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Original Research Article

Mechanism of propofol-induced injury in hippocampal neurons in neonatal rats

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Abstract

Purpose: *To determine the deleterious effect of propofol on hippocampal neurons in developing rats, and the underlying mechanism.*
Methods: *Sixty Wistar rats were randomly assigned to fat emulsion group, low-dose propofol group and high-dose propofol group. Immunohistochemical staining of brain samples was used to detect BrdU-positive (+), nestin+ and GFAP+ cells. The influence of propofol on cellular multiplication, number of neurons in dentate gyrus, activation of astrocytes and microglial activation were determined. The expression levels of Sox2, Akt and ERK signal pathway-associated proteins were assayed by Western blotting.*
Results: *The population of BrdU-positive cells was significantly lower in propofol-exposed rats than in fat emulsion rats, while the levels of Sox2 protein were markedly and dose-dependably lower in propofol-exposed rats than the corresponding levels in fat emulsion rats ($p < 0.05$). The numbers of nestin+ cells, GFAP+ cells and Iba1+ cells in propofol-exposed rats were significantly and concentration-dependently reduced, relative to the corresponding numbers in fat emulsion rats, while p-Akt and p-ERK1/2 in propofol-exposed rats were and dose-dependently reduced, relative to fat emulsion rats ($p < 0.05$).*
Conclusion: *Propofol dose-dependently inhibits the proliferation of hippocampal dentate gyrus stem cells, and suppresses the activation of astrocytes and microglia through regulation of the expressions of Akt/ERK signal pathway-associated proteins. This provides a lead for research and development of new drugs for protecting hippocampal neurons.*

Keywords: *Propofol, Hippocampal neurons, Fat emulsion, Akt/ERK signal pathway, Astrocytes, Microglia, Dentate gyrus stem cell*

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INTRODUCTION

Cognitive function refers to ability to grasp the nature of things and the rules that govern their development [1,2]. At present, learning and memory are regarded as the most crucial aspects of cognitive function, with the hippocampus as the most important brain area that controls them. The development and formation of dentate gyrus in the hippocampus are closely related to multiplication, differentiation, and maturation of neural stem

cells. During the critical period of development, the brain is very prone to interference by external factors, resulting in abnormal brain development and damage to cognitive function.

Every year, thousands of infants and young children undergo treatments involving invasive and imaging-assisted surgery under anesthesia. Clinical general anesthesia is universally applied for surgeries involving infants and children. Recent studies have found that exposure to general anesthesia early at birth may lead to cognitive impairment. However, the relevant mechanisms involved in anesthesia-induced cognitive impairment are poorly understood.

Propofol is an intravenous anesthetic that acts through the activation of the GABA receptor, and it is widely used in clinical anesthesia due to its advantages of rapid effect, complete recovery of patients, and absence of cumulative side effects [3]. Previous studies have shown that propofol exposure and neurological sequelae occurred after surgical anesthesia in infants [4]. It has been reported that propofol anesthesia caused neurodegenerative lesions, neuronal apoptosis, and impairment affecting hippocampal neuronal maturation leading to cognitive dysfunction in neonatal mice, but the relevant mechanisms were not defined [5]. In the present study, 60 healthy 7-day-old Wistar rats were used to investigate the deleterious effect of propofol on hippocampal nerve cells, as well as the mechanisms involved.

EXPERIMENTAL

Animals and ethical approval

Sixty healthy, 7-day-old Wistar rats were selected from the Animal Laboratory, Institute of Field Surgery, Third Military Medical University, Chongqing [license number = SCXK (Chongqing) 2007-0005]. This research was approved by the Animal Ethical Committee of Jingling Hospital (approval no. 20190210), and was conducted according to the guidelines of "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [6].

Animal grouping and treatments

The neonatal rats were randomly assigned to fat emulsion group, low-dose propofol group and high-dose propofol group. Rats in fat emulsion group were intraperitoneally injected with fat emulsion, while those in low-dose and high-dose propofol categories were intraperitoneally injected propofol at doses of 30 and 60 mg/kg body weight, in that order. Rats in the three groups were intraperitoneally injected with equivalent saline volume in place of propofol. After 24 h, all rats were given Brdu solution (50 mg/kg) via the same route. Then, after 2 h, the rats were sacrificed *via* decapitation, and whole brain samples were excised.

Immunohistochemical staining

The whole brain samples were fixed separately in labeled centrifuge tubes for 48 h. The samples were dehydrated with 30 % sucrose solution for 48 h, and put in a refrigerator at -80 ℃ prior to use. Each brain tissue was placed on a sample tray, followed by embedding in OCT and sectioning into brain slices placed in buffer solution to remove the OCT embedding agent. Thereafter, the brain slices were placed in a 24-well plate, washed thrice using PBS, and incubated with H_2O_2 solution for 20 min, followed by washing thrice with PBS and 2-h incubation with 1° antibodies for Brdu, Nestin, GFAP and IBA-1, first at laboratory temperature, and then at 4 ℃ overnight. After rinsing thrice with PBS, the slices were incubated with the corresponding 2° antibody for 2 h at 37 ℃, followed by immunofluorescence and sealing with DPX. Finally, the slides were examined under a light microscope and photographed.

Immunoblotting

Hippocampal protein extraction was done by homogenizing the tissues in a tissue homogenizer, and lysing the homogenate at low temperature. The hippocampal homogenate was centrifuged at 1000 rpm for 10 min in the cold, and protein concentration was estimated with BCA procedure. Following heat denaturation, equal amounts of protein were resolved in SDS-polyacrylamide gel electrophoresis and electro-transferred to PVDF membranes which were subsequently incubated with primary antibodies for SOX2, Akt, p-Akt, Erk1/2 and p-Erk1/2 and beta-actin overnight at 4 °C. This was followed by incubation with secondary antibody conjugated with horse radish peroxidase at room temperature for 2 h. The relative expression of SOX2 was calculated using the software of Carestream instrument, with beta actin as the house keeping gene.

Statistical analysis

All measurement data consistent with normal distribution are presented as mean ± SD. One-way ANOVA was used for comparison amongst multiple groups, while SNK-Q test was used for paired comparisons. The SPSS20.0 was applied

for data processing in this study. Differences were statistically significant at $p < 0.05$.

RESULTS

Dentate gyrus stem cell proliferation in neonatal rats

The numbers of Brdu-positive cells in neonatal rats in propofol-exposed rats were markedly reduced, relative to that in fat emulsion rats. Moreover, the number of Brdu-positive cells in rats given higher level of propofol was markedly decreased, relative to the corresponding number in rats given propofol at the lower level (Table 1 and Figure 1).

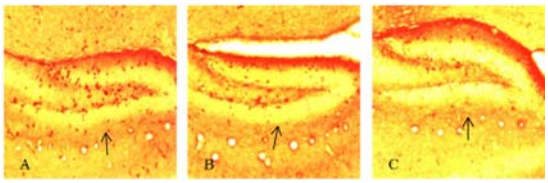

Figure 1: Populations of BrdU+ve cells in hippocampal dentate gyrus of neonatal rats in each group. Images of the BrdU-positive cells in fat emulsion group (A), low-dose propofol group (B), and high-dose group (C)

Table 1: Dentate gyrus stem cell proliferation in each group of neonatal rats

Group	Proliferation (%)
Fat emulsion	108.64±23.64
Low-dose	88.96±20.16[a]
High-dose	67.41±15.34[ab]
F	21.252
P-value	< 0.001

[a]$P < 0.05$, vs fat emulsion rats; [b]$p < 0.05$, vs low propofol dose

SOX2 protein levels in hippocampal dentate gyrus

The SOX2 protein expression levels of neonatal rats were significantly lower in the low-dose and high-dose propofol groups than in fat emulsion group, and SOX2 protein level in the high-dose group was markedly lower than that in the low-dose group ($p < 0.05$; Table 2).

Table 2: Quantitative SOX2 protein expression level in hippocampal dentate gyrus of each group

Group	SOX2 protein level
Fat emulsion	1.00±0.01
Low-dose	0.81±0.03[a]
High-dose	0.62±0.02[ab]
F	1547.142
P-value	<0.001

[a]$P < 0.05$, vs fat emulsion group; [b]$p < 0.05$, vs low propofol dose

Population of Nestin+ cells in rats

There were markedly reduced numbers of Nestin-positive cells in neonatal rats in propofol-exposed rats, relative to the corresponding number in fat emulsion rats, and the number of Nestin-positive cells was markedly lower in high-dose propofol than in low-dose propofol group ($p < 0.05$). These results are shown in Table 3.

Table 3: Population of Nestin+ cells in dentate gyrus of hippocampus of rats in each group

Group	No. of Nestin+ cells
Fat emulsion	125.03±12.56
Low-dose	89.64±10.28[a]
High-dose	71.26±8.79[ab]
F	132.201
P-value	<0.001

[a]$P < 0.05$, vs fat emulsion rats; [b]$p < 0.05$, vs low propofol rats

GFAP+ cells

Table 4 shows that the numbers of GFAP+ cells in neonatal rats in propofol-exposed rats were significantly lower than that in fat emulsion rats, and the number of GFAP+ cells was markedly lower in high-dose propofol rats than in low-dose propofol rats.

Table 4: Number of GFAP- cells in dentate gyrus of hippocampus of rats in each group

Group	GFAP+ cells
Fat emulsion	85.46±9.48
Low-dose	65.39±6.37[a]
High-dose	45.89±5.46[ab]
F	146.794
P-value	＜0.001

[a]$P < 0.05$, vs fat emulsion; [b]$p < 0.05$, vs low propofol

Iba1- cells

The numbers of Iba1-positive cells in neonatal rats in low-dose and high-dose propofol groups were significantly lower than that in fat emulsion group, and the number of Iba1- cells was markedly lower in high-dose propofol group than that in low-dose propofol group ($p < 0.05$, Table 5).

Expression levels of AKT and ERK molecular signaling pathway-related proteins

These proteins were markedly down-regulated in the propofol-exposed neonatal rats, relative to fat emulsion group, but they were markedly lower in

the high-dose propofol-exposed rats. These results are presented in Table 6.

Table 5: Population of Iba1+ cells in dentate gyrus of hippocampus of rats in each group

Group	Tba1(+)
Fat emulsion	84.21±8.41
Low-dose	36.59±4.69[a]
High-dose	28.79±3.15[ab]
F	526.061
P-value	<0.001

[a]$P < 0.05$, vs fat emulsion; [b]$p < 0.05$, vs low propofol

Table 6: Quantitative expression levels of AKT/ERK molecular signaling pathway-associated proteins in each group of rats

Group	Akt	p-Akt	Erk1/2	p-Erk1/2
Fat emulsion	1.02±0.05	1.03±0.04	1.02±0.06	1.00±0.05
Low-dose	0.89±0.05[a]	0.84±0.06[a]	1.05±0.03[a]	0.65±0.04[a]
High-dose	0.93±0.02[ab]	0.62±0.03[ab]	1.09±0.04[ab]	0.41±0.03[ab]
F	49.263	414.102	12.131	1056.404
P-value	<0.001	<0.001	<0.001	<0.001

[a]$P < 0.05$, vs fat emulsion; [b]$p < 0.05$, vs low propofol

DISCUSSION

Every year, tens of thousands of infants and children worldwide undergo real-time surgery or invasive examinations under general anesthesia. Recent animal studies have shown that the use of anesthetics may result in neurotoxic effects on the developing brain, which may lead to long-term neurocognitive impairment [7]. In a study, it was found that the incidence of post-surgery dysfunction in four-year-old children after repeated use of propofol anesthesia was significantly increased, thereby seriously endangering their quality of life [8]. Propofol is widely used as obstetric and pediatric anesthesia in the form of intravenous general anesthesia. This is due to its advantages of fast action, effective controllability of anesthesia state, short maintenance time, rapid awakening, and minimal side effects [9]. In an *in vitro* experiment, it was found that propofol induced apoptosis of brain neurons during the developmental stage, with repeated application increasing the percentage apoptosis of nerve cells, although the mechanism was not elucidated [10].

Mature granulosa cells in the hippocampal granulosa layer are involved in the formation of hippocampal internal circuits, and they participate in cognitive functions such as information combination, short-term memory and spatial exploration [11]. Previous studies have shown that the proliferation of hippocampal neurons during infant development plays an important role in enhancing early and adult cognitive functions [12]. It is known that SOX2, a regulatory factor in neural stem cells, is highly expressed in neural stem cells in hippocampal gyrus [13]. It has been reported that SOX2 maintains the proliferation and self-renewal potential of neural stem cells [14].

In the present study, the population of Brdu-positive cells and SOX2 protein expression levels were determined, and the effect of propofol on the proliferation of hippocampal neurons were investigated. It was found that the number of Brdu-positive cells and SOX2 protein expression levels in neonatal rats in the propofol-exposed rats were markedly decreased, relative to the corresponding values in fat emulsion group. Moreover, the number of Brdu-positive cells and SOX2 protein levels were markedly lower in rats given higher level of propofol. These results suggest that propofol dose-reliantly suppressed the growth of hippocampal neurons.

The growth and development of hippocampal stem cells are highly influenced by the surrounding microenvironment, i.e. surrounding granular neurons, astrocytes, blood vessels and extracellular matrix [15]. Nestin, an intermediate filament type protein, is a characteristic marker of neural stem cells [16]. In this study, it was found that the number of Nestin-positive cells, GFAP-positive cells and Iba1-positive cells in neonatal rats in propofol-treated rats were markedly reduced, relative to values in fat emulsion group. Moreover, the populations of these cells were markedly lower in neonatal rats in high-dose propofol than that in low-dose propofol group. These results suggest that propofol induced microenvironmental changes and ultimately led to neurocognitive toxicity by inhibiting the viability of glial cells in the hippocampus. This finding is consistent with the results reported in an earlier study [17].

The PI3K/Akt is widely present in various cell types, and it is involved in cell growth, proliferation and differentiation. It has been demonstrated that PI3K/Akt is implicated in the etiology of neurological illnesses like AD and ALS [18]. The MAPK/Erk signaling pathway is a neurotrophic protein signaling route. It regulates the proliferation, differentiation, migration and survival of neural stem cells in the developing brain [19]. In the present study, the expressions of p-Akt and P-ERK1/2-associated proteins in neonatal rats in propofol-exposed rats were reduced, relative to levels in fat emulsion group.

Moreover, the expression levels of these proteins were markedly lower in neonatal rats in high-dose group than in low-dose propofol group. These results suggest that the propofol-induced damage to hippocampal neurons during development may be related to the regulation of expressions of proteins associated with the AKT/ERK molecular signaling pathway.

CONCLUSION

Propofol dose-dependently inhibits the proliferation of hippocampal dentate gyrus stem cells and the activation of astrocytes and microglia, and reduces their viability via regulation of the expressions of AKT/ERK molecular signaling pathway-associated proteins. Thus, these findings provide a potential lead for the development of new drugs for protecting hippocampal neurons.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jusheng Wu designed the study, supervised the data collection, analyzed the data and reviewed the draft manuscript. Tao Zhang interpreted the data and prepared the manuscript for publication. Tao Zhang and Jusheng Wu contributed equally to this work and should be considered co-first authors.

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