) and anti-apoptosis (Wei, Cong, & Xian, 2002; Xia, Li, & Zhang, 2001). Meanwhile, gradually growing attention has been paid to polysaccharides characterized as antioxidant natural products. Polysaccharides from plants, animals and fungi are thought to be a promising group of antioxidative compounds (Yang, Gao, Han, & Tan, 2005).

*Potentilla anserine* (*P. anserine*) has been used as an ancient Tibetan natural medicine and food for more than 1500 years in China. In traditional Chinese medicine, *P. anserine* was believed to have a variety of beneficial effects on different body systems including immune function. However, despite its long history and widespread use, the number of rigorous scientific and clinical studies to substantiate the medicinal effects of *P. anserine* is insufficient. In a recent preliminary study, we observed that, compared to the control animals, administration of a polysaccharide isolated from *P. anserine* to mice caused significant changes in the circulatory lymphocytes and indices of thymus and spleen. *P. anserine* polysaccharide could overcome cyclophosphamide induced immunosuppression in mice (Chen, Hu, & Cheng, 2005; Hu, Cheng, & Cheng, 2005) and had a good protective effects on $CCl_4$-induced liver injury in mice (Min, Lu, & Min, 2008). The aim of the present study was to evaluate the protective role of *P. anserine* polysaccharide against hydrogen peroxide induced apoptosis in murine splenic lymphocytes.

## 2. Materials and methods

### 2.1. P. anserine polysaccharide and reagents

*P. anserine* polysaccharide was obtained from the roots of *P. anserine* and identified by the Lanzhou Institute of Animal & Veterinary Pharmaceutics, Chinese Academy of Agricultural Sciences. *P.*

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*anserine* polysaccharide was shown to be homogenous by Sephadex G-100 gel filtration chromatography and DEAE cellulose-52 chromatography. The defined polysaccharide was composed of D-rhamnose, D-arabinose, D-glucose and D-galactose by GC with the molar ratio of 1:1.37:2.94:3.49. Endotoxin was assayed under endotoxin free experimental conditions using a limulus amebocytes lysate (LAL) pyrogen kit (Zhanjiang Adc. Biological Ltd.). The experiments were carried out according to the manufacture's protocol: 100 μL of standards, *P. anserine* polysaccharide or controls were mixed with 100 μL of LAL reagent and incubated for 1 h at 37 °C, each tube was then examined for gelation. From the test, the quantity of endotoxin in *P. anserine* polysaccharide was ⩽0.005 ng/mg. *P. anserine* polysaccharide is a kind of white powder and was dissolved in distilled water in a concentration of 10 g/L and then filtered through a 0.22 μm filter and stored at 4 °C. The solution of *P. anserine* polysaccharide was further diluted to different concentrations (50, 100, 200 or 400 μg/mL) with RPMI-1640 medium in tissue plate separately. Tris(hydroxymethyl) aminomethane, RNaseA, dimethyl sulfoxide (DMSO), *N*-2-hydroxyethyl piperazine-*N*'-2-ethanesuifonic acid (Hepes), Protease K, 2-Mercapto-ethanol and propidium iodide were purchased from Sigma Chemical Co. RPMI-1640 was obtained from Gibco Laboratories. Fetal calf serum was supplied by Sijiqing Institute of Biomaterials, Hangzhou, China. Giemsa dyestuff, $KH_2PO_4$, $MgCl_2$ and $CaCl_2$ were purchased from Sino-American Biotechnology Company. Methanol (spectrum purity) was obtained from Tianjing Biochemicals Reagent Company. All other chemicals used were ultra pure or analytic grade.

### 2.2. Isolation and culture of murine splenic lymphocytes

Murine spleen were collected and purified from BALB/C inbred mice. Splenic cell suspension was filtered through a 10 μm nylon mesh. Recovered splenic cells were resuspended in lysis buffer (0.15 M $NH_4Cl$, pH 7.4) for 5 min to remove erythrocytes and then followed by Ficoll/Hypaque (1.077 g/mL) density gradient centrifugation at 2000 rpm for 30 min. After centrifugation, splenic lymphocytes were collected into a clean tube and washed for three times at 1000 rpm for 10 min with 0.01 mol/L phosphate buffered solution (PBS, pH 7.2), and then resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated (56 °C for 30 min) fetal calf serum (FCS), 100 U/mL benzylpenicillin sodium, 100 μg/mL streptomycin, 2 mmol/L glutamine and 50 μmol/L β-mercaptoethanol. Cell viability was estimated according to the trypan blue exclusion criteria and the purity of lymphocytes was higher than 95%. Three batches of cells were used for morphological observation, DNA agarose gel electrophoresis and flow cytometry analysis respectively.

Murine splenic lymphocytes were set up in 24-well plates ($2 \times 10^6$/mL) and the inhibition of apoptosis was evaluated. To 3.0 mL of suspension of murine splenic lymphocytes in RPMI-1640 medium enriched with PAP at the concentrations of (0, 50, 100, 200 or 400 μg/mL) or catalase (CAT) 100 U/mL, 300 μL of 2.0 mmol/L $H_2O_2$ (in PBS pH 7.2) was added. RPMI-1640 medium with the murine splenic lymphocytes alone was used as normal control. The reaction mixtures were shaken gently and then were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ in air for 12 h. After incubation, the suspensions were centrifuged at 1000 rpm for 10 min and the cell pellets were resuspended in 10 mM phosphate buffered saline (PBS, pH 7.2) in the concentration of $1 \times 10^6$ cells/mL.

### 2.3. Giemsa dyeing and observation with light microscope

About 100 μL of suspension of murine splenic lymphocytes ($1 \times 10^6$ cells/mL) in PBS (pH 7.2) was placed on a clean microscope slide, and fixed with methanol for 1 min, followed by air drying. The cells were treated with *Giemsa* dyeing fluid at room temperature for 5 min, and then the dye was washed off gently and stayed in air at room temperature. The cells were observed under the light microscope.

### 2.4. Observed through laser scanning confocal microscope

After incubation, the cells were harvested and centrifuged at 1000 rpm for 10 min, the sediments were suspended in acridine orange for 30 min and then washed with RPMI-1640 for three times at 1000 rpm for 10 min, the pellets were collected for laser scanning confocal microscope observation.

### 2.5. Measurement of apoptosis by DNA fragmentation

The cells density was adjusted to $2 \times 10^6$/mL and was cultured as the method described above (Section 2.2.). After incubation, the cell suspension from each group was centrifugated at 2000 rpm for 10 min, the cells were collected and placed in 70% alcohol prechilled at −20 °C and stored on ice for 4 h. Then the alcohol was removed by centrifuging at 1000 rpm for 5 min, the cells were washed with PBS and resuspended with 40 μL PC buffer and the cell suspensions were stored at room temperature for 30–60 min and followed by centrifugation at 1000 rpm for 5 min. The supernatants were collected in tubes and concentrated 15 min at vacuum. For each tube, 3 μL 0.25% CA-630 solution and 3 μL RNaseA solution (1 mg/mL) were added and mixed. The solution was kept for 30 min at 37 °C and 3 μL proteinase K (20 mg/mL) was added and then stored for 30 min at 37 °C. Finally, 10 μL of DNA was used to run on a 1.5% agarose gel with 50 V for 90 min.

### 2.6. Apoptosis analyzed by flow cytometry

The cells were cultured as described in Section 2.2. The cells were washed with PBS for two times, then fixed with prechilled 70% alcohol at 4 °C for 24 h. After centrifugation at 1000 rpm for 10 min, the supernatant was abandoned, then the cells were resuspended with 3 mL PBS. Five minutes later, the cell suspension was centrifugated at 1000 rpm for 10 min. Triton X-100 (1.0%) was added and vortex to mix properly. After 10 min, the cell suspension was centrifugated at 1000 rpm for 10 min, then filtered through a 400 screen mesh. The cells were resuspended in 1.0 mL of propidium iodide (PI) buffer (50 μg/mL PI) for 30 min at 4 °C in the dark by gently inverting the tube. The cells were analyzed with a flow cytometry detecting PI emission at 630 nm.

## 3. Results

### 3.1. The morphological changes of the apoptotic cells under light microscope

Morphological assessment of apoptosis was performed with light microscope and laser scanning confocal microscope. Apoptosis occured in murine splenic lymphocytes *in vitro* when the concentration $H_2O_2$ reached to 200 μmol/L. The reduced cell volume, cytoplasm and nucleuses shrinkage appeared. DNA and RNA in non-uniformly stained form were observed; condensed chromatin and formation of apoptotic body were seen in the apoptotic cells (Image B in Fig. 1). Morphology of the cells treated with both $H_2O_2$ and *P. anserine* polysaccharide at concentration of 400 μg/mL did not change when compared with normal control (Image G in Fig. 1).

The nuclei of splenic lymphocytes were blue or amethysts and the luster of chromatins was homogeneous in normal control (Im-

age A in Fig. 1). The nuclei of splenic lymphocyte treated with $H_2O_2$ alone revealed features of apoptosis. The karyopyknosis and side gathered were observed in the apoptotic cells treated with $H_2O_2$ at concentration of 200 μmol/L. The signs were accompanied by cell shrinkage with a accumulation of densely stained chromatin, typically at the edge of the nucleus. The number of membrane-bound apoptotic bodies were observed in the cells treated with $H_2O_2$ alone (Image B in Fig. 1). The damaged state of splenic lymphocytes was attenuated by PAP (Images D–G in Fig. 1). The shape and structure of the cells treated with CAT was found to be nearly identical with those of PAP treatment alone (Image C in Fig. 1).

### 3.2. Results of observing through laser scanning confocal microscope

DNA was dyed in flavo-green fluorescence, RNA and cytoplasm were dyed in tangerine fluorescence in normal control (Images A and H in Fig. 2). The dyeing of cellular nucleus in the cells treated with $H_2O_2$ alone had signs of maldistribution and karyopynosis, with a marked accumulation of densely stained chromatin, particularly at the edge of the nucleus (Images B, I and J in Fig. 2). A number of apoptotic bodies were seen in the cells treated with $H_2O_2$ alone (Image K in Fig. 2). CAT treatment (Image C in Fig. 2) or PAP treatments attenuated apoptosis induced by $H_2O_2$ and the effect of PAP showed a concentration dependent manner increase (Images D–G in Fig. 2).

### 3.3. Results of DNA agarose gel electrophoresis

DNA fragmentation was visualized by agarose gel electrophoresis. In Fig. 3, Lane A exhibited fine DNA laddering of Marker. In Lane B, the DNA appeared unfragmented in normal control; a large band appeared at the edge of the well after electrophoresis, indicating

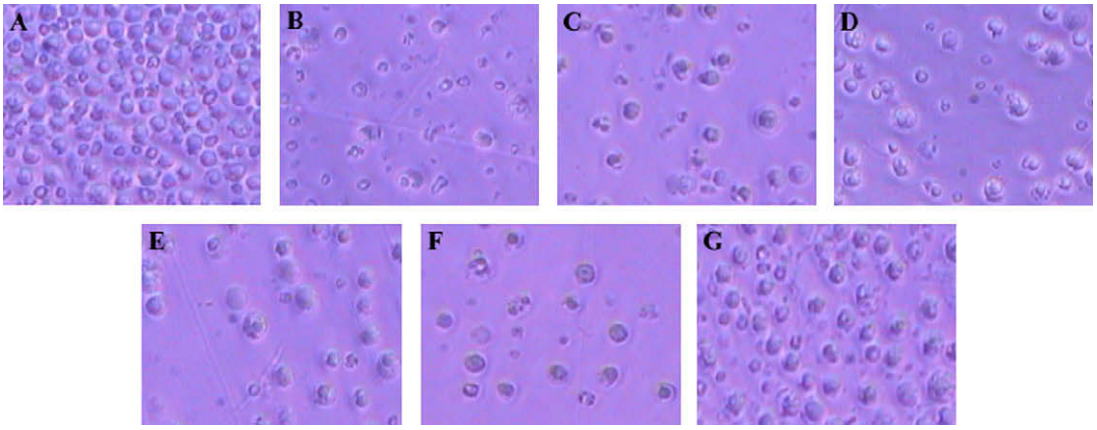


**Fig. 1.** Morphology of apoptotic cells under light microscope. (A) Normal control; (B) $H_2O_2$ treatment (200 μmol/L); (C) CAT (100 U/mL) + $H_2O_2$ (200 μmol/L) treatment. (D) PAP (50 μg/mL) + $H_2O_2$ (200 μmol/L) treatment. (E) PAP (100 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (F) PAP (200 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (G) PAP (400 μg/mL) + $H_2O_2$ (200 μmol/L) treatment.

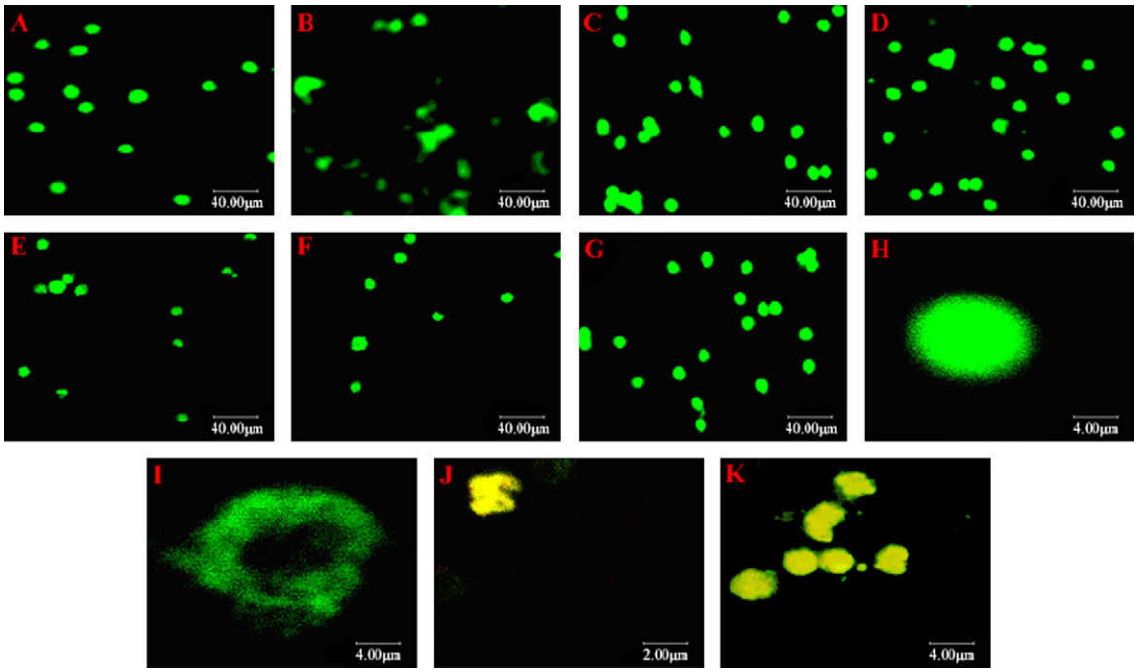


**Fig. 2.** The morphological changes in murine splenic lymphocytes through LCSM. (A) Normal control; (B) $H_2O_2$ treatment (200 μmol/L); (C) CAT(100 U/mL) + $H_2O_2$ (200 μmol/L) treatment. (D) PAP (50 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (E) PAP (100 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (F) PAP (200 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (G) PAP (400 μg/mL) + $H_2O_2$ (200 μmol/L) treatment. (H) Nucleus of normal control; (I) Nucleus of apoptosis; (J) Nucleus of apoptosis; (K) Apoptotic body.

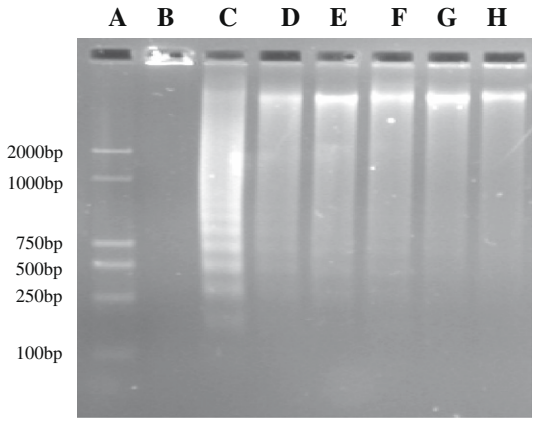


**Fig. 3.** Detection of fragmented DNA by agarose gel electrophoresis. (A) Marker; (B) Normal control; (C) $H_2O_2$ treatment (200 μmol/L); (D) PAP(50 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (E) PAP (100 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (F) PAP (200 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (G) PAP (400 μg/mL) + $H_2O_2$ (200 μmol/L) treatment. (H) CAT (100 U/mL) + $H_2O_2$ (200 μmol/L) treatment.

large amounts of DNA existed. Usually, the band means that sufficient DNA has been extracted. In Lane C, the lymphocytes induced by $H_2O_2$ produced a good fragmentation ladder as a positive control. Meanwhile, unfragmented DNA bands and fragmentation ladder appeared in Lanes D–F, indicating incomplete apoptotic fragmentation in the samples of treatments with both $H_2O_2$ and PAP at concentrations of 50 μg/mL, 100 μg/mL or 200 μg/mL. The DNA in Lane G appeared to be unfragmented, a part of DNA remained near the top of the gel. The DNA in Lane H appeared unfragmented protected by CAT (100 U/mL) (Fig. 3). DNA fragmentation assay showed that ladder bands were reduced obviously when exposed to both $H_2O_2$ and *P. anserine* polysaccharide at concentrations of 50, 100, 200 or 400 μg/mL compared to $H_2O_2$ alone.

### 3.4. Apoptosis detection by Flow cytometry-based methods

Quantitative detection of apoptotic cells and analysis of cell cycle kinetics were performed by flow cytometry. The formed sub-G1 peak and reduced DNA content represented the presence of apoptotic cells. The two major peaks represented the G0/G1 and G2/M phases of the cell cycle. The proportion of apoptotic cells in normal control was 5.60% (Image A in Fig. 4). After treatment with $H_2O_2$ at a dose of 200 μmol/L for 12 h, the proportion of apoptotic cells reached to 45.40% (Image B in Fig. 4). When treated with both $H_2O_2$ (200 μmol/L) and PAP at a dose of 50 μg/mL, 100 μg/mL, 200 μg/mL or 400 μg/mL for 12 h, the proportions of apoptotic cells were 37.80%, 22.70%, 17.70% and 8.50% respectively (Images D–G in Fig. 4). When treated with both $H_2O_2$ (200 μmol/L) and CAT (100 U/mL) for 12 h, the proportion of apoptotic cells was 5.90% (Image C in Fig. 4). Flow cytometry analysis showed that $H_2O_2$ increased the populations of apoptotic sub-G1 (45.4%) compared with 5.6% in normal control. Treatment with *P. anserine* polysaccharide at concentrations of 50, 100, 200 or 400 μg/mL caused decreases of sub-G1 phase respectively in cells compared with $H_2O_2$ treatment alone and the result showed a concentration dependent manner effect (Fig. 4 and Table 1).

## 4. Discussion

Although apoptosis is one character to maintain normal growth and homeostasis in the body with cell proliferation, excessive apoptosis initiated by extracellular agents is a kind of pathologic lesion (Ludwig, Pleschka, Planz, & Wolff, 2006). Reactive oxygen species (ROS) is generated from $O_2$ by multiple pathways. Under physiological conditions, there is a well managed balance between the formation and neutralization of ROS by antioxidant systems. Oxidative stress can occur when ROS production is accelerated or when the mechanisms involved in scavenging ROS is impaired (Li, Zhou, & Li, 2007). Increased production of ROS is thought to occur more frequently than diminished antioxidant defence, and is postulated to play a role in the pathogenesis of several irradiation-induced diseases (Shao, Dai, Xu, Lin, & Gao, 2004). ROS also induce DNA strand breaks and apoptosis (Breen & Murphy, 1995; Sarafian & Bredeson, 1994). Increasing studies proved that ROS mediated apoptosis in many cells as a new kind of second messenger (Clement & Pervaiz, 1999; Inoue & Ogihara, 1998; Joya, Afshin, & Sten, 2000). Oxidative stress and redox chemistry initiate most, if not all, of the pathways leading to apoptosis (Kannan & Jani, 2000).

Reactive oxidizing species initiate apoptosis by stimulating plasma membrane death receptors, but ROS, particularly $H_2O_2$ are also produced as a consequence of activation of these receptors by their ligands, and these latter ROS are also believed to participate in the apoptotic process. $H_2O_2$ can also easily penetrate cell membrane directly into cell nucleus without enzymatic degradation. $H_2O_2$ has been demonstrated to cause lipid peroxidation and DNA damage in cells (Luo et al., 2006). A number of natural antioxidant compounds such as herbal extracts are widely used to reduce damages caused by oxidative stress. Morphological changes to erythrocyte membrane caused by hydrogen peroxide were protected by *mango* peel extract (Ajila & Prasada, 2008). *Sophora subprosrate* polysaccharide overcame cyclophosphamide induced immunosuppression and significantly raised glutathione level, superoxidase dismutase activity and total antioxidant capacity in mice (Chen, Hu, & Zheng, 2007). A polysaccharide isolated from *Cordyceps sinensis*, a traditional Chinese medicine, protected pheochromocytoma PC12 cells from hydrogen peroxide induced injury (Li et al., 2003). *Lycium barbarum* polysaccharides had protective effect on streptozotocin induced oxidative stress in rats (Li, 2007). Many polysaccharides had inhibitory effect on splenic lymphocyte apoptosis (Chen et al., 2008a, 2008b; Luo, Wu, & Yang, 2004; Wu, Yang, & Huang, 2004), but the regulatory mechanism of polysaccharides in occurrence of lymphocyte apoptosis keep unknown till now.

The present study was designed to investigate the protective effect of PAP on apoptosis induced by hydrogen peroxide in murine splenic lymphocytes and to find modulatory mechanism of polysaccharides in cell apoptosis. The result showed that hydrogen peroxide induced apoptosis of murine spleen lymphocytes at concentration of 200 μmol/L. A condensation of the nuclear material at the edge of the nucleus and cell shrinkage with the formation of a number of apoptotic bodies were observed at the light microscopic level in the cells treated with $H_2O_2$ or in the cells treated with both $H_2O_2$ and PAP at concentrations of 50, 100 or 200 μg/mL. PAP at concentration of 400 μg/mL reduced oxidative damage induced by $H_2O_2$ and had an anti-apoptosis function on splenic lymphocytes induced by oxidative stress. Despite considerable progress in the understanding of the mechanistic basis of apoptosis, morphological analysis remains unquestionably the "gold standard" for its assessment and quantitation (Vicki, Peter, & Philip, 2004).

A biochemical hallmark of apoptosis is the cleavage of chromatin into small fragments, including oligonucleosomes, which were described as DNA ladders in the electrophoresed gel. In this study, typical DNA ladder was observed in the cells treated with $H_2O_2$ at concentration of 200 μmol/L. The ladder bands were reduced obviously when splenic lymphocytes were treated with both $H_2O_2$ and PAP at concentrations of 50, 100, 200 or 400 μg/mL compared to the cells treated with $H_2O_2$ alone.

The cellular alterations in lymphocyte apoptosis can be rapidly quantified using flow cytometry. In present study, flow cytometry

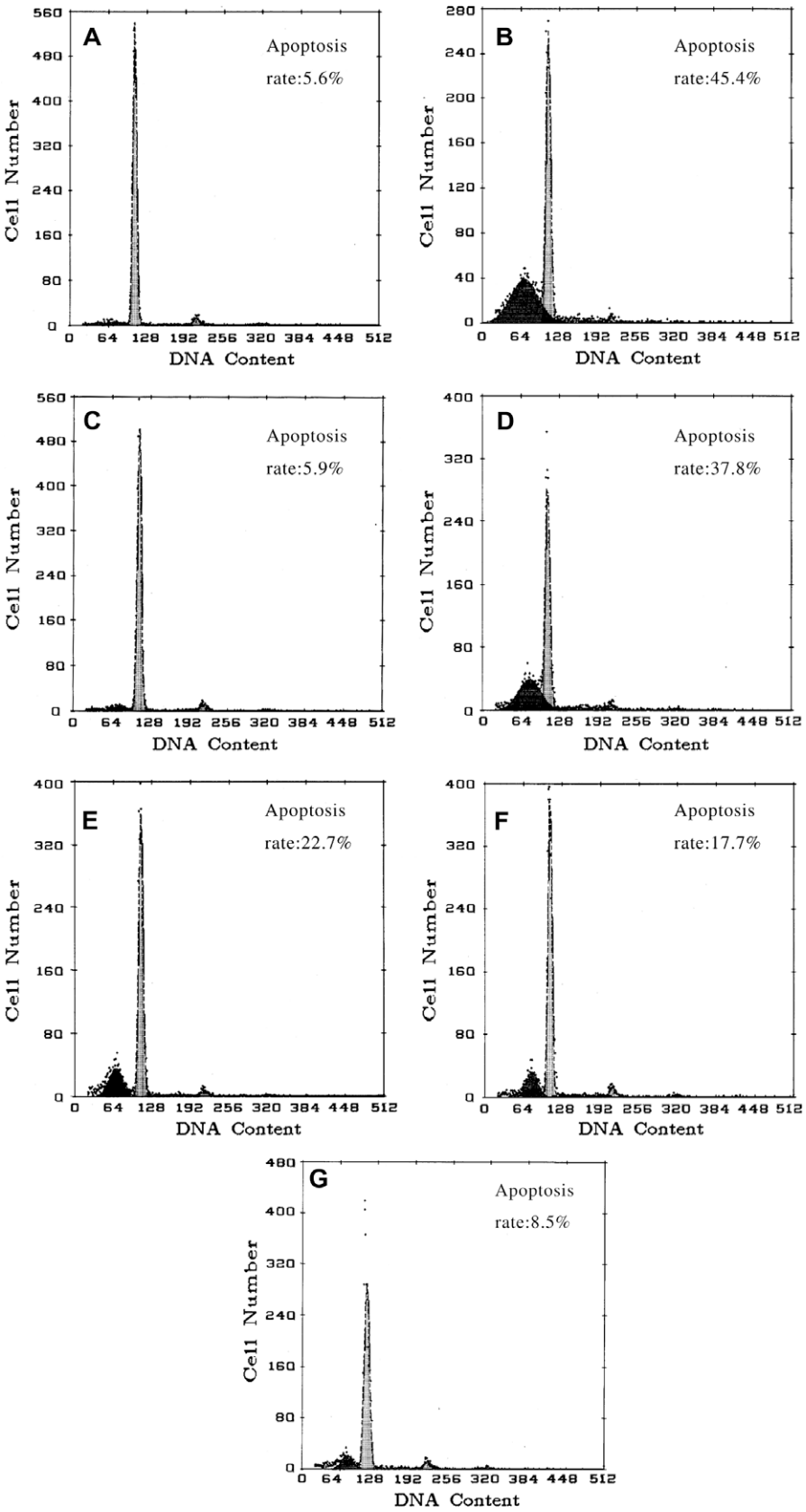


**Fig. 4.** Effects of PAP on murine splenic lymphocyte apoptosis by flow cytometry detection. (A) Normal control; (B) $H_2O_2$ treatment (200 μmol/L); (C) CAT (100 U/mL) + $H_2O_2$ (200 μmol/L) treatment. (D) PAP (50 μg/mL) + $H_2O_2$ (200 μmol/L) treatment. (E) PAP (100 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (F) PAP (200 μg/mL) + $H_2O_2$ (200 μmol/L) treatment; (G) PAP (400 μg/mL) + $H_2O_2$ (200 μmol/L) treatment.

analysis showed that $H_2O_2$ increased the populations of apoptotic sub-G1 compared with that in control. Treatment with both PAP and $H_2O_2$ caused decreases of cells in sub-G1 phase compared with $H_2O_2$ treatment alone.

**Table 1**
The apoptosis rate of murine splenic lymphocytes by flow cytometry.

| Treatments | Apoptosis rate of lymphocytes |
|---|---|
| Normal control | 5.60 ± 0.70 |
| $H_2O_2$ (200 μmol/L) | 45.40 ± 4.30** |
| CAT (100 U/mL) + $H_2O_2$ (200 μmol/L) | 5.90 ± 0.30## |
| PAP (50 μg/mL) + $H_2O_2$ (200 μmol/L) | 37.80 ± 4.90# |
| PAP (100 μg/mL) + $H_2O_2$ (200 μmol/L) | 22.70 ± 2.60## |
| PAP (200 μg/mL) + $H_2O_2$ (200 μmol/L) | 17.70 ± 2.50## |
| PAP (400 μg/mL) + $H_2O_2$ (200 μmol/L) | 8.50 ± 2.10## |

*Notes*. Values are mean ± SD ($n$ = 3 for each treatment).
** $p < 0.01$, compared with normal control.
# $p < 0.05$.
## $p < 0.01$, compared with $H_2O_2$ treatment alone.

In conclusion, PAP reduced intracellular oxidative damages and dose-dependently inhibited apoptosis. This suggests that PAP is a potential anti-apoptotic agent.

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