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Research article

Progesterone alleviates acute brain injury via reducing apoptosis and oxidative stress in a rat experimental subarachnoid hemorrhage model

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HIGHLIGHTS

- Progesterone significantly improved neurological deficits and reduced mortality.
- Progesterone inhibited cell apoptosis and reduced the expression of caspase-3.
- Progesterone significantly reduced the ratio of Bax/Bcl-2 and attenuated the release of cytochrome c from mitochondria.
- Progesterone induced antioxidative effects by elevating the activity of SOD and decreasing MDA content.

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ABSTRACT

This study aimed to investigate the therapeutic effect of progesterone on acute brain injury after subarachnoid hemorrhage (SAH). Subarachnoid hemorrhage was induced in male Sprague–Dawley rats ($n = 72$) by endovascular perforation. Progesterone (8 mg/kg or 16 mg/kg) was administered to rats at 1, 6, and 12 h after SAH. Mortality, neurologic deficits, cell apoptosis, expression of apoptotic markers, the level of malondialdehyde (MDA) and the activity of superoxide dismutase (SOD) were assayed at 24 h after experimental SAH. Mortality, cell apoptosis and the expression of caspase-3 were decreased, and improved neurological function was observed in the progesterone-treated SAH rats. Further, exploration demonstrated that progesterone significantly reduced the ratio of Bax/Bcl-2 and attenuated the release of cytochrome c from mitochondria. Progesterone also induced anti-oxidative effects by elevating the activity of SOD and decreasing MDA content after SAH. Furthermore, dose-response relationships for progesterone treatment were observed, and high doses of progesterone enhanced the neuroprotective effects. Progesterone treatment could alleviate acute brain injury after SAH by inhibiting cell apoptosis and decreasing damage due to oxidative stress. The mechanism involved in the anti-apoptotic effect was related to the mitochondrial pathway. These results indicate that progesterone possesses the potential to be a novel therapeutic agent for the treatment of acute brain injury after SAH.

1. Introduction

Subarachnoid hemorrhage (SAH) is a serious condition resulting in death and disability. Recently, an increasing number of studies have suggested that acute brain injury after SAH appears to play a key role in the poor prognosis of SAH [1–2]. The mechanism of acute brain injury after SAH is a complicated process, including cell death, oxidative stress, blood–brain barrier disruption and inflammation. Moreover, several studies have demonstrated that cell death and oxidative stress are critical to the development of acute brain injury after SAH [3–4]. Therefore, alleviating acute brain injury via various pathways may effectively mitigate the neural damage caused by SAH. Progesterone (PROG) has been reported to be neuroprotective against traumatic brain injury, brain edema, ischemia, and neurodegeneration [5–7]. The original concept of progesterone as a reproduction-restricted hormone has now been modified to incorporate previously unforeseen anti-oxidative, anti-apoptotic, myelinating, and anti-inflammatory effects in the nervous system [7–8]. In our previous study, PROG was found to attenuate SAH-induced brain edema and cell apoptosis [9]. However, the

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neuroprotective effects of PROG in SAH have not been fully investigated. The present study aimed to determine the anti-apoptotic and anti-oxidative effects of PROG on acute brain injury after SAH.

2. Materials and methods

2.1. Animals and experimental design

Male Sprague–Dawley rats (280–300 g) were purchased from the Animal Center of Zhejiang University School of Medicine (Hangzhou, China). Rats were housed in a temperature-controlled room(22 ± 2 °C) and maintained on standardized light/dark (12/12 h) cycles with free access to food and water. Rats ($n = 72$) were randomly divided into four groups: (1) the sham group ($n = 18$) underwent a sham surgery and received saline; (2) the SAH + vehicle group ($n = 18$) was subjected to SAH and treated with a vehicle; (3) the SAH + 8 mg/kg PROG group ($n = 18$) was subjected to SAH and treated with 8 mg/kg dose of PROG; (4) the SAH + 16 mg/kg group ($n = 18$) was subjected to SAH and treated with 16 mg/kg dose of PROG. The initial injection of PROG/vehicle was administered intraperitoneally at 1 h after SAH, and subsequent injections were administered subcutaneously at 6 h and 12 h after SAH according to previous studies [10]. All end points in this study were set at 24 h after SAH. The experimental protocols were approved by the Ethics Committee for the use of Experimental Animals of Zhejiang University and conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

2.2. Rat SAH model

The endovascular perforation model of SAH in rats was performed as previously described [11]. Briefly, general anesthesia was induced with an intraperitoneal injection of pentobarbital (40 mg/kg). After endotracheal intubation was performed, rats were placed on a heating pad to maintain a rectal temperature of 36.0 ± 0.5 °C. The carotid artery was dissected, and a 4-0 monofilament nylon suture was cannulated into the internal carotid artery from the external carotid artery. The suture was advanced to the bifurcation of the anterior cerebral artery and the middle cerebral artery, where a perforation was made to create a SAH. Sham group rats underwent identical procedures except for the perforation. The severity of the SAH was quantified according to a previously published grading scale [12]. The scale was based on the amount of subarachnoid blood in 6 segments of the basal cistern: grade 0, no subarachnoid blood; grade 1, minimal subarachnoid blood; grade 2, moderate blood in the visible arteries; grade 3, blood clots covering all arteries within the segment. A total score ranging from 0 to 18 was obtained by adding the scores from all 6 segments.

2.3. Mortality and neurological examination

The mortality rate of rats was recorded during and after the SAH procedure. At 24 h after SAH, a behavioral test was performed on all rats according to the scoring system of Garcia et al. [13]. Briefly, spontaneous activity, symmetrical movements of four limbs, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch were evaluated. All tests were evaluated by an observer who was blind to the treatment conditions. A total score ranging from 3 to 18 was recorded.

2.4. Histology and TUNEL staining analysis

After the neurological examination, rats ($n = 6$ per group) were euthanized and intracardially perfused with phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (pH 7.4). The fixed brain was immersed in 4% paraformaldehyde at 4 °C for 6–8 h and then immersed in 30% sucrose solution until the tissue sank (2 days). The brains were frozen in tissue-freezing media, and sections were collected on slides (7 μm sections). TUNEL staining was performed according to manufacturer's protocol (Roche Inc., Basel, Switzerland), and sections were examined under a laser scanning confocal microscope (LSM-710; Zeiss). TUNEL-positive cells were calculated in a blind manner. The total number of cells (DAPI+) and the number of TUNEL-positive cells were counted in five separate fields in four different slices. The apoptotic index is defined as the average percentage of TUNEL-positive cells vs. the total number of cells.

2.5. Western blot

Rats ($n = 6$ per group) were euthanized 24 h after SAH. The cerebral cortex, which faced the clot in the subarachnoid space, was isolated and immediately frozen in liquid nitrogen as previously described [13]. Western blots were performed as described previously [9]. Briefly, a cortical sample was homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (Santa Cruz Biotechnology) and centrifuged at $1000 \times g$ for 10 min at 4 °C. The resulting supernatants were further centrifuged, and protein concentrations were measured using a DC protein assay kit (Bio-Rad, Hercules, CA). An equal amount of protein (60 μg) was resuspended in loading buffer and loaded onto sodium dodecyl sulfate–polyacrylamide gels. The proteins were electrophoresed at 80 V for 3 h and transferred to polyvinylidene fluoride membranes at 100 V for 2 h. The membranes were blocked with non fat dry milk buffer for 2 h and then incubated overnight at 4 °C with primary antibodies against caspase-3 (1:500, Cell Signaling Technology, Danvers), Bax (1:500, Abcam, Cambridge), Bcl-2 (1:500, Abcam, Cambridge), cytochrome c (1:500, Abcam, Cambridge), COX-IV (1:400, Santa Cruz Biotechnology, Dallas), and β-actin (1:400, Cell Signaling Technology, Danvers). The membranes were processed with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The protein band densities were detected using X-ray film and quantified using ImageJ software (NIH). The results were normalized to the sham-operated group.

2.6. Assay of MDA content and SOD activity

Malondialdehyde (MDA) is a well-established indicator of lipid peroxidation [14]. When rats ($n = 6$ per group) were sacrificed 24 h after SAH, the level of MDA in brain tissue homogenates was determined using the thiobarbituric acid reaction (TBAR) method with a commercially available kit (LPO-586; Oxis International, Beverly Hills, CA). The MDA levels were expressed as nanomoles per milligram of brain protein [15]. SOD activity was determined by inhibition of the nitroblue tetrazolium reduction due to superoxide anion generation by a xanthine–xanthine oxidase system [16]. Changes in absorbance at 550 nm were monitored with a spectrophotometer, and the SOD levels were expressed as units per mg protein. Commercial kits were used to measure the activity of SOD (706,002, Cayman Chemical).

2.7. Statistical analysis

All data are presented as the means ± SD. Statistical significance was verified using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Differences in mortality among groups were tested using the chi-square test. Differences were considered statistically significant at a value of $P < 0.05$. SPSS 19.0 statistics software (IBM, Armonk, NY, USA) and GraphPad Prism (LaJolla, CA, USA) were used for data analysis.