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Full Length Research Paper

Composition and effect of anti-hypoxia of Chinese medicinal plant mixture

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The *in vitro* and *in vivo* anti-hypoxia activity of shenjiangsuoyangyiqi drug (SJSY, Chinese medicinal plant mixture) was investigated. The compounds ginsenoside rg1, icariin and peoniflorin were detected in mixture by HPLC-DAD. *In vitro* anti-hypoxia activity of the mixture was evaluated for MTT assay against two cell lines (Vero and BHK-21). Compared to the control group (Vero: 48.72% and BHK-21: 35.41%), the survival rates of both cells were increased to 87.33% (Vero) and 83.03% (BHK-21) in high-does group. Arresting of the G1 phase of the cell-cycle under hypoxic condition was inhibited by mixture which was analyzed by FCM. *In vivo* anti-hypoxia activity was studied in mice model.

Key words: Mixture, anti-frigid hypoxia activity, *in vitro, in vivo*.

INTRODUCTION

Ginsenoside Rg1 belongs to the panaxatriol ginsenosides family, has been widely reported to exert neurotrophic and neuroprotective activities on central nervous system (CNS) (Liu et al., 2010). It can protect hippocampal neurons and alleviate neuronal injury induced by oxygen/glucose deprivation (Jiang et al., 2000, 2001; Jiang and Jiang, 2003) and has effect of excitotoxicity insults, traumatic brain injury and cerebral ischemia (Ji et al., 2005; Kim et al., 2007; Leung et al., 2007). Icariin, the main active flavonoid glucoside isolated from *epimedium pubescens*, has been proven to possess a wide range of efficacy, including antioxidative effect, immunoregulatory function, the function of neuroendocrine regulation and postmenopausal women. That icariin can regulate HIF-1 which is essential for regulating the expression of a battery of hypoxia-responsive genes involved in the adaptive and cell death responses (Lee et al., 2007; Vengellur et al., 2005). Peoniflorin is the essential active ingredient of peony which has effects of spasmolysis, anti-irritation, calmness, cooling and anti-oxidation (Wang et al., 1994). The ginsenoside rg1, icariin and peoniflorin were all detective in mixure which had been used for anti-hypoxia hundreds years.

Abbreviations: SJSY, Shenjiangsuoyangyiqi; **MTT,** 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; **FCM,** flow cytometry; **DAPI,** 4', 6-diamidino-2-phenylindole.

Hypoxia is defined as a decrease in available oxygen reaching the tissues of the body. It is linked to the pathology of cancer, cardiovascular disease, and stroke, the leading causes of death. Cells under hypoxic stress either induce an adaptive response that includes increasing the rates of glycolysis and angiogenesis or undergo cell death by promoting apoptosis or necrosis (Lee et al., 2007). The cell-cycle is usually divided into four phases: G1, S, G2 and M. In G1 (G = gap), the cell is not committed to division and the chromosomes do not replicate. Replication of nuclear DNA occurs during the S phase, whereas completion of mitosis occurs in the final M phase. The cell-cycle is the set of events whereby a cell duplicates most of its components, including its chromosomes, in order to undergo division. The interval between DNA replication and division is called the G2 phase. The gap phases G1 and G2 give the cell additional time for growth. But the cell cycles of Vero and BHK-21 under hypoxia condition were not analyzed before (Alarcon et al., 2009).

In the present study, to observe the protective mechanism of the mixture under hypoxia condition, cytotoxicity assays and anti-hypoxia activity of mixture were determined with MTT. Furthermore, flow cytometry (FCM) was used to test the cell cycle treated with the mixture. Additionally, a hypoxia model was set up to investigate anti-hypoxia activity of mixture *in vivo*. Using half death rate time and average survive time to detective

the effect of mixture on mouse models.

MATERIALS AND METHODS

Animals

Mice of Kunming species were housed in groups by 12 mice per cage with free access to standard rodent diet and tap water; they were kept at constant temperature (23 ± 1°C) and humidity (60 ± 10%) under a 12-h light/dark cycle (light on 07:30 to 19:30 h). All animals were obtained from the Animal Service of Health Science Center, Peking University, and adapted to the animal facility for one week before treatment began and treatment protocols were strictly in accordance with the international ethical guide lines and the National Institute of Health Guide concerning the Care (Zu et al., 2010).

Chemicals and reagents

MTT were obtained from Sigma–Aldrich Inc. (St. Louis, MO). Deionized water was used in all experiments. SJSY was provided by 211 Hospital.

Cell culture conditions and mixture prepare

Baby hamster kidney (BHK-21) and African green monkey kidney cells (Vero) were purchased from Harbin Medical University, China. These cell lines were grown and maintained in an incubator at 37°C and in 5% CO_2 atmosphere. DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin was used for cell cultures of Vero and BHK-21. The mixture was SJSY water soluble.

In vitro hypoxia conditions

The *in vitro* model of hypoxia was similar to that described by Yu et al. (1989), Yeon and Hack (1997). Vero and BHK-21were first incubated under hypoxia using a 95% N_2 and 5% CO_2 for 4 h at 37°C in a water-jacketed N_2 /CO_2 incubator (NU-4950: NuAire, Inc.). Then the study of the effects of mixture on the hypoxic damage of Vero and BHK-21, solution of mixture was added directly to the culture medium before placing the culture into the modular incubator chamber. Rhodiola rosea was used as a positive control and negative control was incubated without solution of mixture.

HPLC-DAD analysis

The Chinese medicinal herbs mixture was analyzed by HPLC-DAD. The HPLC system consisted of Waters HPLC (Waters 600 Pump) equipped with a diode array detector (DAD) (Model 2996) (Waters, USA). HPLC conditions were as follows: Solvent A, acetonitrile, and solvent B, 0.05% phosphoric acid in water (*v*/*v*), A: B = 20:80, detection was at 203 nm for ginsenoside rg1. Solvent A, methanol, solvent B, water and C, acetic acid, A: B: C = 54:45: 1, detection was at 270 nm for icariin. Solvent A, acetonitrile, and solvent B, 0.1% phosphoric acid in water (*v*/*v*), A: B = 14:86, detection was at 230 nm for peoniflorin. Flow rate was 1 ml/min, the concentration of mixture was 10 mg/ml and injection volume was 10 µl.

Cytotoxicity assays and anti-hypoxia activity of mixture

Inhibition of cell proliferation by mixture was measured by the MTT assay (Liu et al., 2009). Briefly, Vero and BHK-21 cells were plated in 96-well culture plates (1×10^5 cells/well) separately. After 24 h incubation, cells were treated with mixture (5 mg/ml and 500 µg/mL) for 4 h, MTT solution (5 mg/ml) was then added to each well. After 4 h incubation, the formazan precipitate was dissolved in 100 µl dimethyl sulfoxide, and then the absorbance was measured in an ELISA reader (Thermo Molecular Devices Co., Union City, USA) at 570 nm. The cell viability ratio was calculated by the following formula:

Inhibitory ratio (%) = ((ODcontrol – ODtreated)/(ODcontrol)) × 100% (Vanzyl and Vilioen, 2002).

Flow cytometric analysis of cell cycle

Cell cycle was assayed with CyStain (Jiang et al., 2008). Briefly, 1×10^6 cells/well Vero and BHK-21 cells were seeded in six-well plate and left for 24 h in incubator to resume exponential growth. Cells were exposed to mixture (5 mg/l and 500 µg/l) and incubated for 48 h. Then, the cells were harvested and washed with PBS. After suspension in 800 µL PBS, 200 µL CyStain (Partec GmbH, Germany). The cell cycle distribution of 10,000 cells was recorded by flow cytometry (Partec), and the percentage of cells at G0/G1, S, and G2/M phases was analyzed with FloMax software.

Acute toxicity and anti-hypoxia activity testing on mice

Acute toxicity

48 female Kunming mice weighing 18 to 22 g (six weeks of age) were randomly divided into two groups. Each group was given different dosages with 24 and 12 g/kg, respectively. The mice were observed for 7 days and the death rate of each group was recorded (Cai et al., 2010).

Anti-hypoxia activity of mixture under normal pressure

A total of 60 female ICR mice weighing 18 to 22 g (six weeks of age) were used in the present study. A hypoxia model was set up according to Wu et al. (2008). The mixture were suspended in physiological saline and administered at the designed dosage (30, 20 and 10 g/kg body weight/day). Animals were randomly assigned to five groups, that is negative control group, positive control group (treated with roseroot, 4 g/kg), mixture treatment group (with high-dose, middle-does and low-dose mixture groups). Mixture was administered to mice intragastrically for one week.

Anti-hypoxia activity of mixture under reduced pressure

A total of 60 female ICR mice weighing 18 to 22 g (six weeks of age) were used in the present study. A hypoxia model was set up according to Chen (2000). The mixture were suspended in physiological saline and administered at the designed dosage (20, 10 and 30 g/kg body weight/day). Animals were randomly assigned to five groups, that is negative control group, positive control group (treated with roseroot, 4 g/kg), mixture treatment group (with high-dose, middle-does and low-dose mixture groups). Mixture was administered to mice intragastrically for one week.

Statistical analyses

All results are expressed as F of SD. Statistical significance of

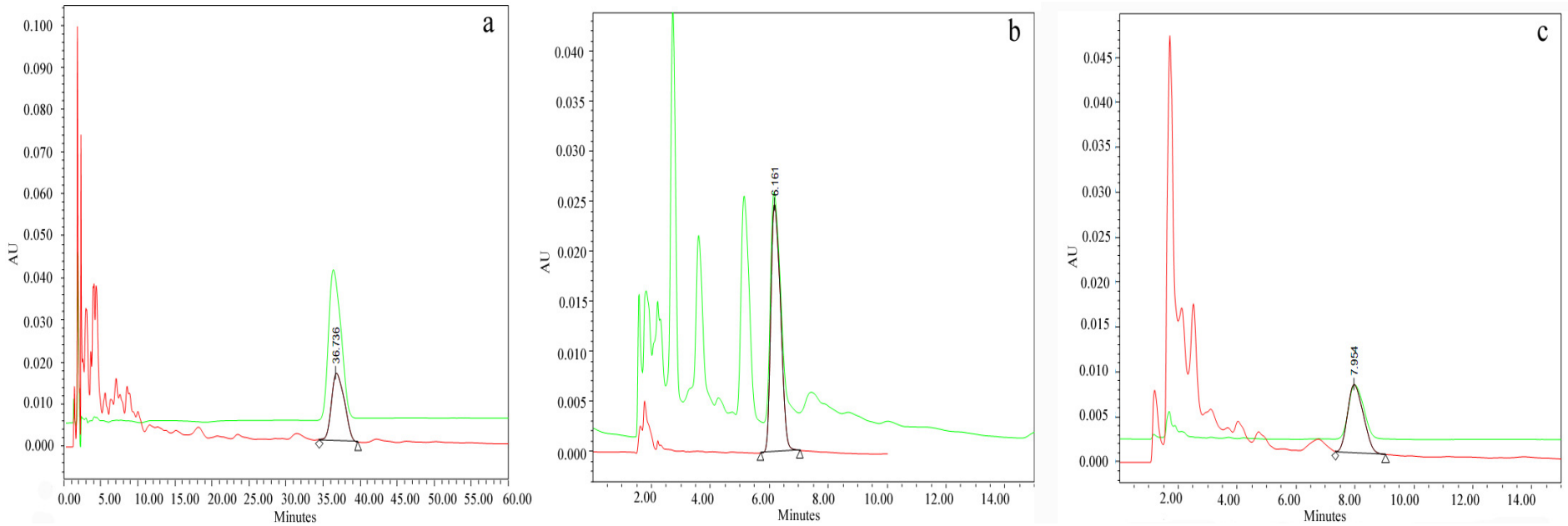

Figure 1. The HPLC-DAD chromatograms of mixture. Conditions were described in the text. a: ginsenoside rg1; b: icariin; c: peoniflorin.

differences between sample populations was evaluated using one-way ANOVA followed by the Dunnet's multiple comparison test for post hoc analysis. Statistical differences of $p \leq 0.05$ were considered to be significant. Alternatively the paired two-tail distribution student t test was applied to compare results with a criterion for statistical significance of a p value of 0.05 or less.

RESULTS

HPLC-DAD analysis

According to the HPLC-DAD analyses, the components of the mixture had weak polarity (Figure 1). In general, the components of mixture were ginsenoside, icariin and peoniflorin.

Cytotoxicity assays Anti-hypoxia activity of mixture

The cytotoxicity and anti-hypoxia activity of mixture onVero and BHK-21 cells was determined by using the MTT reduction assay. The growth inhibitory effects of mixture on two cell lines after 24 h exposure were examined. The cell inhibitory ratio values were shown in (Table 1 and Figure 1) shows the effects of 5 and 0.5 mg/mL mixture on viability of Vero and BHK-21 cell lines after exposure hypoxia for 2 h. The Vero cell survival rate value was 87.33% after high-does (5 mg/ml) mixture, 66.58% after low-does (0.5 mg/ml) mixture treatment compared with control group (48.72%). The BHK-21 cell survival rate value was 83.03% after high-does (5 mg/ml) mixture, 51.46% after low-does (0.5 mg/ml) mixture treatment compared with control group (35.41%). Cell survival rates of mixture towards hypoxia on Vero and BHK-21 cells in a dose-dependent manner.

Flow cytometric analysis of cell cycle

Based on the results of anti-hypoxia activity assay, we examined cell cycle to describe the mechanism of effect on hypoxia of mixture (Figure 2). To evaluate the cell cycle distribution of Vero and BHK-21 cells with or without mixture treatment, the DNA content was measured by flow cytometry (Figure 3). Exposure Vero to hypoxia caused an increase of the G1/M phase population

Table 1. Cell survival rate of mixture towards hypoxia (mg/ml).

	Survival rate (%)	
	Vero	**BHK-21**
Hypoxia damage group	48.72 ± 2.91	35.41 ± 1.89
High-does group (5)	87.33 ± 3.98	83.03 ± 4.27
Low-does group (0.5)	66.58 ± 1.84	51.46 ± 1.12

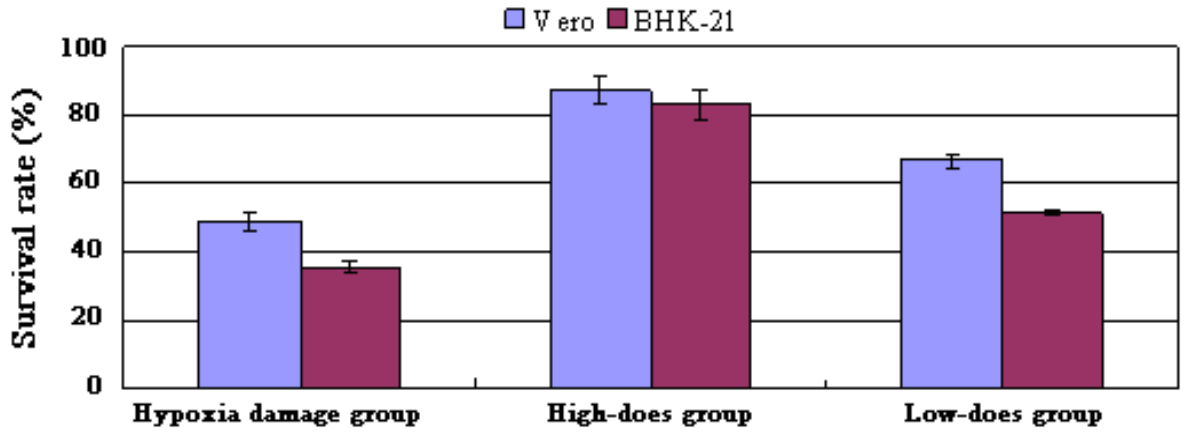

Figure 2. Effect of the mixture on the hypoxic Vero and BHK-21.The concentrations used correspond to high-does (5 mg/mL) and low-does (0.5 mg/mL); the hypoxia damage group did not contain mixture; $p<0.05$.

to 83.81%, as compared to 64.34 and 69.85% of G1 phase cells in mixture treated samples and 59.82% of G1 phase cells in untreated control samples. Hence, mixture exerted anti-hypoxia effects via resume G1 phase arrested by hypoxia in a concentration-dependent manner. A similar objection applies to BHK-21, the G1 phase population from 66.96 and 71.59% in mixture treated samples, as compared to 82.73% of G1 phase cells in hypoxia damage control.

Anti-hypoxia activity testing on mice

Results of mixture towards normal pressure and reduced pressure hypoxia survival time on mice are shown in (Tables 2 and 3). The mixture administrated beforehand could prolong survival time of mice under normal pressure and reduced pressure hypoxia. Mice were towards normal pressure and reduced pressure hypoxia and were either treated with the *Rhodiola rosea* (positive control) or mixture at three concentrations (intragastrically with 30, 20 or 10 mg/kg BW/day). Then the animal's survival time were tested (Tables 2 and 3). The results had shown the effects of 5 and 0.5 mg/ml mixture on the mice after exposure hypoxia. Under normal pressure hypoxia (Table 2), the half death rate of time value was 22.42 min in high-does group, 21.17 and 20.02 min in middle-does group and low-does group compared with negative control (17.27 min). The effects of mixture on the half death rate of time were shown in a dose-dependent manner. But average survival time was not shown in a dose-dependent manner. Under reduced pressure hypoxia (Table 3), the half death rate of time value was 17.08 min in high-does group, 11.98 and 15.17 min in middle-does group and low-does group compared with negative control (16.25 min), high-does group and low-does group were better than positive control (13 min). The same situation was according to average survive time. The different results were found between *in vitro* and *in vivo*, accordingly, further studies are urgently needed for identification of the reason for it.

In conclusion, results in the present study of Chinese medicinal herbs mixture have significant anti-hypoxia activities, both *in vitro* and *in vivo*. It is likely that mixture might also work on athletic hypoxia. However, further studies are urgently needed for identification of the active components in mixture with enhanced anti-hypoxia activity and for testing them against athletic hypoxia.

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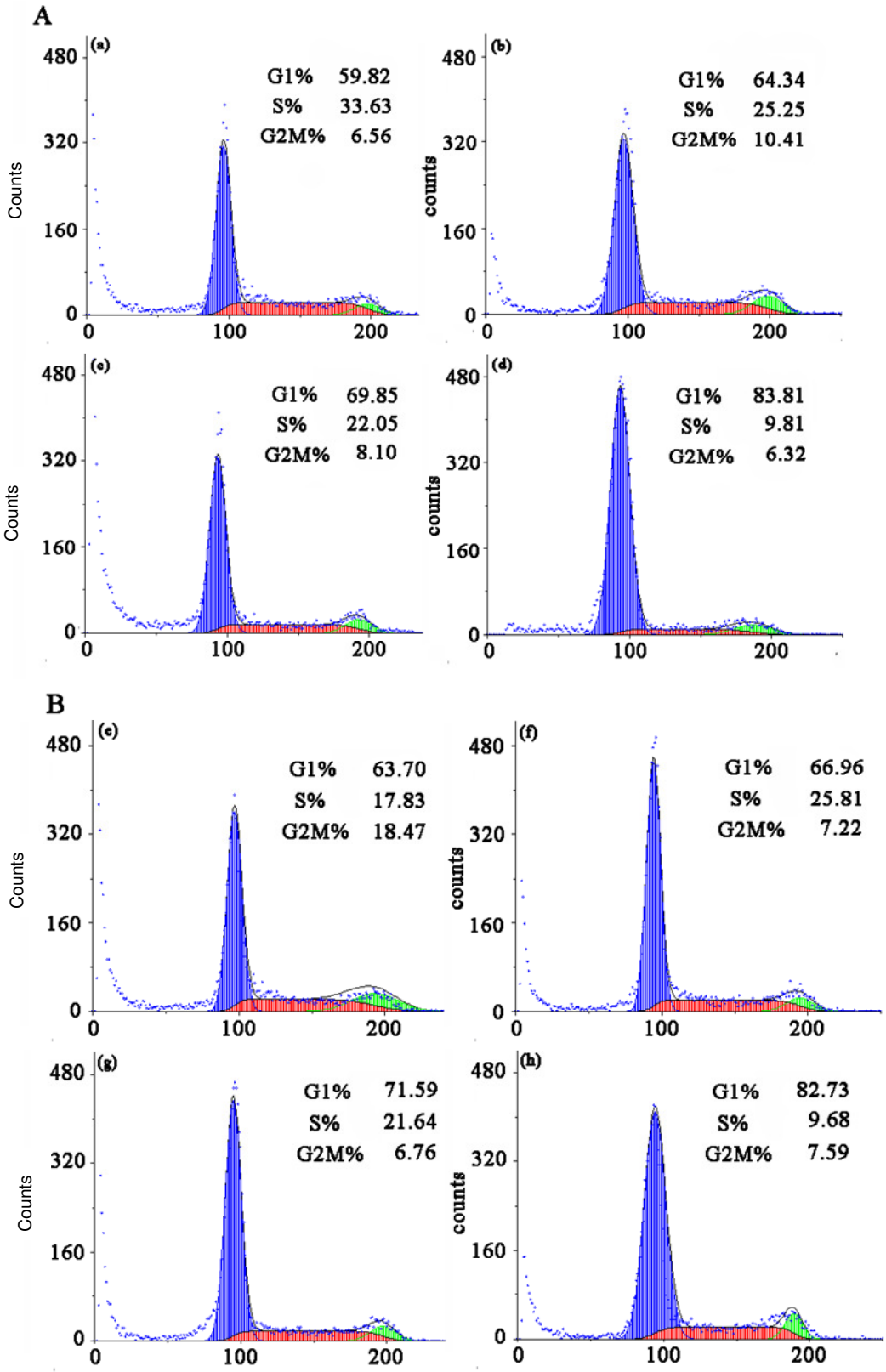

Figure 3. A: Flow cytometry histograms of Vero cell-cycle in the presence of mixture stained with DAPI. a: normal control; b: high-does group (5 mg/ml) treatment; c: low-does group (0.5 mg/ml) treatment; d: hypoxia damage group. B: Flow cytometry histograms of BHK-21 cell-cycle in the presence of mixture stained with DAPI. e: normal control; f: high-does group (5 mg/ml) treatment; g: low-does group (0.5 mg/ml) treatment; h: hypoxia damage group.

Table 2. The effects of mixture on survival time of normal pressure hypoxia mice (min) ($\bar{x}$ ± S.D., n = 10).

Group	Half death rate of time	Average survive time
Negative control	17.27	18.73±2.91
High-does group	22.42	22.73±3.76
Middle-does group	21.17	20.99±4.31
Low-does group	20.02	21.24±2.43
Positive control	22.22	23.24±1.77

Data were presented as mean ± S.D. P < 0.05, significantly different compared with the model group.

Table 3. The effects of mixture on survival time of reduced pressure hypoxia mice (min) ($\bar{x}$ ± S.D., n = 10).

Group	Half death rate of time	Average survive time
Negative control	16.25	16.43 ± 0.86
High-does group	17.08	17.57 ± 1.72
Middle-does group	11.98	12.20 ± 1.23
Low-does group	15.17	15.42 ± 0.92
Positive control	13	14.53 ± 3.70

Data were presented as mean ± S.D. P < 0.05, significantly different compared with the model group.

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