

Original Article

Cattle encephalon glycoside and ignotin injection promoted synthesis of glial cell line-derived neurotrophic factor by astrocytes that protected against neuronal damage induced by AAPH

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Abstract: Cattle encephalon glycoside and ignotin injection offers neural protection after cerebral apoplexy clinically, but the exact mechanism of the protective effect is unclear. We performed primary culture of neurons and astrocytes and simulated ischemic brain damage using AAPH and discussed the action mechanism of cattle encephalon glycoside and ignotin injection. Results showed that cattle encephalon glycoside and ignotin injection was effective against the neuronal damage induced by AAPH through promoting the secretion of glial cell line-derived neurotrophic factor (GDNF) by the astrocytes.

Keywords: Cattle encephalon glycoside and ignotin injection, astrocytes, neuron, glial cell line-derived neurotrophic factor (GDNF), AAPH

Introduction

Cerebral apoplexy is among the major causes of death in China with high disability rate and high mortality, and ischemic cerebral apoplexy accounts for 60-80% [1]. Apart from thrombolysis in acute stage, neural protectants also play a crucial role in the recovery from cerebral apoplexy [2]. Cattle encephalon glycoside and ignotin injection is a compound preparation and mixture of muscle extract from healthy rabbits and cattle brain gangliosides. The main ingredients are polypeptides, various gangliosides, free amino acids and nucleic acids. Cattle encephalon glycoside and ignotin injection is the commonly used neural protectant. It is important to understand the mechanism of neural protection offered by cattle encephalon glycoside and ignotin injection after cerebral apoplexy, so as to develop new neural protectants for cerebral apoplexy.

Previous studies on neural protection mainly focus on neural cells, and the use of astrocytes for such researches is gaining popularity in recent years. As the main cell type in the brain, astrocytes outnumber neurons by over 5 times and play an important role in maintaining the physiological functions of neurons [3]. Since astrocytes can relieve cerebral edema and the damage by free radicals, they attract increasing attention for the effect of cerebral apoplexy [4]. In the present study, we discussed the mechanism of protective action of cattle encephalon glycoside and ignotin injection on neurons through experiment with astrocytes.

Materials and methods

Materials

Experimental animals: SPF Sprague-Dawley rats were purchased from Vital River Laboratory Animal Technology Co., Ltd. Rats at gestational

age of 16-18 age were used for primary neuronal culture, while suckling mice at 24 hours of age were used for primary astrocyte culture.

Reagents and equipment: DMEM, Neurobasal Medium, fetal bovine serum and 0.25% trypsin were purchased from Invitrogen (USA). Cell culture plate, cell culture flask and centrifuge tube were purchased from Corning Corporation (USA). Mouse anti-neurofilament monoclonal antibody, mouse anti-GDNF monoclonal antibody, rabbit anti-GFAP polyclonal antibody, 2, 2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) and propidium iodide were purchased from Sigma (USA). Alexa Fluor 568-conjugated goat anti-rabbit antibody and Alexa Fluor 488-conjugated goat anti-mouse antibody were purchased from Molecular Probes (USA). Western Blot reagents were purchased from Applygen Technologies Inc. CCK-8 assay kit was purchased from Dongren Company.

Experimental methods

Primary culture of neurons: Rats at gestational age of 16-18 days were anesthetized with 10% chloral hydrate and immobilized at the ultra-clean workbench. The embryos were harvested under sterile conditions and placed in Neurobasal Medium. The cortex was separated and the meninges and blood vessels were removed. After washing with culture medium twice and cutting the tissues into pieces, the cells were dispersed by blowing and digested with 0.25% trypsin for 10 min. All tissue blocks were removed by filtering. The cells were suspended in DMEM containing 10% fetal bovine serum and inoculated to 24-well plate. The culture medium was removed the next day and replaced by neuron culture medium, which was Neurobasal Medium containing 2% B-27. The culture medium was replaced every other day for 7 days.

Primary culture of astrocytes: SD suckling mice at 24 hours of age were subjected to hypothermic anesthesia and disinfected by soaking in 75% alcohol for 5 min. With the removal of skulls under sterile conditions, the brain tissues were harvested and placed in sterile glass dish containing pre-cooled DMEM. After washing for 3 times, the meninges and blood vessels were stripped under the dissecting microscope using microsurgical forceps. The tissue blocks were loaded into the centrifuge tube containing 5 ml of 0.25% trypsin and digested

for 30 min at 37°C in water bath. The digestion was terminated by adding complete culture medium (10% FBS+DMEM+double antibodies). Cell suspension was prepared by blowing for 20-30 times using a straw. The tissue blocks were removed by filtering through 70 µm sieve, and the resultant cell suspension was centrifuged at 1500 rpm for 5 min. With the supernatant removed, the cells were resuspended, transferred to the culture flask and cultured in a 5% CO₂ incubator at 37°C. The culture medium was replaced 2-3 days for 7-9 days.

Immunofluorescence staining: After primary culture, the neurons and astrocytes were fixed in 4% paraformaldehyde for 30 min and washed with PBS buffer. The cell membrane permeability was increased by adding 0.2% Triton-X-100. The antigen loci were sealed with 10% goat serum. Mouse anti-neurofilament monoclonal antibodies and rabbit anti-GFAP polyclonal antibodies (1:500) were added into the neuron and astrocytes, respectively, and incubated overnight at 4°C. Then the cells were washed and added with Alexa Fluor 488-conjugated goat anti-mouse antibodies and Alexa Fluor 568-conjugated goat anti-rabbit antibodies, respectively, for cell incubation at room temperature for 1 h. The cells were observed under the fluorescence microscope and images were collected.

AAPH-induced cell damage and cell viability assay: AAPH was dissolved in DMEM to prepare 1 mM, 5 mM, 10 mM, 20 mM and 40 mM solutions, which were added into neurons and astrocytes, respectively. In the meantime, CCK-8 solution was added and the absorbance at 495 nm was detected using a spectrophotometer 4 hours later. The influence of AAPH on cell viability was determined.

Influence of cattle encephalon glycoside and igiton injection on AAPH-induced astrocyte damage: The astrocytes were purified by several rounds of passage and 40 mM AAPH was added to induce cell damage for 4 h. Then the culture medium was replaced and 0.025 µg/ml, 0.05 µg/ml and 0.1 µg/ml cattle encephalon glycoside and igiton injection was added to treat the cells for 24 h, respectively. Finally CCK-8 solution was added.

Preparation of conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection: The astrocytes were first added with 40 mM AAPH to induce cell

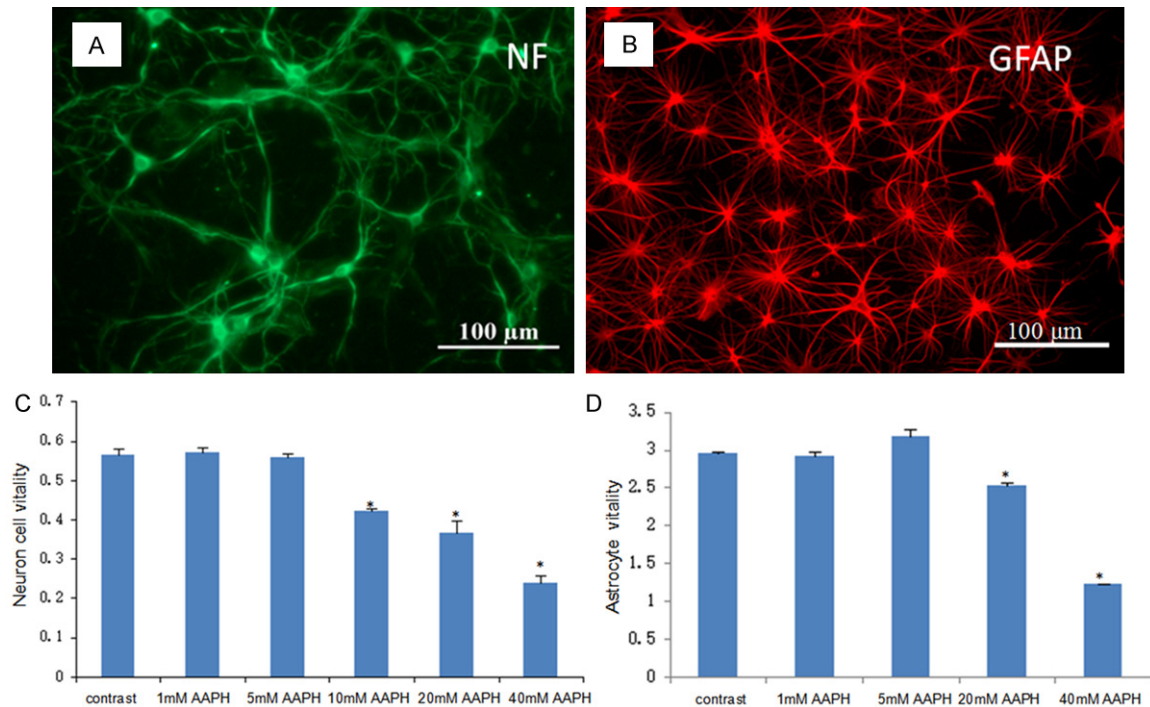


Figure 1. AAPH simulated neuron and astrocyte damage that can be induced by OGD. Note: After primary culture, the neurons had a small size with few long protrusions and were able to express NF specifically (A). In contrast, the astrocytes had many short protrusions and specifically expressed GFAP under immunofluorescence staining (B). AAPH could simulate cerebral ischemia and 10 mM AAPH induced neuronal damage (C). But AAPH did not induce damage to astrocytes unless under the concentration of 20 mM (D) (“*” indicated statistically significant difference compared with the control).

damage for 4 h. Culture medium was replaced and the cells were treated by 0.1 $\mu\text{g}/\text{ml}$ cattle encephalon glycoside and ignotin injection for 24 h. Then culture supernatant was collected and passed through 0.22 μm filter.

Western blot

The astrocytes were added with 40 mM AAPH to induce cell damage for 4 h. Culture medium was replaced and the cells were treated by 0.025 $\mu\text{g}/\text{ml}$, 0.05 $\mu\text{g}/\text{ml}$ and 0.1 $\mu\text{g}/\text{ml}$ cattle encephalon glycoside and ignotin injection for 24 h, respectively. After lysis, fragmentation and centrifugation, the proteins were quantified using Bradford method. The final concentration of the proteins was adjusted to 20 $\mu\text{g}/\text{ml}$, and 20 μL of the sample was taken for electrophoresis and membrane transfer. The membrane was sealed with 5% defatted milk powder at room temperature for 1 h and added with GDNF antibodies and the corresponding secondary antibodies (1:1000) to incubate the cells at room temperature for 2 h. After washing

with PBS and addition of ECL reagent for 1-5 min, the protein expressions were observed and analyzed by using Image J.

Statistical analysis

All data were statistically analyzed using SPSS15.0 software and expressed as mean \pm standard deviation. ANOVA method was used to compare the difference among groups and LSD method was utilized to carry out the inter-group comparison. $P < 0.05$ indicated statistically significant difference.

Results

AAPH can induce neuron and astrocyte damage that occurs in cerebral apoplexy

After primary culture, the neurons and astrocytes were stained by specific markers. The cells expressed neurofilament (NF) and glial fibrillary acidic protein (GFAP) specifically, so the cells were identified as neurons and astrocytes, respectively (**Figure 1A** and **1B**). Trea-

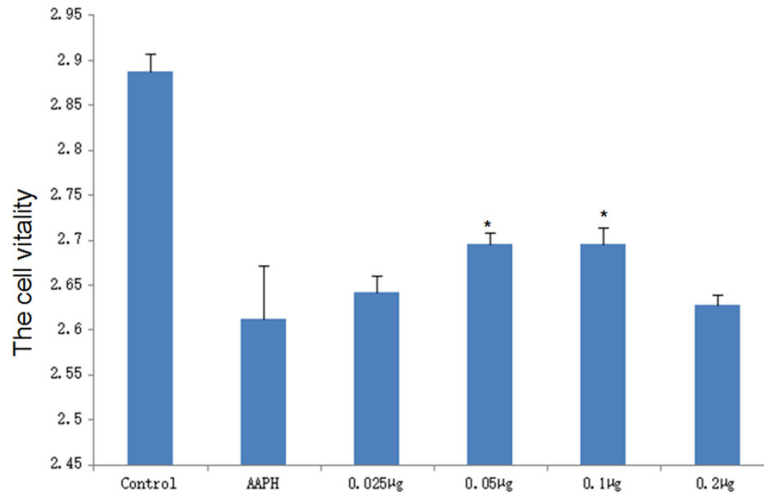


Figure 2. Cattle encephalon glycoside and igiton injection relieved the AAPH-induced damage to astrocytes. Note: 40 mM AAPH caused obvious damage to astrocytes, and 0.025 μg/mL, 0.05 μg/mL and 0.1 μg/mL cattle encephalon glycoside and igiton injection alleviated the cell damage. The protective effect offered by 0.05 μg/mL and 0.1 μg/mL concentration was considerably different (“*” indicated $P \leq 0.05$). But 0.2 μg/mL concentration did not show a protective effect.

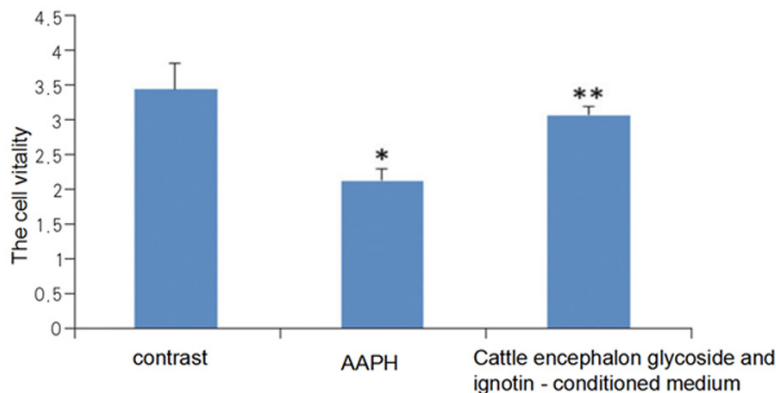


Figure 3. Conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection relieved AAPH-induced neuronal damage. Note: Compared with the control group, 40 mM AAPH could induce neuronal damage, which was relieved by treatment with conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection; moreover, the cell viability was restored considerably (“*” indicated a great reduction of cell viability induced by AAPH compared with the control, $P \leq 0.05$; “**” indicated that conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection relieved AAPH-induced neuronal damage; the effect was significantly different, $P \leq 0.05$).

tment with different concentrations of AAPH induced damage to neurons and astrocytes, and the cell viability decreased with the increase of AAPH concentration (Figure 1C and 1D). Among these concentrations, 40 mM AAPH induced greatest damage to neurons and astrocytes.

Cattle encephalon glycoside and igiton injection relieved AAPH-induced damage to astrocytes

At 4 h after cell damage induction by AAPH, the astrocytes were incubated with 0.025 μg/mL, 0.05 μg/mL, 0.1 μg/mL and 0.2 μg/mL cattle encephalon glycoside and igiton injection for 24 h, respectively. Then cell viability assay was performed. Results showed that all concentrations of cattle encephalon glycoside and igiton injection relieved AAPH-induced damage to astrocytes. The protective effect offered by 0.05 μg/mL and 0.1 μg/mL concentration showed a significant difference (Figure 2). However, 0.2 μg/mL cattle encephalon glycoside and igiton injection did not exert a protective action on the astrocytes.

Conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection relieved AAPH-induced neuronal damage

We found that 0.1 μg/mL cattle encephalon glycoside and igiton injection greatly reduced the AAPH-induced astrocyte damage. Therefore, 40 mM AAPH was used to induce damage to astrocytes and 0.1 μg/mL cattle encephalon glycoside and igiton injection was added subsequently to treat the astrocytes for 24 h. The supernatant was collected as the conditioned medium. After induction of neuronal damage with 40 mM AAPH, the conditioned medium was added to treat the cells for 24 h and neuronal viability was detected. Results showed that the conditioned medium from astrocytes inoculated with cattle enceph-

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Cattle encephalon glycoside and igiton neurons damage

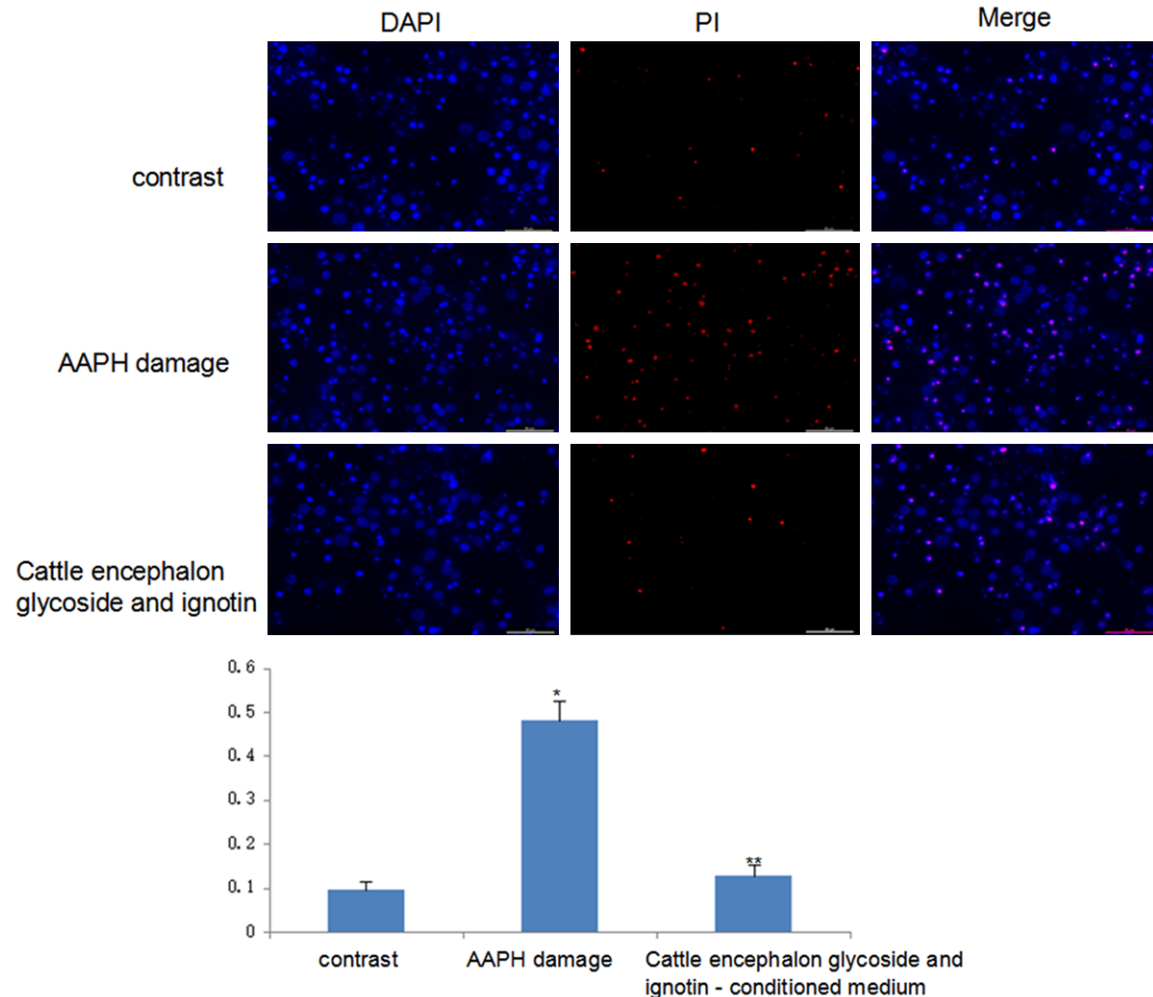


Figure 4. Conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection relieved AAPH-induced neuronal apoptosis. Note: PI can label the apoptotic cells. After treatment with AAPH, the number of apoptotic cells increased greatly. The trend was reversed by adding conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection (“*” indicated a great increase of apoptotic cells induced by AAPH compared with the control, $P \leq 0.05$; “**” indicated that conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection significantly reduced cell apoptosis, $P \leq 0.05$).

alon glycoside and igiton injection reversed AAPH-induced neuronal damage, and the results showed significant difference (Figure 3).

Conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection relieved AAPH-induced neuronal apoptosis

Propidium iodide (PI) is a DNA-binding dye that stains damaged DNA. The normal cells are not stained under the fluorescence microscope, while the damaged and apoptotic cells emit red fluorescence. Our experiment showed that after treatment with conditioned medium from astrocytes inoculated with cattle encephalon

glycoside and igiton injection for 24 h, the number of apoptotic neurons decreased considerably compared with that before treatment (Figure 4).

Cattle encephalon glycoside and igiton injection promoted the synthesis of GDNF by astrocytes

After primary culture, the astrocytes were passaged and inoculated to 25 cm² culture flask. Cell damage was induced by AAPH for 4 h, and 0.025 µg/mL, 0.05 µg/mL and 0.1 µg/mL cattle encephalon glycoside and igiton injection was added to incubate the cells for 24 h, respectively. The cells were collected and lysed

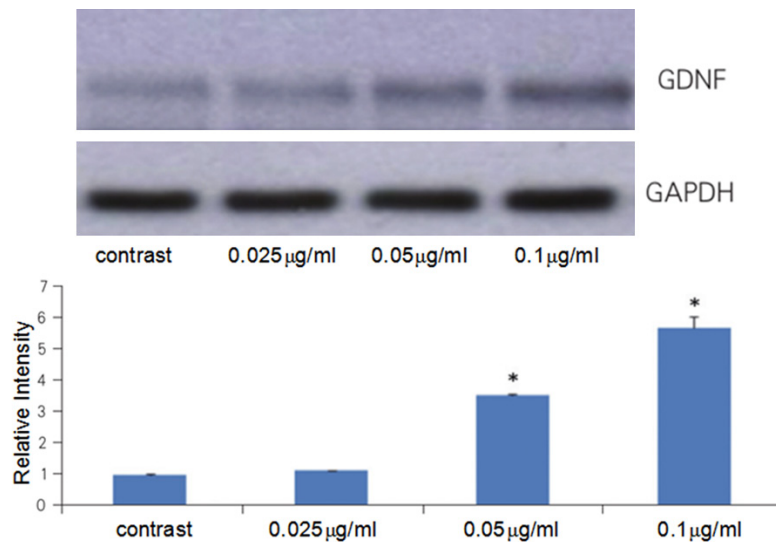


Figure 5. Cattle encephalon glycoside and igiton injection promoted the synthesis of GDNF by astrocytes. Note: Cattle encephalon glycoside and igiton injection promoted the synthesis of GDNF by astrocytes. GDNF expressions increased in all groups after treatment with cattle encephalon glycoside and igiton injection for 24 h. Compared with the control group, the GDNF expressions in 0.05 µg/mL and 0.1 µg/mL treatment group showed a significant difference ($P \leq 0.05$).

and GDNF expression was detected. Western Blot revealed that cattle encephalon glycoside and igiton injection promoted the synthesis of GDNF by astrocytes in a dose-dependent manner. GDNF expression in 0.1 µg/mL group was obviously higher than that of AAPH-induced damage group, 0.025 µg/mL group and 0.05 µg/mL group (Figure 5).

Discussion

Astrocytes are the new targets of neural protection, accounting for the largest proportion in the central nervous system. It is generally believed that astrocytes provide nutrients and support to neurons. Recent studies show that mature astrocytes after damage can dedifferentiate into radial glial cells which restore neural function following brain damage [5]. Ethidium bromide (EB) can bind specifically to DNA and RNA in glial cells, thereby interfering with the synthesis of nucleic acids and proteins. This may lead to inhibited proliferation of astrocytes in cerebral ischemia model and the expansion of infarct loci. Astrocytes offer a protective effect against neuronal damage after cerebral ischemia [6] and therefore are qualified as the target cells in the development of neural protectants.

AAPH can generate oxygen free radicals in cells and therefore simulate the cell damage caused by free radicals in cerebral apoplexy. Our experiments indicated that AAPH could induce damage to neurons. However, the treatment with AAPH for a short period of time increased the viability of astrocytes. This indicated the greater tolerance of astrocytes to free radicals after ischemic brain damage compared with the neurons. The culture supernatant collected from the treatment with cattle encephalon glycoside and igiton injection relieved AAPH-induced neuronal damage [7].

Existing researches show that cattle encephalon glycoside and igiton injection works through the anti-inflammatory mechanism and provides protection for neurons. The symptoms of cerebral apoplexy and the neural functions can be improved by cattle encephalon glycoside and igiton injection clinically. We found that conditioned medium from astrocytes inoculated with cattle encephalon glycoside and igiton injection reduced neuronal apoptosis induced by AAPH and enhanced the neuronal viability.

Cattle encephalon glycoside and igiton injection may exert the protective effect by promoting the synthesis of GDNF by astrocytes. GDNF secreted by astrocytes has an obvious protective effect on neurons [8]. Injection of GDNF into the lateral ventricle effectively prevents neuronal apoptosis following cerebral ischemia [9]. The administration of GDNF to the model of permanent cerebral ischemia also reduced infarct loci, relieves cerebral edema and inhibits neuronal apoptosis [10]. We found that cattle encephalon glycoside and igiton injection not only reduced the damage to astrocytes induced by AAPH, but also acted on astrocytes in which damage was induced by AAPH. Cattle encephalon glycoside and igiton injection was protective against neuronal damage by promoting GDNF synthesis by astrocytes.

Conclusion

Cattle encephalon glycoside and igiton injection provides neural protection probably through the promotion of synthesis of BDNF by astrocytes. We found that astrocytes were the targets of neural protection and the recovery of patients with cerebral apoplexy could be facilitated by protecting neurons through the protection of astrocytes.

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Disclosure of conflict of interest

None.

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