African Journal of Pharmacy and Pharmacology Vol. 6(31), pp. 2354-2358, 22 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.360
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

The protective effect of *Acanthopanax senticosus* B on apoptosis in rat adrenal pheochromocytoma cell line (PC12) cell induced by sodium cyanide (NaCN)/glucose deprivation

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Accepted 28 May, 2012

The protective effect of *Acanthopanax senticosus* B (ASB) on apoptosis in Rat adrenal pheochromocytoma cell line (PC12) cell induced by sodium cyanide (NaCN)/glucose deprivation was studied. The PC12 cell was cultured in RPMI-1640 medium supplemented with 10% newborn calf serum at 37℃ in a 5% CO_2 incubator. NaCN/glucose deprivation was conducted to establish an oxidative-stress injury model *in vitro*. The protective effect of ASB was evaluated by using this model. We compared the lactate dehydrogenase (LDH) release rate and malondialdehyde (MDA) content of PC12 cells in different treatment group. The cell apoptosis was detected by using flow cytometry (FCM) and the cell proliferation by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. These results showed that pretreatment with ASB could improve cell growth and proliferation in a concentration dependent manner, reduce the release of LDH and MDA content, and inhibit the NaCN/glucose deprivation-induced apoptosis of PC12 cells. Therefore, these results suggested that ASB possessed the protective activity of PC12 cells injury by NaCN/glucose deprivation *in vitro*.

Key words: *Acanthopanax senticosus* B, PC12 cell, sodium cyanide, glucose deprivation, protective effect.

INTRODUCTION

Acanthopanax senticosus (Rupret Maxim) Harms is a shrub of the family Araliaceae, which is commonly grown in the forests in South-East Russia, North-East China, Korea, South-East Asia and Japan (Deyama et al., 2001; Jiang et al., 2006). Since 1971, the triterpenoid saponins in the leaves of this plant have been well known (Frolova et al., 1971; Shao et al., 1988). Up to now, more than 25 species of *A. senticosus* saponins have been isolated from the leaves of *A. senticosus* (Shao et al., 1989; Melek et al., 2002; Jin et al., 2004). These saponins have been used widely in traditional Chinese medicine (TCM) for treatment of many diseases. *A. senticosus* saponins can inhibit the production of nitric oxide and reactive oxygen species in murine macrophages and show antioxidant and antiglycation activity in diabetes mellitus (Lin et al., 2008; Lin et al., 2007; Xi et al., 2008). Moreover, *A. senticosus* saponins can also resist serum malondialdehyde (MDA) in postmenopausal women (Young et al., 2008), In addition, *A; senticosus* has been reported to treat the following diseases, such as brain ischemia, chronic renal failure, diabetes mellitus, chronic bronchitis, hypertension, gastric ulcers, anti-rheumatic, hypertension, anti-stress, gastric ulcer and insulin resistance (Bu et al., 2005; Tsang-Pai et al., 2005; Nishibe et al., 1990; Fujikawa et al., 1996; Davydov and Krikorian, 2000; Cha et al., 2003).

A. senticosides B (ASB) is a monomer of *A. senticosus* saponins. It was reported that ASB was a mitochondrial

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adenosine triphosphate (ATP)-sensitive potassium channels agonist ventricular myocytes (Zhou et al., 2005). Meanwhile, several Chinese scholars also found that ASB could protect PC cells from apoptosis induced by different ways, such as hypoxia and MPP^+ (Ji et al., 2006; Wang et al., 2007). However, the effects of ASB on rat adrenal pheochromocytoma cell line (PC12) cells apoptosis induced by sodium cyanide (NaCN)/glucose deprivation are still unclear.

In our present study, we studied the effect of ASB on apoptosis induced by NaCN/glucose deprivation in PC12 cell lines. The lactate dehydrogenase (LDH) release rate, MDA content, cell apoptosis and proliferation of PC12 cells in different treatment group was evaluated by using LDH release assay, thiobarbituric acid assay, flow cytometry (FCM) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, respectively.

MATERIALS AND METHODS

Reagents

ASB was obtained from China pharmaceutical and biological products inspection (Lot: 111616-201002). NaCN, glucose, MTT, Tris and Australia phenol blue were purchased from Sigma (USA). RPMI-1640 mediu and Dulbecco's minimum essential medium (DMEM) were obtained from Gibco (USA). Newborn calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Company (Hangzhou, China). Trypsin, Tween20, and dimethyl sulfoxide (DMSO) were obtained from Amresco (USA). LDH and MDA kits were purchased from Nanjing Jiancheng Bioengineering Research Institute (China).

Cell culture and treatment

PC12 cells (rat adrenal pheochromocytoma cells) were taken from Experimental Animal Center of Harbin Medical University (Harbin, China). Cells were cultured in RPMI-1640 medium supplemented with 10% newborn calf serum at 37℃ in a 5% CO_2 incubator. When cells were closed to 80% confluence, new media with newborn calf serum were added before the compounds treatment. We used 10 mM of glucose-free medium with NaCN to establish an oxidative-stress injury model *in vitro*, and evaluate the effect of ASB by using this model. When needed, cells were incubated for 30 min with different concentrations of ASB (0.1, 1, 2, 5, 10 and 20 M) and then exposed to 10 mM of glucose-free medium with NaCN for 24 h.

Detection of LDH release of PC12 cells

When PC12 cells were closed to 80% confluence, new media with newborn calf serum was added before the compounds treatment. Cells were treated with ASB for 30 min followed by the addition of glucose-free medium with NaCN to a final concentration of 20 μM, and incubated for 24 h. The medium was collected to a 1 ml EP tube. At the end of treatments, PC12 cells were treated with 10% Triton X-100, and the media which contained detached cells were collected and centrifuged at 800 g at 4℃ for 2 min. The supernatant was used for the assay of LDH release. The enzyme was determined by using an assay kit according to the manufacturer's protocol. The absorbance of the samples was read at 440 nm. The LDH release was in proportional to the number of damaged PC12 cells. Reagent blanks were subtracted.

Detection of MDA content of PC12 cells

The PC12 cells were cultured in 6-well plates and pretreated with varying concentrations of ASB for 1 h prior to exposure to glucose-free medium with NaCN. After 24 h, cells were collected in 1.5 ml of tubes in 0.5 ml phosphate-buffered solution (PBS). Cell lysis was performed by means of three cycles of freezing and thawing. MDA content was measured using a thiobarbituric acid assay according to the manufacturer's instructions.

FCM with propidium iodide (PI) staining

The PC12 cells were treated in the same way as previously described. Cells were collected, digested with 0.25% trypsin and made into a single cell suspension by RPMI-1640 medium supplemented with 10% newborn calf serum. The single cell suspension was centrifuged at 1000 rpm for 5 min at 4℃. Then the supernatant was removed, washed with cold PBS, centrifuged at 1000 rpm for 5 min at 4℃. The cell pellets were resu spended in 1 ml binding buffer (10 mM of Hepes/NaOH, pH 7.4; 140 mM of NaCl; 2.5 mM of $CaCl_2$) and incubated for 15 min in the dark with Annexin V-FITC (20 μg/ml) and PI (50 μg/ml) at 4℃. Fluorescence was analyzed with an FCM.

MTT assay

Logarithmic growth phase cells were seeded in 96-well plates at a density of 5×10^3/ ml. Cells were cultured for 24 h at 37℃ with 5% CO_2, treated in the same way as previously described. Each group was set up three parallel holes. Cells were cultured for 24 h, followed by incubation with 0.5 mg/ml MTT, 200 μl serum-free medium for 4 h. Finally, 100 μl of DMSO was added and absorbance at 570 nm wavelength (A_{570}) was measured by means of enzyme-linked immunosorbent instrument. Relative cell proliferation inhibition rate (IR) = (1 - average A_{570} of the experimental group/average A_{570} of the control group) $\times$ 100%.

Statistical analysis

The database was set up with the SPSS 16.0 software package for analysis. Data were represented as mean ± SD. The means of multiple groups were compared with One-way analysis of variance (ANOVA), after the equal check of variance, and the two-two comparisons among the means were performed by Student's *t*-test. $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of ASB on LDH release from NaCN/glucose deprivation-induced PCl2 cells

After PC12 cells were treated with 10 mM of glucose-free medium with NaCN for 24 h, the release of LDH significantly increased from (26.45 ± 1.37 U/ml) to (88.23 ± 4.32 U/ml). When 0.1, 1.0, 2, 5, 10 and 20 μM of ASB were added to the assay, the release of LDH reduced to (78.42 ± 3.92 U/ml), (68.47 ± 3 .56 U/ml), (63.89 ± 3.39

Table 1. Collection of culture medium and analysis of LDH release.

Group	LDH release (U/ml)
Medium	26.45± 1.37**
NaCN/glucose deprivation injury	88.43 ± 4.32
0.1 M ASB	78.22 ± 3.92*
1 M ASB	68.47 ± 3.56*
2 M ASB	63.89 ± 3.39**
5 M ASB	53.45 ± 3.51**
10 M ASB	42.89 ± 2.78**
20 M ASB	35.54 ± 1.98**

Data shown represent the average of three experiments. ***P*<0.001 versus NaCN/glucose deprivation injury group, **P*<0.01 versus NaCN/glucose deprivation injury group.

Table 2. Collection of culture medium and analysis of MDA content.

Group	MDA content (nmol/mg)
Medium	3.91 ± 0.34**
NaCN/glucose deprivation injury	10.32 ± 1.18
0.1 M ASB	8.38 ± 0.49*
1 M ASB	6.75 ± 0.34*
2 M ASB	6.24 ± 0.29**
5 M ASB	5.88 ± 0.22**
10 M ASB	4.65 ± 0.28**
20 M ASB	4.02 ± 0.21**

Data shown represent the average of three experiments. ***P*<0.001 versus NaCN/glucose deprivation injury group, **P*<0.01 versus NaCN/glucose deprivation injury group.

U/ml), (53.45 ± 3.51 U/ml), (42.89 ± 2.78 U/ml) and (35.54 ± 1.98 U/ml) in a concentration dependent manner. The results showed that ASB could significantly inhibit the LDH release of NaCN/glucose deprivation-induced PC12 cells in a concentration dependent manner (Table 1).

Effects of ASB on MDA content from NaCN/glucose deprivation deprivation-induced PCl2 cells

After PC12 cells were treated with 10 mM of glucose-free medium with NaCN for 24 h, the MDA content significantly increased from (3.91 ± 0.34 nmol/mg) to (10.32 ± 1.18 nmol/mg). When 0.1, 1.0, 2, 5, 10 and 20 μM of ASB were added to the assay, the MDA content reduced to (8.38 ± 0.49 nmol/mg), (6.75 ± 0.34 nmol/mg), (6.24 ± 0.29 nmol/mg), (5.88 ± 0.22 nmol/mg), (4.65 ± 0.28 nmol/mg) and (4.02 ± 0.21 nmol/mg) in a concentration dependent manner. The results showed that ASB could significantly inhibit the MDA content of NaCN/glucose deprivation-induced PC12 cells in a concentration dependent manner (Table 2).

Effects of ASB on apoptosis rate of NaCN/glucose deprivation deprivation-induced PCl2 cells

FCM results showed that the apoptosis rate of PC12 cell line treated with 10 mM of glucose-free medium with NaCN for 24 h was 34.39 ± 4.25%, which was significantly higher than that of medium group (2.14 ± 0.24%). When 0.1, 1.0, 2, 5, 10 and 20 μM of ASB were added to the assay, cell apoptosis rate was reduced to 25.48 ± 3.67%, 19.45 ± 2.86%, 15.88 ± 2.27%, 13.42 ± 1.82%, 10.54 ± 1.76% and 8.49 ± 1.21% in a concentration dependent manner. This indicated that ASB could significantly inhibit the NaCN/glucose deprivation-induced apoptosis of PC12 cells (Table 3).

Effects of ASB on proliferation IR of NaCN/glucose deprivation deprivation-induced PCl2 cells

The MTT assay demonstrated that the IR of cells significantly increased to 92.14 ± 7.27% after PC12 cells were treated with glucose-free medium with NaCN. When PC12 cells were treated with 0.1, 1.0, 2, 5, 10 and 20 μMof ASB, the apoptosis rate of cells reduced to (57.35 ± 4.25%), (67.24± 3.96%), (73.58 ± 5.21%), (81.82 ± 4.02%), (86.59 ± 8.38%) and (92.33 ± 7.25%), respectively. These results indicated that ASB could significantly inhibit NaCN/glucose deprivation-induced inhibition of PC12 cells in a concentration dependent manner (Table 4).

DISCUSSION

NaCN is known to be a blocker in electron transport chain of various biological functions. Under such conditions, a series of metabolic stress responses can be triggered within the injured cells, which include the elecation of extracellular glutamate, LDH activity. The accumulation of glutamate causes toxic effects to cells. Meanwhile, glutamate activated NMDA receptor and opens its ion channel in cell membrane. The large influx of calcium into cells could cause cell death. This NaCN/glucose deprivation injury mimics the oxidative stress-related neurodegenerative disorder such as Alzheimer's disease, Down syndrome (Shimohama, 2000; Yuan et al., 2000).

PC12 cells, a rat adrenal pheochromocytoma cell line, can extend processes similar to those produced by sympathetic neurons when exposed to nerve growth factor and these cells exhibit a single phenotype, with stable features that can be sub-cultured (Cheng and Zhang, 2008; McLaurin et al., 2000). PC12 cells are extremely similar to neurons in cell morphology, structure

Table 3. FCM showing that all genistein derivatives can decrease apoptosis rate of NaCN/glucose deprivation-induced PC12 cells.

Group	Apoptosis rate (%)
Medium	2.14 ± 0.24**
NaCN/glucose deprivation injury	34.39 ± 4.25
0.1 M ASB	25.48 ± 3.67*
1 M ASB	19.45 ± 2.86*
2 M ASB	15.88 ± 2.27**
5 M ASB	13.42 ± 1.82**
10 M ASB	10.54 ± 1.76**
20 M ASB	8.49 ± 1.21**

Data shown represent the average of three experiments. **P<0.001 versus NaCN/glucose deprivation injury group, *P<0.01 versus NaCN/glucose deprivation injury group.

Table 4. MTT assay showing that all genistein derivatives can increase proliferation rate of NaCN/glucose deprivation-induced PC12 cells.

Group	Cell viability (%)
Medium	92.14 ± 7.27**
NaCN/glucose deprivation injury	25.44 ± 4.78
0.1 M ASB	57.35 ± 4.25*
1 M ASB	67.24± 3.96*
2 M ASB	73.58 ± 5.21**
5 M ASB	81.82 ± 4.02**
10 M ASB	86.59 ± 8.38**
20 M ASB	92.33 ± 7.25**

Data shown represent the average of three experiments. **P<0.001 versus NaCN/glucose deprivation injury group, *P<0.01 versus NaCN/glucose deprivation injury group.

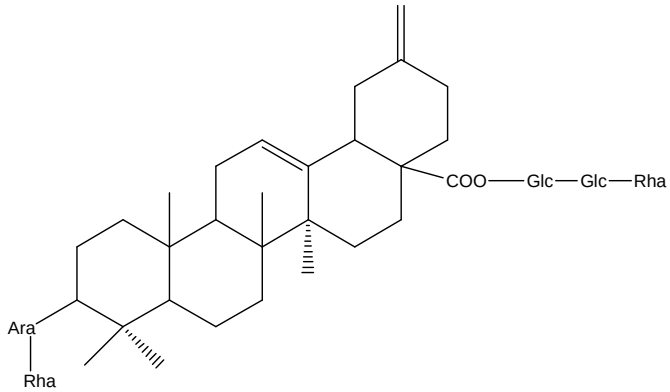

Figure 1. The chemical structure of ASB.

and function. Therefore, it has been widely used as a cell model for study of neuron cells (Saito et al., 2003).

LDH is a stable cytoplasmic enzyme that is present in all cells and is rapidly released into the culture supernatant when the plasma membrane is damaged; thus, it can be used as a reliable biochemical index for damage of the plasma membrane (Zhao et al., 2002). Our results showed that ASB could decrease LDH release of PC12 cells injured by NaCN/glucose deprivation. MDA is the end product of free radical- initiated lipid peroxidation and thus, reflects the level of lipid peroxidation. Our results also showed that NaCN/glucose deprivation may reduce the MDA content in PC12 cells, thus suggesting that its neuroprotective effects are potentially due to its antioxidant property (Xu et al., 2008; Mills et al., 1996). MTT assay results con-firmed that cell growth and proliferation were suppressed when PC12 cells were treated with glucose-free medium with NaCN for 24 h, while ASB could effectively decrease the suppression. FCM with PI staining results showed that ASB could reduce the NaCN/glucose deprivation-induced apoptosis of PC12 cells in a concentration dependent manner (Figure 1).

Conclusion

In our present work, we established the model of injury and apoptosis induced by NaCN/glucose deprivation to evaluate different concentration of ASB against oxidative stress injury in PC12 cells lines. ASB could inhibit LDH release, the MDA content, the suppression of cell proliferation and NaCN/glucose deprivation-induced apoptosis of PC12 cells in a concentration dependent manner. These findings suggest that ASB might have the protective effect against oxidative stress injury induced by NaCN/glucose deprivation in PC12 cells. However, further study in this field is needed.

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