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Original Article

Long-term action of propofol on cognitive function and hippocampal neuroapoptosis in neonatal rats

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Abstract: Propofol is a short-acting anesthetic and generally is utilized for the induction and maintenance of anesthesia in pediatrics and adults. However, whether repeated use of propofol affects long-term cognitive function remains unclear. This study investigated the effects of propofol on cognitive function and hippocampal neuroapoptosis in neonatal rat. A total of 112 male newborn 7-day old Sprague-Dawley rats were randomly divided into 8 groups (n=14 rats per group) and intraperitoneally injected either with saline or propofol at 50, 100, and 150 mg/kg/day for 5 consecutive days. Four non-surgical groups were assigned as Con1, P50, P100, and P150. Four surgical groups were received an appendicectomy under propofol anesthesia and assigned as Con2, SP50, SP100, SP150. After 2 months raising, cognitive function, hippocampal neuroapoptosis, and intracephalic inflammatory cytokines were evaluated. There was no obvious effect on the cognitive function and neuroapoptosis after repeated use of propofol at a low dose for 5 days, whereas repeated use of propofol at a middle/high dose significantly increase the expression of apoptotic factors (caspase-3 and Bax), pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), and impair the cognitive function. Thus, our data suggest that repeated use of propofol at a low dose may be safe during the period of brain growth spurt. Using propofol at a recommended or higher dose for anaesthesia may lead to the cognitive defects, attributed to hippocampal neuroapoptosis and the overexpression of pro-inflammatory cytokines in the brain.

Keywords: Propofol, neonatal rat, hippocampus, neuroapoptosis, inflammatory cytokines, cognitive dysfunction

Introduction

Propofol, an intravenous anesthetic, is a short-acting agent. Because of its fast induction and short recovery time, propofol is generally used for the induction and maintenance of anesthesia in adults [1, 2]. It is also widely used for the induction and maintenance of anesthesia in pediatrics and obstetrics due to its short context-sensitive half-life along with rapid reawakening and its antiemetic properties. Previous studies have shown that propofol has several mechanisms of action by affecting γ-aminobutyric acid (GABA)-A receptor and N-methyl-D aspartic acid (NMDA) receptor [3, 4] and property as a sodium channel blocker [5]. Furthermore, a binding site of propofol was identified on a transmembrane domain of the beta subunit of the (GABA)-A receptor in the brain [6]. Because the nerve system is extremely sensitive to inner and outer environment change [7], the certain dose of propofol may induce neurotoxicity [8]. Indeed, the propofol-induced toxicity in embryonic neural stem cells has been demonstrated [9]. Recent studies have also been shown that anesthetics can induce neuroapoptosis [10, 11] and may impair children's learning memory and cognitive functions during the period of brain growth spurt [12]. However, the impact of propofol in the immature brain repeatedly exposed to anesthetics and the potential mechanisms of the deleterious effects induced by propofol anesthesia remain obscure [13]. Since some newborns and infants inevitably have to receive surgery and anesthesia for assured disease and since the end phase of gestation and early phase of postnatal are important periods for learning memory and cognitive function during the development of the brain, it is necessary to

investigate the potential mechanisms of the deleterious effects of propofol *in vivo* and *in vitro*. The present study intends to observe the long-term action of propofol on cognitive function in the immature hippocampal neuron and to explore its underlying mechanism by repeatedly exposing propofol at different doses to neonatal rats.

Materials and methods

Experimental animals

A total of one hundred and twelve male newborn 7-day (7 d) old Sprague-Dawley (SD) rats were provided by Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China). The animal rooms were SPF grade and kept neat and uncluttered. Animals were maintained 5 per cage in suckling period raised by maternal rats under a 12/12 h light/dark cycle at 20-25°C and 58% humidity and received food and water ad libitum. All the experimental protocols were performed in compliance with the Institutional Animals Care and Use Committee of Fudan University.

Groups and propofol administration

Propofol was obtained from AstraZeneca (Diprivan, 20 ml, AstraZeneca UK Limited, Cheshire, UK). Neonatal 7 d SD rats were randomly divided into 8 groups (4 non-surgical groups and 4 appendicectomy groups; n=14 per group) and intraperitoneally injected either with saline or propofol at different doses for 5 consecutive days. Four non-surgical groups were Con1 (control group 1 with saline injection), P50 (propofol at 50 mg/kg/day), P100 (propofol at 100 mg/kg/day), P150 (propofol at 150 mg/kg/day). Animals in four surgical groups were received an appendicectomy under 100 mg/kg propofol anesthesia on the last day of treatment and divided as Con2 (control group 2 with saline injection), SP50 (surgical group with propofol at 50 mg/kg/day), SP100 (surgical group with propofol at 100 mg/kg/day), SP150 (surgical group with propofol at 150 mg/kg/day).

Behavioral test

After 2 months raising, rats in 8 groups received Morris water maze behavioral test which included two parts: the positioned sailing test and space exploration test. Positioned sailing test was made with the Morris water maze task based on the method described previously [14, 15]. The water maze was a circular swimming pool (100 cm in diameter, 30 cm in depth) with black ABS engineering plastic walls. The pool was divided into four equal quadrants and a white escape platform was placed in the center of the target quadrant (1.5 cm below the water level). The temperature in floor and water was maintained at 24-26°C. The experimental conditions such as light and room temperature were constant throughout the experiments. After training that consisted of the place navigation test 4 times a day (training time: 60 s each) with 1 hour interval for 5 consecutive days, the exploration experiment was performed by recording the latent period of finding the platform and the time of crossing the platform by rats on the 6th day. The swimming paths of the rats were recorded for 60 s and the animal activity was videotaped by a video-camera mounted above the center of the pool and analyzed with Behavioral Analyzing System provided by Shanghai Duoyi Science Information Co. Ltd (Shanghai, China).

Immunohistochemical staining of caspase-3 and Bax

After behavioral test, the animals were decapitated. The hippocampal CA3 area was separated and dissected into small blocks which were fixed in 4% paraformaldehyde and embedded in paraffin for sectioning. After deparaffinization and antigen retrieval, the tissue sections (4 μm thick) were blocked in a blocking solution (1% goat serum with 0.3% Triton 100, Sigma, USA) for 10 min, followed by incubation with rabbit anti-caspase 3 or anti-Bax antibody (1:200 dilution, Abcam, Cambridge, UK) overnight at 4°C. After wash with PBS for 3 times (5 min each), the sections were incubated with HRP-IgG secondary antibody for 30 min at 37°C. After washed with PBS, the sections were added with DAB coloration reagent. The negative control was conducted by replacing the primary antibody with PBS. Sepia or claybank color appeared in the cytoplasm, cell nucleus, or cytomembrane was considered as a positive cell. Five fields were randomly chosen with a high objective (×40). At least 200 cells per field were counted and an average percentage of positive cells were calculated.

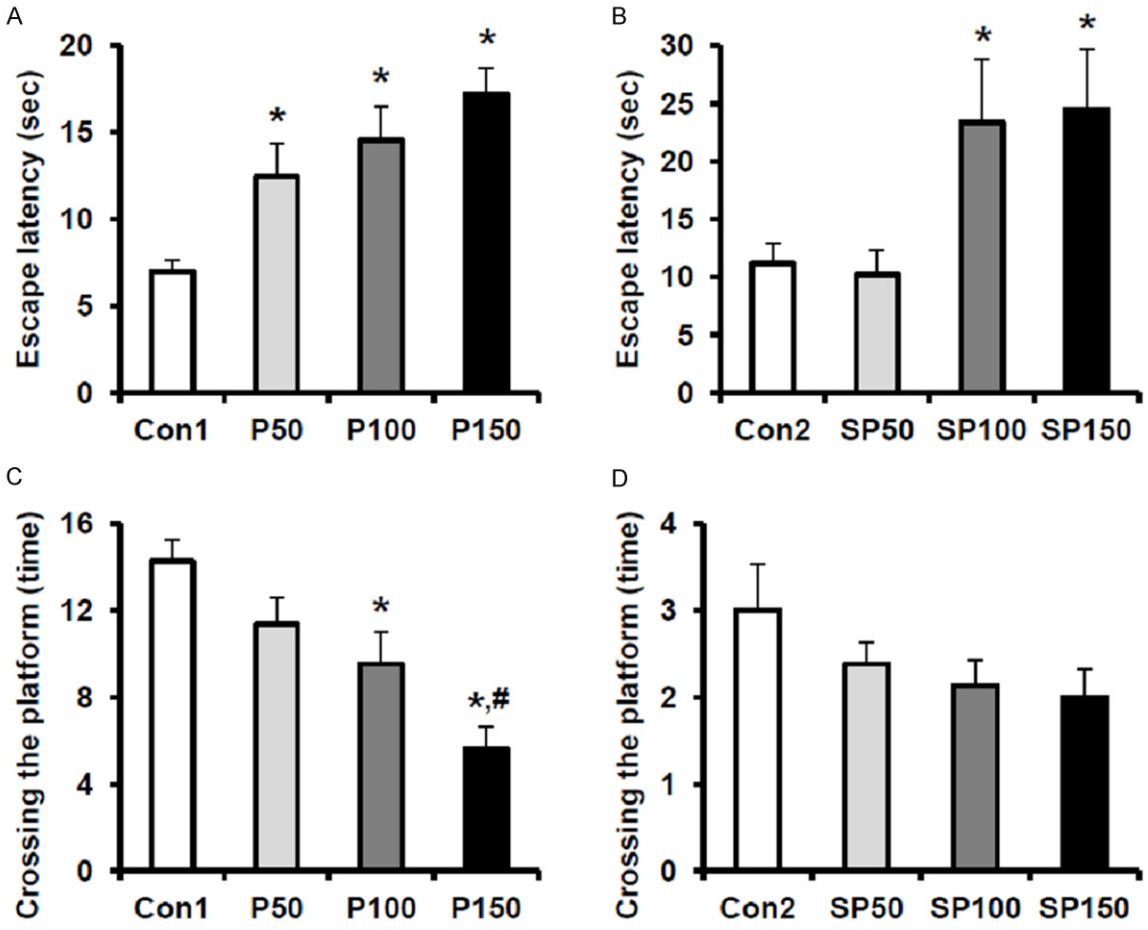

Figure 1. Influence of propofol on behavior change in rats. The escape latency was examined in non-surgical groups (A) and surgical groups (B). The time of crossing the platform was measured in non-surgical groups (C) and surgical groups (D). Data present as mean ± SEM (n=8 per group). Con1 or Con2, control group 1 or 2; P50 or SP50, 50 mg propofol; P100 or SP100, 100 mg propofol; P150 or SP150, 150 mg propofol. $^{*}P<0.05$ compared with Con1 or Con2; $^{\#}P<0.05$ compared with P50.

Measurement of cytokines

The levels of the cytokine IL-1β, TNF-α, IL-6, IL-10 of the brain were measured by Bio-Plex Suspension Assay using the Bio-Plex 200 System with Bio-Plex Pro Reagent Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Briefly, the tissue of the hippocampal CA3 was homogenized with PBS. After spinning the crude homogenate, the supernatant was collected. Each sample (25 μl) was then mixed with 100 μl assay buffer and placed into the well of a plate. After adding 25 μl magnetic beads, the mixture was incubated for 16 h on swing bed at 4°C. After wash 3 times, a detection antibody (50 μl/well) was added and incubated for 1 hour at room temperature. After wash, 50 μl Streptavidin-Phycoerythrin was added and incubated for 30 min at room temperature. Finally, after 100 μl sheath fluid was added into each well, the signals were detected and analyzed by MFI (mean of fluorescence intensity).

Statistical analysis

The data were analyzed using SPSS 11.5 software. All data are presented as mean ± standard error of the mean (SEM). The one-way ANOVA was used for a multiple-group comparison. A P value <0.05 denotes a significant difference.

Results

Influence of propofol on long-term spatial learning memory

We first examined rats in the positioned sailing test. In non-surgical groups, the escape latency

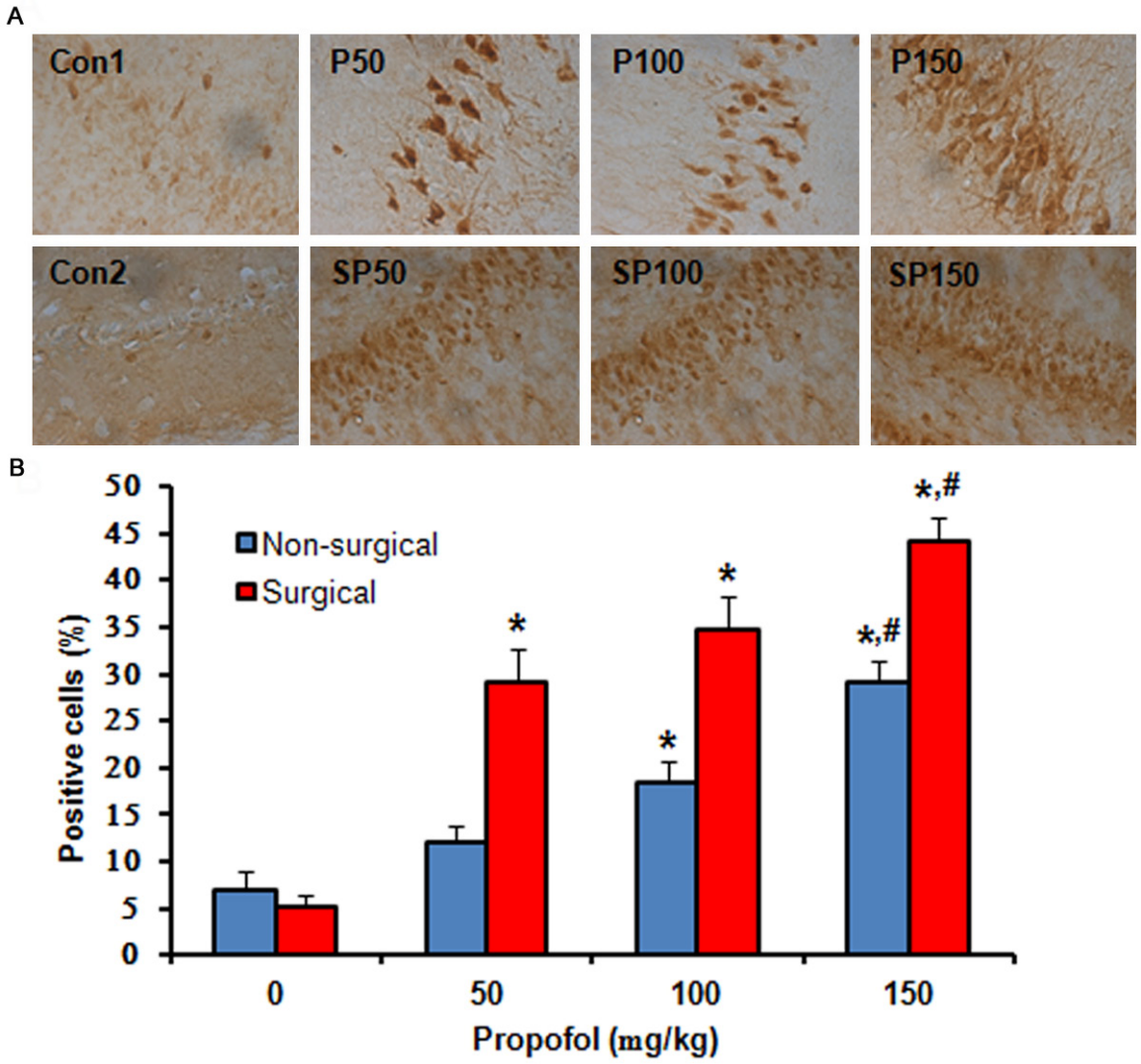

Figure 2. Immunohistochemistry staining of caspase-3 in the brain of rat. The expression of caspase-3 was detected in the hippocampal CA3 area by immunohistochemical staining. Representative images are shown (A). Top panel, non-surgical groups; bottom panel, appendicectomy groups. Original amplification ×400. Quantitative assessment of caspase-3 in non-surgical groups and surgical groups was shown (B). Data present as mean ± SEM (n=6 per group). Con1 or Con2, control group 1 or 2; P50 or SP50, 50 mg propofol; P100 or SP100, 100 mg propofol; P150 or SP150, 150 mg propofol. $^{*}P<0.05$ compared with Con1 or Con2; $^{\#}P<0.05$ compared with P50 or SP50.

was significantly increased in P50, P100 and P150 groups compared with Con1 and showed as in a dose-dependent manner (**Figure 1A**, $P<0.05$). In surgical groups, the escape latency in SP100 and SP150 groups was significantly increased compared with Con2 or SP50 group (**Figure 1B**, $P<0.05$). However, there was no difference of escape latency between SP50 and Con2 groups ($P>0.05$).

Next, we examined rats in the space exploration test. The frequency (number of time) of crossing the platform in non-surgical groups and surgical groups was measured. Compared with Con1 group, there was no obvious difference of the time of crossing the platform between P50 group and Con1 group (**Figure 1C**, $P>0.05$). The time of crossing the platform in P100 and P150 groups was significantly decreased compared with Con1 group ($P<0.05$). The significance was also observed between P50 and P150. In surgical groups, however, there was no significant difference of the time of crossing the platform between groups (**Figure 1D**, $P>0.05$). Conversely, the frequency of the time of crossing the platform was significantly different between non-surgical and surgical groups (Con1 *vs.* Con2, $P<0.05$), indi-

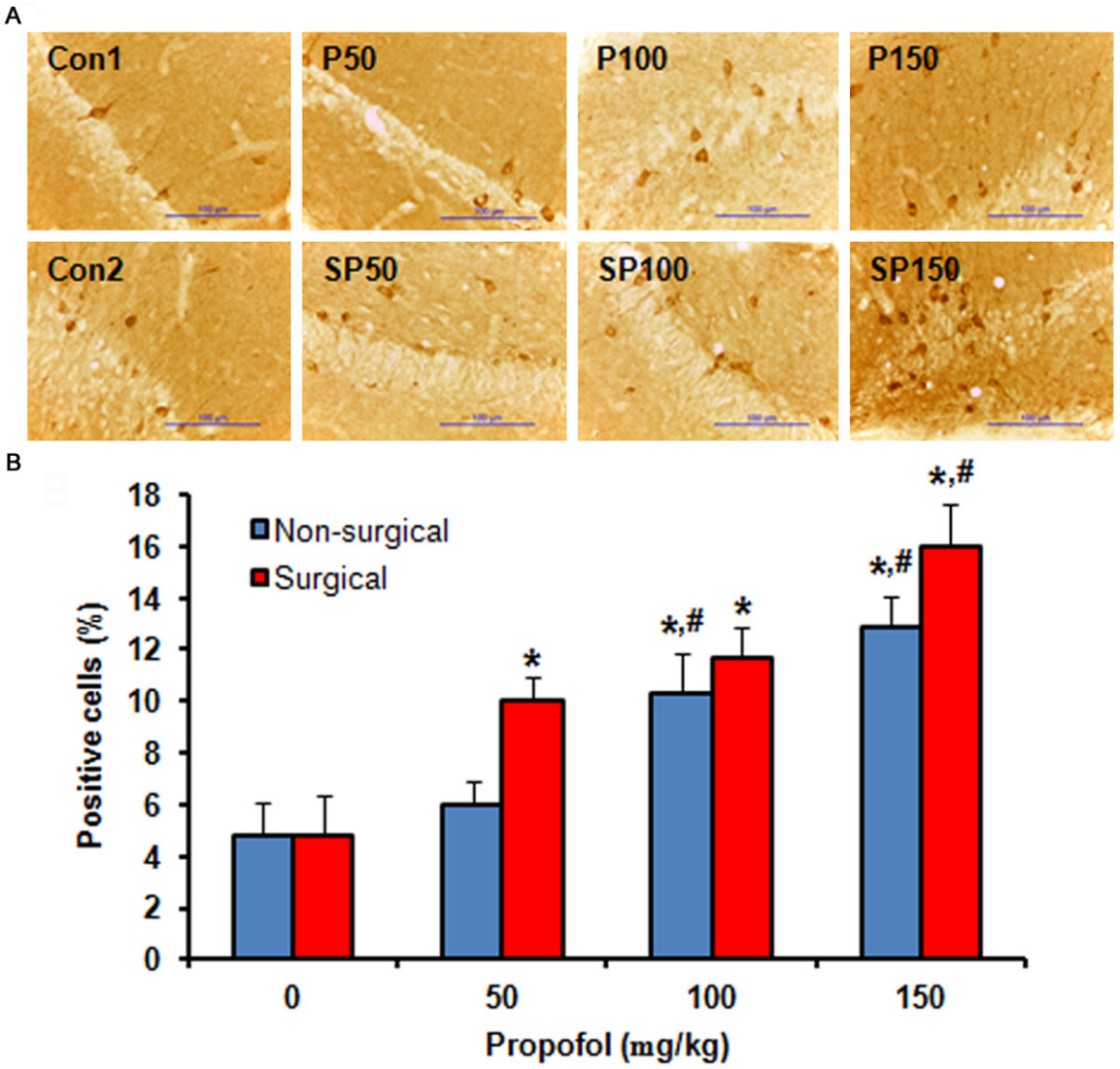

Figure 3. Immunohistochemistry staining of Bax in rat brain. The expression of Bax was detected in the hippocampal CA3 area by immunohistochemical staining. Representative images are shown (A). Top panel, non-surgical groups; bottom panel, appendicectomy groups. Scale bar, 100 μm. Quantitative assessment of Bax in non-surgical groups and appendicectomy groups was shown (B). Data present as mean ± SEM (n=6 per group). Con1 or Con2, control group 1 or 2; P50 or SP50, 50 mg propofol; P100 or SP100, 100 mg propofol; P150 or SP150, 150 mg propofol. $^{*}P<0.05$ compared with Con1 or Con2; $^{\#}P<0.05$ compared with P50 or SP50.

cating that propofol used as an anaesthetic affected the leaning memory in a surgical group (Con2).

Induction of neuroapoptosis by propofol in hippocampal CA3 area

Neonate rats were administrated with either saline (control group) or propofol (P or SP group) at different dose every day for 5 consecutive days. The neuroapoptosis in hippocampal CA3 area of rat was measured by the detection of caspase-3 and Bax, 2 apoptotic factors, by immunohistochemistry. In non-surgical groups, propofol at 50 mg/kg (P50) did not increase caspase-3-positive apoptotic cells, whereas it at 100 and 150 mg/kg did (**Figure 2A** top panel and **Figure 2B**, $P<0.05$). In surgical groups, repeatedly administrating propofol at 3 indicated doses significantly increased the expression of caspase-3 compared with Con2 group and the increase of apoptosis was dose-dependent (**Figure 2A** bottom panel and **Figure 2B**, $P<0.05$).

Furthermore, in non-surgical groups, propofol at 50 mg/kg (P50) did not increase Bax expression compared with Con1 group, whereas

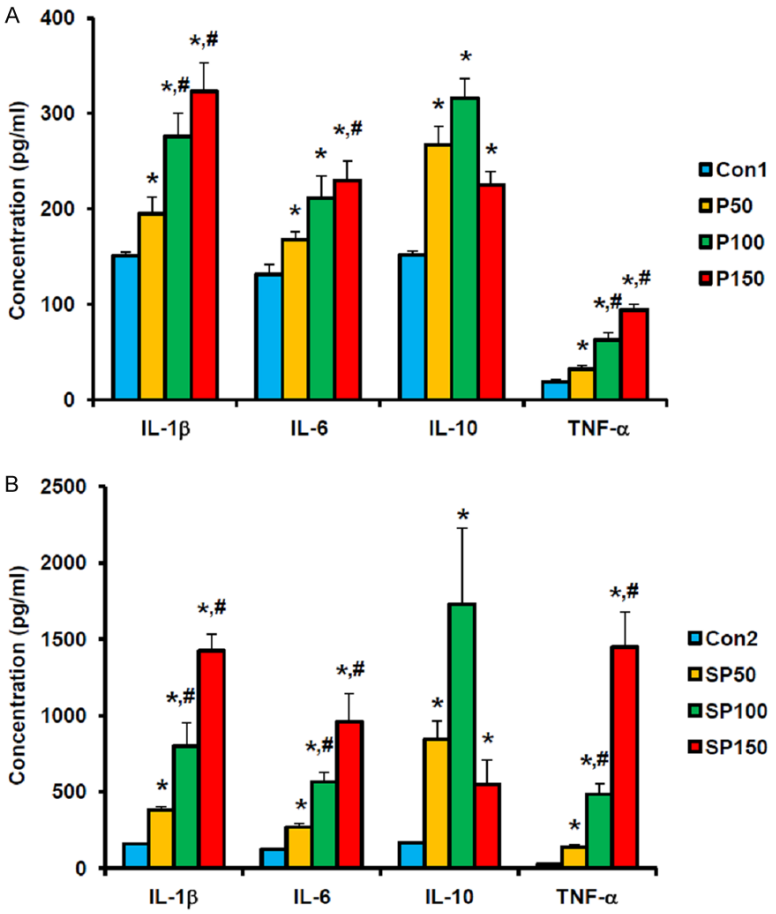

Figure 4. Effect of propofol on inflammatory cytokines in the hippocampal CA3 area of rat. The pro-inflammatory cytokines IL-1β, IL-6, and TNF-α and anti-inflammatory cytokine IL-10 were detected in non-surgical groups (A) and appendicectomy groups (B) using Bio-Plex Suspension Assay. Data present as mean ± SEM (n=8 per group). Con1 or Con2, control group 1 or 2; P50 or SP50, 50 mg propofol; P100 or SP100, 100 mg propofol; P150 or SP150, 150 mg propofol. *P<0.05 compared with Con1 or Con2; #P<0.05 compared with P50 or SP50.

it at 100 and 150 mg/kg did (**Figure 3A** top panel and **Figure 3B**, P<0.05). In surgical groups, repeatedly administrating propofol at 3 indicated doses significantly increased Bax expression compared with Con2 group and the increase of apoptosis was dose-dependent (**Figure 3A** bottom panel and **Figure 3B**, P< 0.05). These data indicated that propofol at middle/high dose indeed induced hippocampal neuroapoptosis.

Effect of propofol on inflammatory cytokines

In non-surgical groups, propofol significantly increased pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (**Figure 4A**, P<0.05, *vs.* Con1) in a dose-dependent manner. Propofol increased anti-inflammatory cytokine IL-10, but the highest level of IL-10 was observed at P100.

In surgical groups, the expression of IL-1β, IL-6, IL-10, and TNF-α was significantly increased in propofol-treated groups compared with Con2 group (P<0.05). Compared with SP50 group, IL-1β, IL-6, and TNF-α, but not IL-10, were significantly increased in SP100 and SP150 groups (P<0.05).

Discussion

Current study evaluated the effect of propofol on hippocampal neuroapoptosis, the expression of inflammatory cytokines in the brain, and long-term cognitive function during the period of brain growth spurt in rats. In general, our data provided evidence that propofol at a low dose (50 mg/kg) with a single administration daily repeated for 5 consecutive days was safe in rat, whereas propofol at a middle/high dose impaired cognitive function during the development of brain.

How much and how long propofol should be used as an anaesthetic was critical. Clinically, the dosage of anaesthetics by intraperitoneal injection is 2 times higher than those by intravenous injection. The efficacy of 50 mg/kg propofol by intraperitoneal injection was generally similar to it by intravenous injection at 1-2 mg/kg [16]. In children, the amount of propofol for the induction of anesthesia is 2-3 mg/kg, which was tantamount to 100 mg/kg propofol by intraperitoneal injection in rats. Current study used 3 different doses of propofol and demonstrated that a middle/high dose of anaesthetic influenced cognitive function, especially related with post-surgery cognitive dysfunction.

It has been shown that anesthesia with 3% sevoflurane for 3 days induced cognitive im-

pairment and neuroinflammation in young but not in adult mice [17]. Generally, propofol at a low dose did not influence on long-term spatial learning memory, whereas propofol at a middle/high dose impaired cognitive function as shown in our Morris water maze test. This cognitive defect complied with hippocampal neuroapoptosis and the overexpression of pro-inflammatory cytokines. In non-surgical group, we found that propofol at 50 mg/kg did not induce hippocampal neuroapoptosis. However, hippocampal neuroapoptosis was observed after increasing a dose. We also found that the induction of neuroapoptosis was dose-dependent in the rats that received appendicectomy under propofol anesthesia. After the intraperitoneal injection of 50 mg/kg propofol, the rat showed its sleeping status which lasted for 1 h. Although the surviving rate of neonatal rat was comparatively high, in some operations there may not be enough time to maintain disappearing rig-hting reflex while satisfying anesthesia requirement. In general, the induction and maintenance of anaesthesia with propofol at a dose lower than a recommended dose can satisfy short-time operation requirement. It might induce the hippocampal neuroapoptosis in some cases, but would not affect long-term spatial learning memory. As shown in this study, using a recommended dosage or higher for anaesthesia increased hippocampal neuroapoptosis, leading to the impairment of long-term spatial learning ability.

Some anesthetics exhibit neurotoxicity in the immature but not mature brains. The mechanism of propofol inducing neuroapoptosis in the immature brain is still not clear. In inhalation anesthetics, the excitotoxicity injury of the immature brain is through the activation of GABA receptor [18] and the activation of p75 neurotrophin receptor [19]. The down-regulation of nerve growth factor (NGF) expression in the cortex and thalamus and a decrease of phosphorylated Akt were observed at 1 and 24 h post-propofol treatment [20]. Furthermore, the extrinsic apoptotic pathway was induced by the overexpression of tumor necrosis factor (TNF) which led to the activation of caspase-3 in the developing rats [20]. General anesthetics results in a reduction in inhibitory synaptic transmission, an increase in excitatory synaptic transmission, and an increase in the amplitude of T-type calcium currents in neurons of the nucleus reticularis thalami [21], possibly leading to neuronal degeneration and long-term cognitive dysfunction.

In the present study, propofol changed the expression level of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α and anti-inflammatory cytokine IL-10 indicating that neuroinflammation may exist. It must be point out that the effectof propofol on neuroinflammation depending on the dose and time of anaesthesia. Previous report showed that propofol anaesthesia in Wistar rat does not have a major impact onthe expression profiles of pro-inflammatory cytokines in the developing central nervous system during the brain growth spurt [22], which was different with our observation. There were two major points in difference. First, their Wistar rats received 6-h propofol anaesthesia at postnatal day 10 or 20 and pro-inflammatory cytokines were examined subsequently, whereas our SD rats received propofol daily for 5 consecutive days started at postnatal day 7 and pro-inflammatory cytokines were examined 2 months later at day 67. Second, they used a quantitative real-time PCR to detect IL-1β and IL-6 mRNA, whereas we used Bio-Plex to detect IL-1β, IL-6, and TNF-α protein. It seems that the timing after anaesthesia is critical for the examination of the effect of propofol since no changes of IL-1β and IL-6 at mRNA level were detected following drug exposure at early developmental stages (<20 days). According to the brain development extended [23], our 67-day SD rat is tantamount to preschoolers in human. These data suggest that an early exposure of anesthetics to neonates can cause the long-term damage of brain, while inflammation was induced.

Current study was to measure the neuroapoptosis and neuroinflammation in hippocampal CA3 region at post-anaesthesia 2 months later. We cannot exclude that these changes might peak in the different time period of brain development and also occur in other brain regions. It has been shown that brain regional vulnerability to neuroapoptosis induced by anaesthetics shifts with age at exposure and extends into adulthood for some regions [24].

In summary, neuroapoptosis and inflammatory response are involved in cognitive dysfunction caused by anesthetics in the developing brain. Present study suggests that repeated use of a low dose propofol may be safe during the brain growth spurt, whereas using recommended or

higher dose propofol for anaesthesia can induce hippocampal neuroapoptosis and neuroinflammation, ultimately leading to the cognitive defects.

Disclosure of conflict of interest

None.

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