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Effect of rhynchophylline on apoptosis induced by dopamine in NT2 cells[1]

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KEY WORDS dopamine; rhynchophylline; NT2 cells; apoptosis

ABSTRACT

AIM: To study the effect of rhynchophylline (Rhy) on the apoptosis induced by dopamine (DA) in NT2 cell line. **METHODS**: Release of lactate dihydrogenase (LDH) from cultured cells was determined by enzyme-linked immunosorbant assay; apoptotic NT2 neurons by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling method; DNA fragmentation by agaros gel electrophoresis; Bcl-2 protein expression by Western blotting. **RESULTS**: DA 100 – 500 μmol/L increased release of LDH, number of apoptotic NT2 neurons, and DNA fragmentation ($P < 0.01$). NT2 cells survival rate was decreased. Rhy 5 and 50 μmol/L reduced the percentage of apoptosis after treated with DA 100 μmol/L for 6 h in both with and without *bcl*-2 gene neurons ($P < 0.05$, $P < 0.01$, respectively). The expression of Bcl-2 protein was not affected by Rhy. **CONCLUSION**: Rhy protected NT2 neurons from the damage induced by DA.

INTRODUCTION

Dopamine (DA) is a neurotransmitter under physiologic conditions. However, accumulating evidences indicated that DA might also served as a neuron-toxin and thereby participated in the neurondegenerative process such as apoptosis[1]. Rhynchophylline (Rhy) is an alkaloid extracted from Chinese herb *Uncaria rhynchophylla* (Miq) Jackson. Rhy possessed pharmacological effects of hypotension, vascular relaxation, and bradycardia[2-4]. It could also inhibit platelet aggregation and thrombosis, regulate the content and release of DA in rats brain, and protect primary culture striatal neurons of rats from the damages induced by excitative acids[5-8]. In present study, the effect of Rhy on the apoptosis induced by DA in NT2 cell line, which is derived from a human teratocarcinoma and represents a committed precursor stage of differentiation, was observed.

MATERIALS AND METHODS

Materials Rhynchophylline (white crystal, purity >98 %) was provided by Guangxi Institute of Traditional Chinese Medicine and Materia Medica. It was dissolved in hydrochloride 0.1 mol/L and diluted with distilled water to pH 6.5 before use. The fetal calf serum (FCS) and Opti-MEM were purchased from Life Technologies, Sweden. Penicillin/streptomycin (P/S) was Sigma product. The phenazine methosulfate (PMS) and 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) were purchased from Promega (USA).

Cell culture NT2 precursor cell line (Stratagene, Cambridge, UK), was effectually transfected with *bcl*-2 gene, and cultured in 100 mm dish containing 10 mL Opti-MEM supplemented with 10 % FCS and 1 % P/S in a humidified incubator at 37 ℃ with 5 % – 6 % CO_2. Two days later the cells were washed twice with 10 mL sterile PBS. Trypsin-edetic acid 0.05 % 1 mL was added into cell culture dish and incubated with cell suspension for 2 min at 37 ℃. The NT2 cells sheet was dislodged by striking the side of dish with palm several times. Opti-MEM 4 – 5 mL was added to suspense the cells.

Viability assays[9,10] NT2 cell suspension 0.2 mL was removed to 96-well plate containing about 1×10^4 NT2 cells per well. The cells were then cultured for 24 h. Rhy 0.5, 5, and 50 μmol/L was added and incubated with cells for 6 h at 37 ℃ with 5 % – 6 %

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CO_2, into control and DA 50 – 500 μmol/L treated wells, respectively. Medium from each well 50 μL was removed into another 96-well plate and the release of LDH was measured by standard enzyme-linked immunosorbant assay at 490 nm after incubating the cells with 20 μL PMS reagent for 30 min at 37 ℃. Then cells were lysed by 20 μL 1 % Triton X-100 at 37 ℃ for 45 min to determine the total LDH. The ratio of LDH release to total LDH was as an index of the viability of cells. Another assay was quantified by adding MTS to the cultured 96-well plate and the procedure was the same with PMS. Both of the determination was performed quintuplicate and each experiment was repeated at least three times.

Detection of apoptotic NT2 neurons[11] Cells were cultured for 5 weeks with retinoic acid 10 μmol/L in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS and 1 % P/S. The cells were then replated at 1∶6 ratio. Two days later, cytosine arabinoside 1 μmol/L, a mitotic inhibitor, was added to the medium to inhibit the division of non-neuronal cells. The differentiated NT2 neurons were separated from non-neuronal cells, which was achieved with mechanical dislodgment of the cells by gentle striking the flask for 2 min after treatment with 0.05 % trypsin-edetic acid. The purified NT2 neurons were plated on Matrigel coating cover in the 24-well plate. The neurons were treated with Rhy 0.5, 5, and 50 μmol/L and DA 100 μmol/L simultaneously. The neurons on the cover were fixated with 4 % paraformaldehyde. The apoptotic nuclear morphology was examined and quantified by [Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling] (TUNEL) staining with *in situ* Cell Death Detection Kit (Boehringer Mannheim, USA). The apoptotic NT2 neurons were quantified according to the percentage in a visual field under a light microscope.

DNA fragmentation[12] The native NT2 cells was cultured in 6-well plate for 48 h. A fresh Opti-MEM with 10 % FCS and 1 % P/S was added, and the cells were treated with DA 250 μmol/L and Rhy 5 and 50 μmol/L as described above. Cells were harvested by gentle scraping and collected by centrifugation at 1000 × *g* for 5 min at 4 ℃. The pellet was resuspended in TE buffer (Tris-HCl 5 mmol/L, pH 8.0, edetic acid 20 mmol/L) containing 0.5 % (v/v) Triton X-100 for 20 min at 4 ℃. The preparation was centrifuged at 1000 × *g* for 5 min at 4 ℃. To remove the DNA with high molecular weight, the supernatant were centrifuged at 16 000 × *g* for 30 min in the presence of 0.1 % SDS. The pellet was resuspended in PK buffer (Tris 10 mmol/L, edetic acid 10 mmol/L, NaCl 150 mmol/L, SDS 0.5 %) containing RNase 10 mg/L and proteinase K 20 mg/L, and incubated for 1 h at 37 ℃. The sample was then extracted with equal volumes of equilibrated phenol and chloroform/isoamylalcohol (24∶1, v/v). Residual phenol was removed with equal volume of chloroform/isoamylalcohol. DNA was precipitated by ethanol, air-dried, and dissolved in TE buffer. The samples were run on 1.5 % agarose gels containing ethidium bromide 0.4 mg/L, and visualized under UV illumination.

Expression of Bcl-2 protein[13] NT2 cells with or without *bcl*-2 gene were cultured in medium with or without Rhy 5 μmol/L for 24 h. The harvested cells were lysed in a buffer (NaCl 150 mmol/L, NP-40 1 %, Tris 50 mmol/L, pH 8.0), and protein concentration was determined. Equal amount of protein extract was resolved by 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, Sweden), and transferred to nitrocellulose membranes. Monoclonal mouse anti-human Bcl-2 antibody (1∶800, Clone 124, DAKO, Denmark) and horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody were used. Detection was performed with silver staining.

RESULTS

Viability of NT2 cells After exposure to different concentrations of DA for 6 h, a great release of LDH was observed at higher DA level (100, 250, and 500 μmol/L, $P<0.01$). Rhy 5 and 50 μmol/L inhibited LDH leakage induced by DA. The cell damage, however, was not completely blocked by pretreatment with Rhy (Fig 1). The MTS (Owen's reagent) can be bioreduced by cells into a formazan that is soluble in cell culture medium. The conversion of the MTS into the aqueous soluble formazan is accomplished by dehydrogenase enzyme found in metabolically active cells. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. Rhy 5 and 50 μmol/L inhibited the decrease of absorbance induced by DA (100, 250, and 500 μmol/L, $P<0.01$), which indicated that Rhy facilitated the survival of NT2 cells (Fig 2).

Apoptosis of NT2 cells After TUNEL staining, the apoptotic characteristics of NT2 neurons induced by

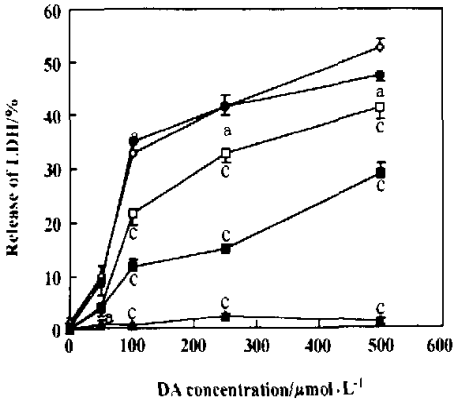

Fig 1. Effect of Rhy on the release of LDH induced by DA. (◇) DA; (●) DA + Rhy 0.5 μmol/L; (□) DA + Rhy 5 μmol/L; (■) DA + Rhy 50 μmol/L; (▲) Rhy 50 μmol/L. $n = 4$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* DA control.

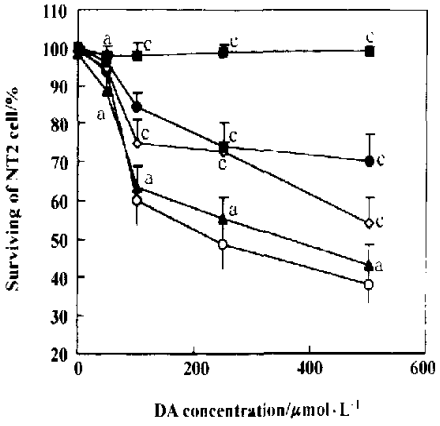

Fig 2. Effect of Rhy on the survival rate of NT2 cells. (○) DA; (▲) DA + Rhy 0.5 μmol/L; (◇) DA + Rhy 5 μmol/L; (●) DA + Rhy 50 μmol/L; (■) Rhy 50 μmol/L. $n = 4$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* DA control.

DA 100 μmol/L were shown as shrinkage and condensed nuclei and Rhy 50 μmol/L prevented NT2 neurons against apoptosis (Fig 3). Exposure to DA 100 μmol/L for 6 h induced marked apoptosis in both neurons with or without *bcl*-2 gene. The percentage of apoptotic neurons was 6.2 % and 13.5 %, respectively, which were higher than that in Rhy-treated and normal control neurons ($P < 0.05$, $P < 0.01$, respectively).

However, the apototic percentage of neurons with *bcl*-2 gene was more little than that of neurons without *bcl*-2 gene in normal control, DA, and Rhy-treated groups (Tab 1). In the cultures treated with Rhy, no marked DNA fragmentation was observed (Fig 4).

Effect of Rhy on the expression of Bcl-2 protein The expression of Bcl-2 protein was not affected by Rhy (Fig 5). But there was no remarkable expression of Bcl-2 protein in the native NT2 cells (untransfected with *bcl*-2 gene), and Rhy did not induce

Tab 1. Effect of Rhy on apoptosis rate of NT2 neurons induced by DA. $n = 4$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* normal control. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* dopamine 100 μmol/L.

	Apoptotic neurons. %	
	With *bcl*-2	Without *bcl*-2
Normal control	0.60 ± 0.25	5.2 ± 2.3
Dopamine (100 μmol/L)	6.2 ± 1.3^e	14 ± 3^e
Rhy (5 μmol/L) + DA (100 μmol/L)	4.3 ± 0.9^d	12.6 ± 2.9^d
Rhy (50 μmol/L) + DA (100 μmol/L)	2.5 ± 1.1^f	7.2 ± 2.2^f
Rhy (50 μmol/L)	0.9 ± 1.1^a	6.0 ± 1.2^a

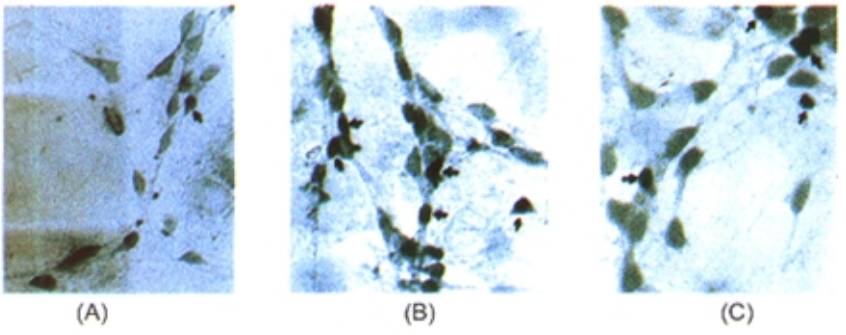

Fig 3. Effect of Rhy on apoptosis after exposure to DA 100 μmol/L for 6 h by TUNEL staining. The apoptotic charateristics of neurons were shown as shrinkage and condensed nuclei (arrows). (A) Normal control; (B) DA control; (C) DA + Rhy 50 μmol/L. × 200.

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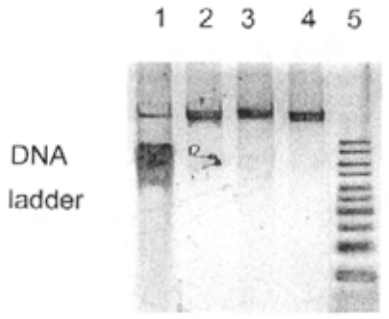

Fig 4. Effect of Rhy on DNA fragmentation induced by DA in NT2 cells. (1) DA 250 μmol/L; (2) DA + Rhy 5 μmol/L; (3) DA + Rhy 50 μmol/L; (4) Rhy 50 μmol/L; (5) DNA Marker.

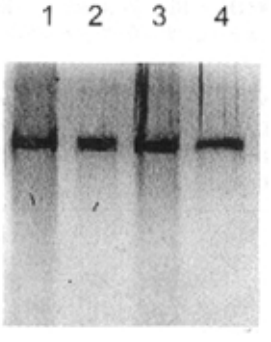

Fig 5. Effect of Rhy and DA on Bcl-2 protein expression in NT2 cells transfected with *bcl*-2 gene. (1) Control; (2) DA 250 μmol/L; (3) Rhy 5 μmol/L; (4) Marker.

or promote Bcl-2 protein expression either in native NT2 cells or in NT2 cells with *bcl*-2 gene.

DISCUSSION

It has been reported that DA is highly cytotoxic to some cell lines, especially to neurons[14,15]. Results presented here showed that DA, at concentrations of 100 – 500 μmol/L overlapping the physiological extent, induced a marked damage to the NT2 cells. The damage was demonstrated as apoptosis morphologically although the DNA fragmentation was not significant.

Neuron degeneration resulted from different factors, in which apoptosis was widely studied. The oxidative stress, p53 gene inducement, and *bcl*-2 gene inhibition were understood as the trigger of apoptosis. The trigger for DA-induced apoptosis may be DA itself and/or its metabolites. It has been reported that many agents, including DA, induce apoptosis by free radicals, in which the free radical scavengers and antioxidants decreased apoptosis[16-18]. Previous study showed that Rhy could protect primary culture striatal neurons of rats from the damages induced by excitatory amino acids[8]. In current experiment, Rhy reduced the NT2 cells/neurons damage (apoptosis) induced by DA. This result confirmed further the protective effects of Rhy on the neurons. Previous study showed that DA could induce a DNA ladder in 293 cells after exposure to DA 200 – 500 μmol/L for 24 – 30 h[16]. In present study the DNA ladder induced by DA was not significant, which maybe due to short time (6 h) for DA exposure although this exposure time was suitable to the viability experiments. However, Rhy decreased the apoptotic neurons by TUNEL staining. Current results suggested that protective effect against NT2 cells damage may be due to inhibiting the apoptosis induced by DA. The percentage of apoptotic neurons without *bcl*-2 gene was more than that in neurons with *bcl*-2 gene, which approved *bcl*-2 gene against the apoptosis. However, Rhy did not induce Bcl-2 protein expression in both cells with or without *bcl*-2 gene, which indicated that Bcl-2 protein expresion was not contributed to the protective effect of Rhy on NT2 cells. Accumulative evidences showed that intracellular Ca^{2+} overload was an important pathway of apoptosis induced by free radicals and excitative acids. Previous experiments suggested that Rhy was calcium blocker and antioxidant[7,8,19]. It is surmisable that the protective effect of Rhy on neuron damage induced by excitative acids and DA resulted from calcium antagonization. Furthermore, it was also reported that the potassium channel openers inhibited apoptosis. The evidence of Rhy regulating potassium channel may be another interpretation[20], which remained to be further studied.

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钩藤碱对多巴胺诱导的 NT2 细胞凋亡的影响[1]

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关键词 多巴胺; 钩藤碱; NT2 细胞; 细胞凋亡

目的: 观察钩藤碱(rhnchophylline, Rhy)对多巴胺(dopamine, DA)诱导 NT2 细胞凋亡的防护作用. **方法**: 以 LDH 的漏出率反映细胞的生存率; 用 TUNEL 染色法和 DNA 琼脂糖凝胶电泳法观察 NT2 神经元凋亡情况; 用 Western blotting 法测定 Bcl-2 蛋白表达. **结果**: Rhy 在 5 和 50 μmol/L 的浓度下能显著地抑制由 DA 所致的乳酸脱氢酶的漏出, 以及明显地提高以 PMS 试剂转化为指标的生存率($P<0.05$, $P<0.01$); 在分化的 NT2 细胞神经元中, 转染 *bcl-2* 基因的神经元凋亡率明显低于未经 *bcl-2* 基因转染的神经元, 而 Rhy 使 DA 诱导的转染 *bcl-2* 基因神经元和未转染 *bcl-2* 基因神经元的凋亡率均明显减少; Rhy 能抑制 DA 所致的 DNA 降解, 但 Rhy 对 NT2 细胞 Bcl-2 蛋白表达无明显影响. **结论**: Rhy 能对抗 DA 诱导的 NT2 细胞的损伤.

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