



# Effects of hypothermia during propofol anesthesia on learning and memory ability and hippocampal apoptosis in neonatal rats

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## Abstract

**Objective** At present, the harm of hypothermia to the central nervous system has received a great attention from scholars. The present study aimed to investigate the effects of hypothermia on learning and memory abilities and hippocampal apoptosis in neonatal rats and the role of p-ERK and p-CREB in anesthesia.

**Methods** In this study, 60 Sprague Dawley newborn rats (age 7-day-old) were randomly divided into 3 groups ( $n=20$ ), including Control Group (Group C), Anesthesia Group (Group A), and Anesthesia Hypothermia Group (Group AH). Group C was intraperitoneally injected with 0.1 ml saline, and rectal temperature was maintained in the range of 38–39 °C; Group A was intraperitoneally injected with 25 mg/kg of propofol (0.1 ml), the 1/2 initial dose was added per each period of 20 min, anesthesia was maintained for 2 h, and rectal temperature was kept in the range of 38–39 °C. The anesthesia mode and duration of Group AH were as same as Group A, room temperature was set to 23 °C, which caused body's temperature naturally dropped down. After the anesthesia recovered, each group randomly involved five rats for analyzing by Western blot to detect the expression level of p-ERK and p-CREB, and other five rates were also analyzed by flow cytometry assay to detect hippocampal apoptosis rate. The remaining 10 rats in each group were kept up to 30 days for conducting the Morris water maze test, five rats were tested for detecting the expression level of p-ERK and p-CREB, as well as hippocampal apoptosis rate in each group.

**Results** Compared with Group C and Group A, the rectal temperature of Group AH was decreased significantly ( $P<0.05$ ); At the age of 7 days, compared with Group C and Group A, apoptosis rate of hippocampal tissue in Group AH was increased ( $P<0.05$ ), the expression level of p-ERK and p-CREB proteins in Group AH was significantly reduced ( $P<0.05$ ), and there were no significant differences between Group C and Group A. At the age of 36 days, there were no significant differences in the results of behavioral test, apoptotic rates, and expression level of the proteins.

**Conclusion** Our findings suggest that hypothermia during anesthesia can increase the apoptosis rate in the hippocampus of neonatal rats, whose mechanism may be related to the downward adjustment of p-ERK and p-CREB. However, it has no obvious influence on the long-term learning and memory abilities.

**Keywords** Hypothermia · Apoptosis · Neonatal rats · Hippocampus

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## Introduction

Since various factors may affect the body's temperature-regulation system or the operative time, general anesthesia patients are prone to hypothermia. In the anesthesia for infants, because the center of the body's temperature is not well-developed, the ability to regulate body' temperature is poor, and the body surface heat dissipation area of infants is large, newborns are more likely to take hypothermia than adults [1]. The neonatal central nervous system is in the peak of development, so that it is more sensitive to outside stimulation and the harm caused by hypothermia will be more

serious. Because of the complexity of clinical research and the particularity of infants, the clinical study of the effect of hypothermia on neurological impairment during the period of anesthesia needs to be further explored.

Although hypothermia is often used as a protective tool in craniocerebral injuries and surgical applications, especially in the cases of hypoxia and ischemia, hypothermia has a special influence on treatment. There is a controversy about the body's latent injury and central nervous function caused by hypothermia, which should be seriously taken into account. There have been studies confirming that it can be further developed by oxidative damage when the body is subjected to cold injury: induced brain edema, secondary injury, and apoptosis [2]. Prolonged cold exposure may cause impairment of learning and memory in rats [3], and may lead to functional impairment in the hippocampus region [4]. To date, the harm of hypothermia to the central nervous system has received more and more attention by scholars, however, there is no research reporting whether hypothermia during the period of anesthesia also has a negative influence on the central nervous system during the peak of development. Therefore, we selected 7-day-old newborn rats, and established a low-temperature model induced by general anesthesia. We tested animal behavior, detected apoptosis in hippocampal tissue, and the protein expression in p-ERK (phospho-ERK) and p-CREB (phospho-CREB) to investigate the effects of hypothermia on the long-term learning, memory ability, and apoptosis of hippocampal tissue in rats during the period of anesthesia, and defined the role of p-ERK and p-CREB in them as well.

## Methods

### Animals and treatments

Here, 60 healthy 7-day-old newborn rats were obtained from Guizhou Medical University (Guiyang, Guizhou province, China). The animal models have been approved by Medical Science Ethics Committee of Guizhou Medical University. The animals were weighed 12–16 g. The animals were randomly divided into three groups: Control Group (Group C), Anesthesia Group (Group A), Anesthesia-Hypothermia Group (Group AH), and each group included 20 rats.

The rats in Control Group were intraperitoneally injected 0.1 ml saline. In Anesthesia Group and Anesthesia-Hypothermia Group, the rats were intraperitoneally injected 0.1 ml propofol with anesthetic doses of 25 mg/kg, the 1/2 initial dose was added per each period of 20 min, and anesthesia was maintained for 2 h. The rats in Anesthesia Group were equipped with heating pads to keep their body's temperature at 38–39 °C. However, the temperature of their body in Anesthesia-Hypothermia Group were not

controlled during anesthesia and was allowed to spontaneously decrease.

After the anesthesia recovered, each group randomly involved ten rats, the rats were immediately killed by cervical dislocation, five rats were analyzed by Western blotting to detect expression of p-ERK and p-CREB, five rats were analyzed by flow cytometry assay to detect hippocampal apoptosis rate. The remaining ten rats in each group were kept up to 30 days for the test of their spatial learning and memory ability, five rats were analyzed respectively for the purposes of expression of p-ERK and p-CREB and hippocampal apoptosis rate in each group.

### Morris water maze task

The Morris water maze test was used to examine spatial learning and memory ability of rats [5]. In brief, each rat was forced to finish a swim test every day subsequently from each of the four quadrants per day for 5 days from each of the four quadrants in a circular water pool (colored with black ink, 100 cm in diameter, 50 cm in height) with a 30 cm depth of water and a hidden circular platform (12 cm in diameter) at 2 cm below the water surface. The temperature of water was at the range of 21–23 °C. The animals were forced to swim to find the hidden platform within a maximum time-period of 120 s. The time that each rat spent to find the hidden platform for the first time on the 5th day of the trials (latency) was recorded to evaluate the learning ability of the rats. A video camera on the ceiling was used to record the performances of the rats related to swimming ability.

In addition, 24 h after the last training session, the platform was removed from the tank. The rats were allowed to swim freely for 120 s as a probe trial. The number of times that a rat crosses over the exact location of the platform and the time spent on searching in the safety quadrant within 120 s were recorded as well.

### Flow cytometry assay

Five rats in each group were killed at the given time points. The brains were fully removed and placed on ice, and the hippocampus was rapidly isolated. The hippocampus was cut into pieces, placed in a glass tube, and incubated with 1.5 ml 2.5% EDTA free trypsin at 37 °C for 30 min. Next, the sample was gently blown through a straw to filter into a 1.5 ml Eppendorf tube with a 300 mesh strainer and was centrifuged at 1000 r/min for 5 min. The supernatant was discarded, and the concentration of cells was adjusted to  $1 \times 10^6$ /ml with  $1 \times$  Annexin V Binding Buffer. The sample was then dyed with an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). A FACSAria II Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ,

USA) was used to analyze the cells. FlowJo 10 was used to analyze the apoptotic ratio.

## Western blotting

The Western blotting was performed using antibodies specific for phosphorylated extracellular signal-regulated kinase (p-ERK, 1:700 dilution; GeneTex, Irvine, CA, USA), phosphorylated cyclic AMP response element-binding protein (p-CREB, 1:300 dilution; Santa Cruz, Dallas, TX, USA) and GAPDH (1:10,000; Proteintech Group, Wuhan, Hubei province, China). For Western blotting, the rats were killed and hippocampus tissue proteins were extracted, and the cellular proteins were prepared as described previously. Protein concentrations were determined by Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), and subsequently, the proteins were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein blots were transblotted onto polyvinylidene difluoride membranes (PVDF, Millipore, USA), which were then blocked and incubated with the specific primary antibodies at 4 °C overnight, and horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Immunoreactive protein bands were visualized by a chemiluminescence reaction, and the data were analyzed using the Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

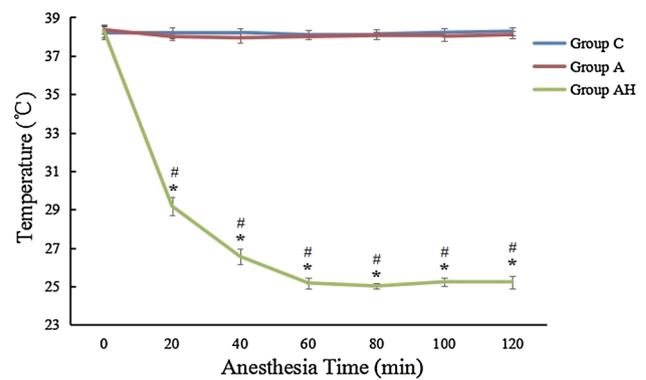
## Statistical analysis

The SPSS 20.0 software (IBM, Armonk, NY, USA) was used for conducting the statistical analyses. Results were expressed as mean  $\pm$  standard deviation (SD) ( $\bar{x} \pm s$ ). The differences between two groups and among more than two groups were analyzed using Tukey's test and analysis of variance (ANOVA), respectively.  $P < 0.05$  was statistically considered significant.

## Results

### Animal body temperature

There was no significant difference in body's temperature before anesthesia among three groups of rats, and there was no statistically significant difference in the body's temperature between Group C and Group A during the whole anesthesia. The body's temperature of Group AH was gradually reduced after beginning anesthesia, and rectal temperature was significantly lower in Group AH than Group C and Group A ( $P < 0.05$ ; Fig. 1).



**Fig. 1** Comparing the temperature in three groups of rats during anesthesia. \* $P < 0.05$  compared with Group C. # $P < 0.05$  compared with Group A

### Animal performance

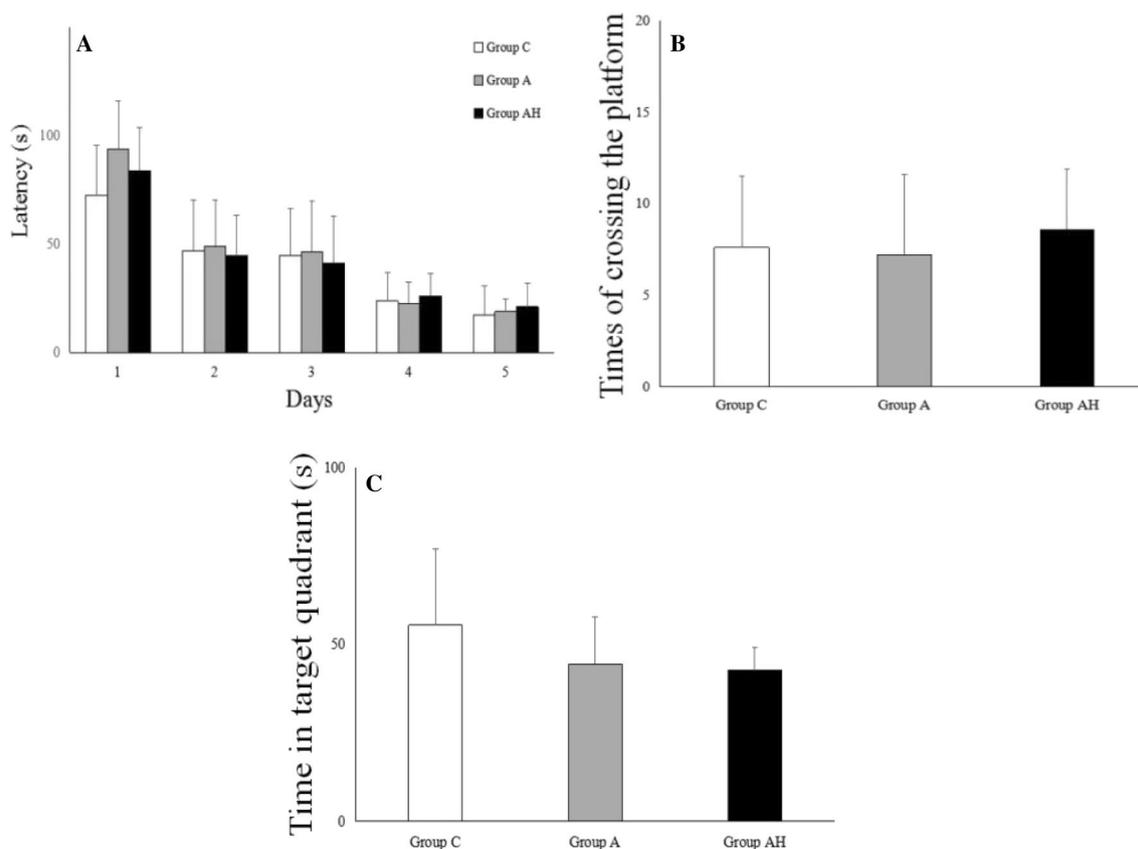
With the increase of training days, the avoidance latency of three groups of rats was gradually shortened. The results of comparison among groups showed that there was no statistically significant difference in avoidance latency. After removing the underwater platform, there was no statistically significant difference in the number of crossing platforms and the time in target quadrant among the three groups of rats (Fig. 2).

### Apoptosis rate of hippocampal tissue

At 7-day-old, cells in Group C and Group A were mainly distributed in the Q4 Quadrant, with only a small number of cells distributed in the Q2 Quadrant and Q3 Quadrant. No significant difference was found on the early and late apoptosis rates of cells between the two groups. Compared with Group C and Group A, the rate of apoptosis in Group AH was increased ( $P < 0.05$ ) (Fig. 3). When the rats grew up to 36-day-old, there was no statistical significance in the rates of cell apoptosis between three groups (Fig. 4).

### Protein expression

At 7-day-old, the protein expression levels of p-ERK and p-CREB in Group AH were significantly lower than Group C and Group A ( $P < 0.05$ ). There was no significant difference in the expression level of the two proteins between Group C and Group A (Fig. 5). No statistically significant difference was found in protein expression level among three groups of rats at 36-day-old (Fig. 6).



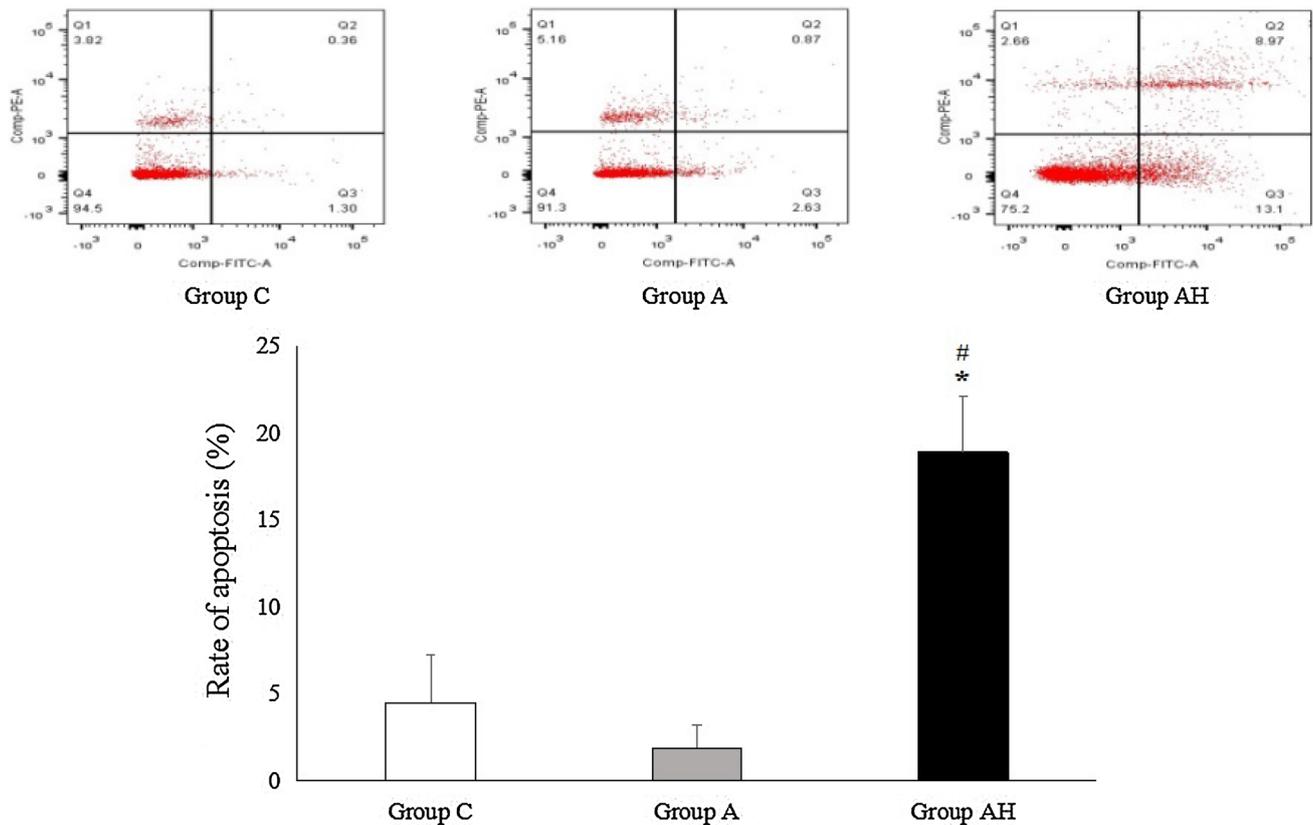
**Fig. 2** When the rats grew up to 30-day-old, we carried out the Morris water maze test. The avoidance latency (a), the duration of crossing platform (b), and the time duration in target quadrant (c) were compared among groups

## Discussion

ERK is an important member of mitogen-activated protein kinase family, playing an important role in regulating the growth, proliferation, differentiation, and survival of nerve cells in the family. As the upstream signaling molecule of CREB, ERK is activated by phosphorylation and is transferred to the nucleus, causing that the CREB to also be phosphorylated and become in an active form [6]. CREB is a protein that regulates gene transcription and can participate in the regulation of downstream gene transcription and protein synthesis, and its active form is a key factor in memory formation and the normal survival of neurons [7]. It has been shown that sevoflurane can damage the learning and memory abilities by inhibiting the expression of p-ERK protein in rats, while it has no significant impact on the content of total ERK protein [8]. Chronic aluminum exposure can also lead to learning and memory impairment, and may inhibit the expression of p-CREB protein, but not the total protein content of CREB [9]. It can be seen that as an active form of the two proteins, its effect on memory does not entirely depend on the total protein contents of ERK and CREB [10]. Therefore, only two types of phosphorylated

proteins were selected in this study, and the protein expression level of p-ERK and p-CREB was detected by Western blotting to detect the molecular mechanism of the influence of hypothermia on learning memory ability or apoptosis rate during anesthesia.

The 7-day-old SD rats were equivalent to 36 weeks of gestational age or full-term newborn of human [11]. At this point, the central nervous system of rodents is at a peak of development. When the central nervous system of a rat is stimulated by external factors, it is very easy to have nerve cell injury, leading to the decrease of memory [12]. Therefore, we herein chose 7-day-old newborn rats. According to the existing literature [13] and the results of the preliminary experiment, when propofol is injected with a 25 mg/kg dose, the newborn rat's reflex can disappear and obtain a state of anesthesia. Therefore, the dosage of propofol was selected for anesthesia and anesthesia lasted for 2 h. In this study, the ambient temperature of the laboratory was about 23 °C, the temperature of neonatal rats could be reduced to about 25 °C without other heat preservation measures, and it may remain at this level until the end of anesthesia. Although it is lethal to humans when the body's temperature drops to 25 °C, the lethal critical temperature of adult rats is 14–20 °C [14].



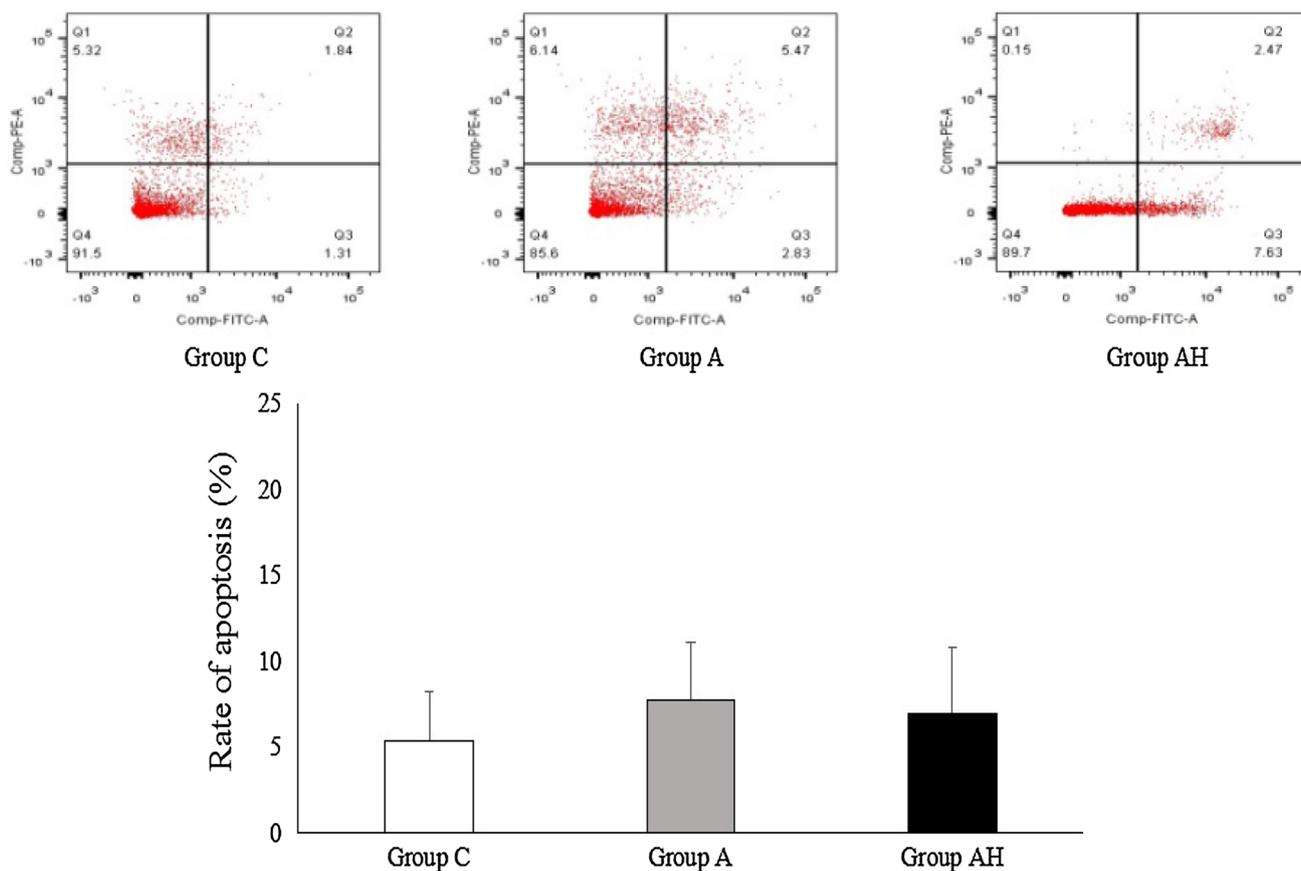
**Fig. 3** Comparing the apoptotic rate in four groups of 7-day-old rats was made by flow cytometry. The apoptosis rate was the sum of early apoptosis and late apoptosis, which was used to indicate the apoptosis of hippocampal tissue. Q1: annexin V-/PI+, necrotic cells; Q2:

annexin V+/PI+, late apoptosis cells; Q3: annexin V+/PI-, early apoptosis cells; Q4: annexin V-/PI-, normal cells. \* $P < 0.05$  as compared with Group C. # $P < 0.05$  as compared with Group A

Besides, newborn rats are stronger than adult rats in term of their ability to tolerate hypothermia [15]. Although it can withstand at a lower temperature, it does not mean that newborn rats can ignore the body's damage due to hypothermia.

In our study, at 7-day-old, the apoptosis rate of hippocampal tissue in AH group was increased. However, in both groups with normal body temperature, no significant apoptosis occurred in the hippocampus. This suggests that it is not an anesthetic, however, hypothermia during anesthesia may be the main cause of the increase in hippocampal tissue apoptosis. In this experiment, the expression of p-ERK and p-CREB in the Group AH at the 7-year-old rats was inhibited. This is consistent with the results of Whittington et al. study [13]. It shows that hypothermia in anesthesia can also induce changes in hippocampal protein expression in rats. Studies have pointed out that when rodents are stimulated by cold, the content of intracranial ATP and ATP enzyme activity decrease [16, 17], thus that may lead to the membrane ion pump dysfunction, thereby resulting in energy metabolism disorders and intracellular calcium overload, and ultimately can induce cell apoptosis. In addition, ATP not only is an energy carrier, but also an important signal molecule, which

can regulate the activation of multiple signaling pathways and ERK [18]. Hence, we speculate that when a rat is in a hypothermic state, the expression of p-ERK in hippocampus is inhibited by the decrease of the molecular content of the upstream signal. ERK is the upstream protein of the CREB. When its expression decreases, the phosphorylation of the Ser133 residue of CREB also weakens, which results in the same decrease of p-CREB expression. In our study, the increase of apoptosis rate in hippocampus of rats may be related to insulin resistance in neurons of rat induced by hypothermia during anesthesia. At the same time, when the nerve cells have insulin resistance, the activation of ERK can be inhibited by decreasing the activity of Ras-ERK signaling pathway [19, 20]. In addition, there is a wide range of gap-connected communication between the nerve cells, which can transmit several kinds of signals and substances. This function is closely associated with cell proliferation, apoptosis, and other life-dependent activities. When hypothermia is attenuated, the communication function is weakened and the nutrients are prevented from passing through the cells. Thus, it could also be one of the causes of nerve cell apoptosis [21]. In this study, it was found that the use of



**Fig. 4** Comparing the apoptotic rate in four groups of 36-day-old rats was made by Flow cytometry

anesthetics had no significant effect on the apoptosis rate and protein expression of hippocampus in the two groups of the same body's temperature, which may be due to lower dosage of propofol injection or shorter duration of anesthesia. Some studies have shown that propofol at anesthetic doses of 25 mg/kg has no significant impact on neuroapoptosis and memory ability of rats [22, 23].

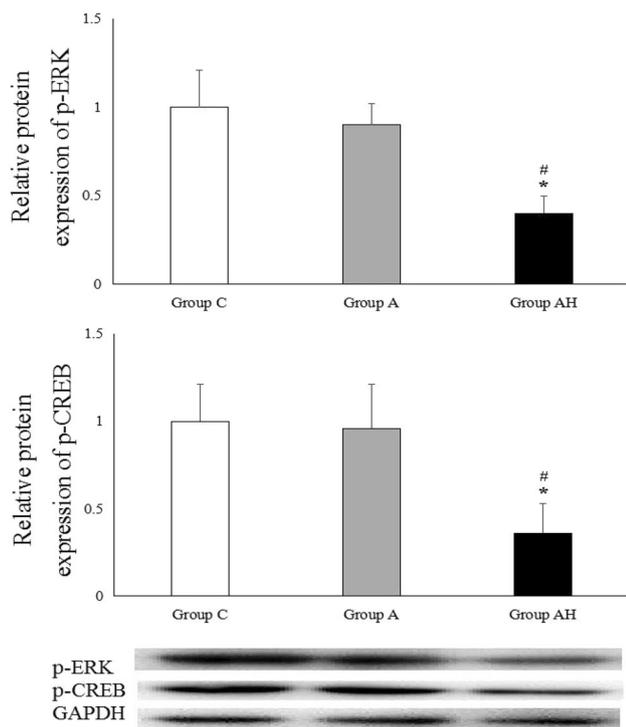
To study the effect of hypothermia on the long-term learning and memory abilities during anesthesia and the possible mechanism of action, the newborn rats were fed to 30-day-old, and animal behavioral studies, and detection of apoptosis rate and protein expression were carried out. In this study, the results of animal behavior, expression of protein, and hippocampal tissue apoptosis showed that there is no significant difference among the three groups of 36-day-old rats. This may be due to the fact that hypothermia has a reversible effect on the central nervous system in a short time-period. In addition, its effects will disappear as body's temperature recovers. Similar reports showed that compared

with normal control group, there was no significant difference in the animal behavior test and the expression of memory-related protein after rats restored from hypothermia during anesthesia to normal temperature during a few days [24].

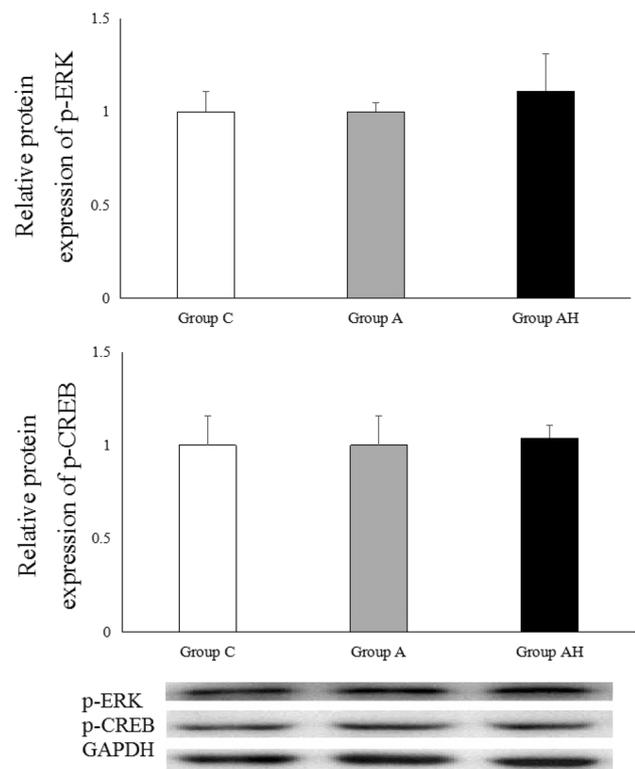
The findings of this study provided some experimental reference data for the clinical anesthesia of infants and children requiring attention to strengthen the heat preservation measures.

## Conclusion

Our findings suggest that hypothermia during anesthesia can increase the apoptosis rate in hippocampus of neonatal rats, whose mechanism may be related to the downward adjustment of p-ERK and p-CREB. However, it has no obvious influence on the long-term learning and memory ability.



**Fig. 5** The expression of p-ERK and p-CREB in 7-day-old rats was determined by Western blotting. \* $P < 0.05$  as compared with Group C. # $P < 0.05$  as compared with Group A



**Fig. 6** The expression of p-ERK and p-CREB in 36-day-old rats was determined by Western blotting

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