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Inhibition of serum deprivation-induced PC12 cell apoptosis by tanshinone Ⅱ A

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KEY WORDS tanshinone Ⅱ A; PC12 cells; serum-free culture media; apoptosis; DNA fragmentation; flow cytometry; neuroprotective agents; antioxidants

ABSTRACT

AIM: *To study the effect of tanshinone* Ⅱ A (Tan Ⅱ A) on PC12 cell apoptosis induced by serum deprivation. **METHODS**: PC12 cell survival was measured by MTT assay. The DNA content and percentage of apoptosis were monitored by flow cytometry and DNA fragmentation was analyzed by agarose gel electrophoresis. **RESULTS**: Serum-free (12 h) medium induced apoptosis in PC12 cells. When the cells had been treated with Tan Ⅱ A (0.1 and 1 $\mu mol \cdot L^{-1}$) for 12 h, the percentage of PC12 cell apoptosis was grealy decreased to 25.71 % and 4.89 % from 96.07 % in serum deprivation alone group, and DNA fragmentation was prevented. Tan Ⅱ A (0.01 – 10 $\mu mol \cdot L^{-1}$) attenuated the cytotoxic effect of sodium cyanide (20 $mmol \cdot L^{-1}$), glutamate (0.5 $mmol \cdot L^{-1}$), and sodium nitroprusside (0.5 $mmol \cdot L^{-1}$). **CONCLUSION**: Tan Ⅱ A prevented PC12 cells from apoptosis induced by serum-free medium.

INTRODUCTION

Apoptosis is a prominent feature of normal nervous system development and is as well a disastrous consequence of a number of neurodegenerative disorders and various nervous system insults or injuries[1]. Several apoptosis-inducing agents including oxidative stress have now been recognized[2]. The potential antiapoptotic value of antioxidants has prompted investigators to search for compounds with potent antioxidant activity but low cytotoxicity[3].

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The rhizome of *Salviae Miltiorrhiza* Bunge, also known as "Tanshen", is an important herb for promoting the circulation of blood and eliminating stasis in Chinese traditional medicine. The chemical composition of Tanshen has been actively investigated and utilized to treat coronary heart disease in clinic. Tanshinone Ⅱ A (Tan Ⅱ A) isolated from this herb is a derivative of phenanthrenequinone. Its pharmacological effects have been reported in preliminary studies on myocardial ischemia-reperfusion and antioxidation[3,4]. However, there has been little basic information on the neuroprotective actions of Tan Ⅱ A.

To provide sufficiently homogeneous material for experiment, we used cultured PC12 cells, which have proved to be useful for studying mechanisms of neuronal apoptotic death[5]. In the present study, we observed the effect of Tan Ⅱ A on PC12 cell apoptosis induced by serum-free medium. Moreover, the effects of Tan Ⅱ A on sodium cyanide (NaCN)-, glutamate (Glu)-, and sodium nitroprusside (SNP)-induced cytotoxicity were also examined.

MATERIALS AND METHODS

Materials Tan Ⅱ A, prepared and kindly gifted by Prof MIN Zhi-Da (Department of Phytochemistry, *China Pharmaceutical University*), was dissolved in dimethylsulfoxide (Me_2SO). The concentration of Me_2SO in the final culture media was $\leqslant 0.1$ % (v/v), which had no toxic effect on PC12 cells[6]. 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Fluka, and Dulbecoo's modified Eagle's medium(DMEM) was the product of Gibco. All other chemicals were of analytical grade.

Cell culture PC12 cell line was obtained from Shanghai Institute of Cell Biology. The cells were cultured at 37 ℃ in a humidified CO_2 (5 %) incubator in DMEM containing 10 % heat-inactivated fetal bovine serum, benzylpenicillin (100 $kU \cdot L^{-1}$) and streptomycin

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(100 mg·L^{-1}). Cells in exponential growth were used. For the experiments in serum-free medium, cells were washed three to four times with serum-free DMEM. Cells (5×10^4 cells in 100 μL medium each well) in a 96-well plate were used for MTT assay. For DNA fragmentation and flow cytometry, cells (5×10^6 cells in 2 mL medium each well) were grown in 6-well plates.

Viability assay Cell viability was measured using MTT assay[7]. PC12 cells were treated with different concentrations of Tan Ⅱ A (0.01 – 10 μmol·L^{-1}) for 30 min, then NaCN (20 mmol·L^{-1}), Glu (0.5 mmol·L^{-1}), or SNP (0.5 mmol·L^{-1}) was added to each well. After 20 min, the medium was separated from the wells and the cells were washed three times with serum-free medium. The fresh serum-free medium was appended to each well and incubated at 37 ℃ for 20 h, then MTT solution (0.5 g·L^{-1}) was added. After incubation for additional 4 h, the formazan crystals were dissolved in 150 μL of Me_2SO. The absorbance at 570 nm was then measured using an ELISA plate reader (Hua Dong Electronic Co, Nanjing, China).

Flow cytometry At 12 h following withdrawal of serum support, PC12 cells were harvested for flow cytometry[8]. Briefly, cells were fixed in 70 % ethanol at 4 ℃ overnight. Subsequently, cells were treated with Tris-HCl buffer (pH 7.4) containing 1 % RNase A and were stained with propidium iodide 5 mg·L^{-1} (PI). Distribution of cells with different DNA contents was determined by flow cytometry (Facscalibur, Becton Dickinson) and the data were analyzed by multicycle DNA content and cell cycle analysis software (Modfit LT 2.0).

DNA fragmentation Experiments were performed as described by Troy *et al*[9]. In brief, PC12 cells were washed and plated in serum-free DMEM medium with or without indicated additives. After incubation for 12 h at 37 ℃, fragmented DNA was analyzed by electrophoresis. The cellular DNA was extracted, dialyzed, electrophoresed in 1.2 % agarose gel, and visualized under UV light after staining with ethidium bromide.

Statistics The results were expressed as $\bar{x} \pm s$ and evaluated by t-test.

RESULTS

Effects of Tan Ⅱ A on NaCN-, Glu-, and SNP-induced cytotoxicity in PC12 cells The exposure of PC12 cells to NaCN (20 mmol·L^{-1}), Glu (0.5 mmol·L^{-1}), or SNP (0.5 mmol·L^{-1}) for 20 min and the following incubation with serum-free medium for 24 h can produce an obvious decrease in the cell viability, as assayed by MTT. When cultures were pretreated with Tan Ⅱ A for 30 min, and then exposed to NaCN, Glu, or SNP, the cell damage was greatly attenuated (Tab 1).

Tab 1. Effect of Tan Ⅱ A on NaCN-, Glu-, or SNP-induced cytotoxicity in PC12 cells. Cells in serum-free medium were pretreated with or without different concentrations (0.01 – 10 μmol·L^{-1}) of Tan Ⅱ A for 30 min and then exposed to NaCN (20 mmol·L^{-1}), Glu (0.5 mmol·L^{-1}), or SNP (0.5 mmol·L^{-1}) for 20 min. $n = 4$ (4 wells in each experiments). $\bar{x} \pm s$. $^cP < 0.01$ *vs* control group. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* model group.

Drug/μmol·L^{-1}	A_{570}	Inhibition of cell damage/%
Control	1.26 ± 0.13	
NaCN	0.78 ± 0.08^c	
Tan Ⅱ A 0.01 + NaCN	0.81 ± 0.03^d	6.2
Tan Ⅱ A 0.1 + NaCN	0.89 ± 0.03^d	22.9
Tan Ⅱ A 1 + NaCN	0.96 ± 0.04^e	37.5
Tan Ⅱ A 10 + NaCN	1.04 ± 0.09^f	54.2
Control	1.28 ± 0.11	
Glu	0.74 ± 0.07^c	
Tan Ⅱ A 0.01 + Glu	0.78 ± 0.09^d	7.4
Tan Ⅱ A 0.1 + Glu	0.82 ± 0.08^d	14.8
Tan Ⅱ A 1 + Glu	0.92 ± 0.10^e	33.3
Tan Ⅱ A 10 + Glu	1.04 ± 0.11^f	55.6
Control	1.46 ± 0.11	
SNP	0.89 ± 0.09^c	
Tan Ⅱ A 0.01 + SNP	0.92 ± 0.10^d	5.3
Tan Ⅱ A 0.1 + SNP	0.97 ± 0.11^d	14.0
Tan Ⅱ A 1 + SNP	1.09 ± 0.13^e	35.1
Tan Ⅱ A 10 + SNP	1.19 ± 0.12^f	52.6

Flow cytometry assay When treated with serum-free medium, the percentage of apoptotic cells with fractional DNA content was 96.07 % and a large amount of debris was observed, indicating a pronounced degree of cell disruption. When Tan Ⅱ A was added to serum-free treated cells, at concentration 0.1 μmol·L^{-1} and 1 μmol·L^{-1}, the percentage of apoptotic cells was significantly reduced (25.71 % and 4.89 % respectively, Fig 1).

DNA fragmentation Detection of internucleosomal DNA fragmentation provides a sensitive means to monitor early events of apoptosis. PC12 cell cultures were deprived of serum and different concentrations of Tan Ⅱ A (0.1 μmol·L^{-1} and 1 μmol·L^{-1}) were added.

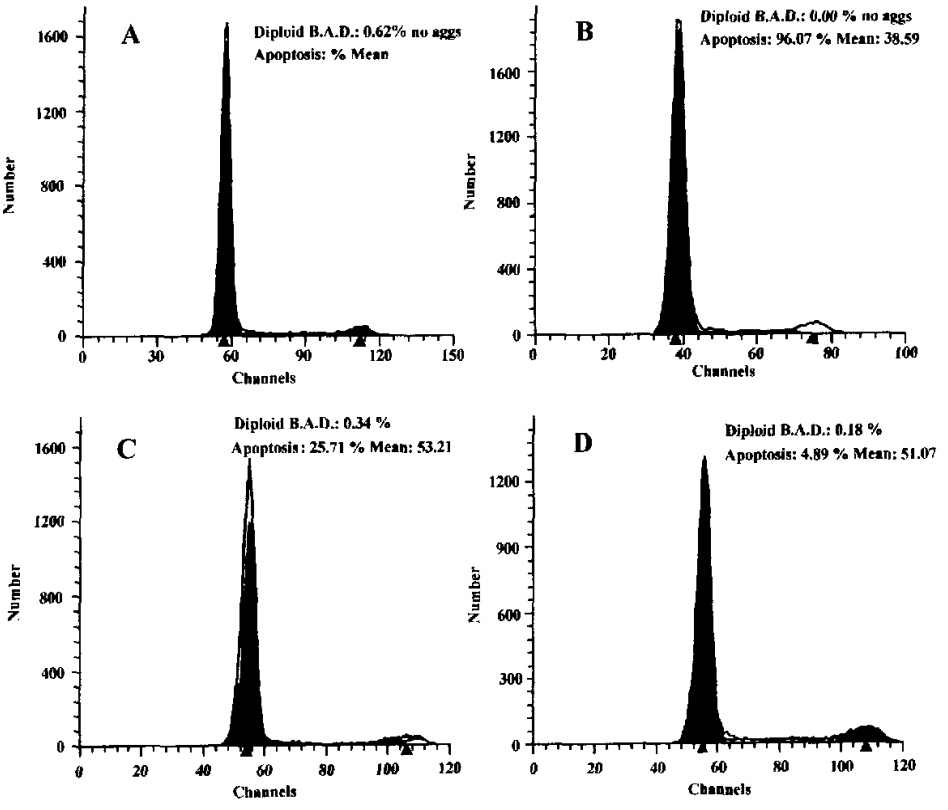

Fig 1. *Flow cytometric assay of* **PC12 cells. A) Control; B) Serum-free medium; C) Serum-free medium plus Tan Ⅱ A 0.1 μmol·L^{-1}; D) Serum-free medium plus Tan Ⅱ A 1 μmol·L^{-1}.**

After 12-h incubation, DNA fragmentation was observed to be prevented. In untreated cells, spontaneous DNA fragmentation was observed, as evidence of the characteristic "DNA ladder" profile that was considered the hallmark of apoptosis (Fig 2).

DISCUSSION

In the present study, it was shown that Tan Ⅱ A prevented PC12 cell apoptosis when these cells were treated *in vitro* with serum-free medium as analyzed using flow cytometry and DNA agarose gel electrophoresis. Furthermore, our study revealed that NaCN-, Glu-, and SNP-induced PC12 cell damage could be attenuated by Tan Ⅱ A pretreatment. Serum-free cultures of PC12 cells are a useful model system for studying the neuronal cell death which occurs after neurotrophic factor deprivation. Serum-deprived PC12 cells undergo apoptotic internucleosomal DNA cleavage, which is detected within 3 h of serum withdrawal and several hours before any morphological sign of cell degeneration[10,11]. Our results are consistent with these studies.

Although the mechanisms by which neurotrophins suppress apoptosis are not fully understood, apoptotic death of trophic factor-deprived PC12 cells appears to be regulated by many pathways[5,12-14]. Assay of cell apoptosis in PC12 cells has led to identification of a wide variety of agents that confer protection from loss of trophic support. Our results also demonstrated that Tan Ⅱ A prevented PC12 cells apoptosis caused by serum-free culture. Despite the efficacy of these treatments, the relative positions at which they block the apoptotic pathway has been unclear. Strong evidence has demonstrated that Tan Ⅱ A has antioxidant properties[4,15], but whether or not the ability of Tan Ⅱ A to scavenge lipid peroxidation and to depress DNA damage contributes towards its neuroprotective effect is yet to be ascertained.

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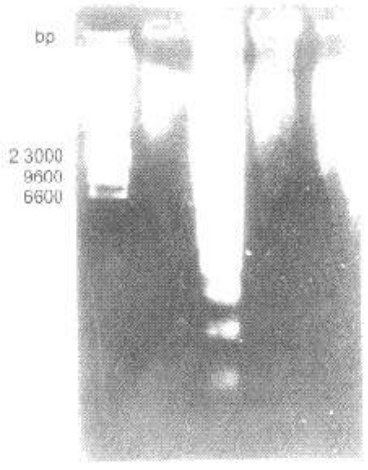

Fig 2. Effect of Tan Ⅱ A on prevention of serum-free induced DNA internucleosomal cleavage in PC12 cells. Lane M, molecular weight; Lane A, control; Lane B, cells incubated with serum-free medium for 12 h; Lane C, cells incubated with serum-free medium and Tan Ⅱ A 0.1 μmol·L^{-1}; Lane D, cells incubated with serum-free medium and Tan Ⅱ A 1 μmol·L^{-1}.

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丹参酮Ⅱ A 抑制去血清培养诱导的 PC12 细胞凋亡

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关键词 丹参酮Ⅱ A；PC12 细胞；无血清培养基；细胞凋亡；DNA 断片；流式细胞术；神经保护剂；抗氧化剂

目的：研究丹参酮Ⅱ A 对无血清培养诱致 PC12 细胞凋亡的抑制作用。**方法**：以噻唑兰（MTT）法测定 PC12 细胞存活率；用流式细胞术检测 DNA 含量及凋亡细胞百分率；DNA 琼脂糖电泳法观察 DNA 断裂。**结果**：去血清培养（12 h）可诱导 PC12 细胞凋亡（细胞凋亡率 96.07 %）。丹参酮Ⅱ A（0.1，1 μmol·L^{-1}）显著降低凋亡细胞百分率（细胞凋亡率分别为 25.71 % 和 4.89 %），并减少 DNA 断裂。丹参酮Ⅱ A（0.01–10 μmol·L^{-1}）抑制氰化钠（20 mmol·L^{-1}），谷氨酸钠（0.5 mmol·L^{-1}）和硝普钠（0.5 mmol·L^{-1}）所致的细胞毒性。**结论**：丹参酮Ⅱ A 可抑制去血清培养引起的 PC12 细胞凋亡。

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