

# Effects of Dexmedetomidine on the Expression of Cytochrome C Oxidase in Neonatal Mouse Primary Hippocampal Neurons Cultured in Propofol

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## Yang *et al.*: Expression of Cytochrome C in Primary Hippocampal Neurons

To observe the effects of propofol and dexmedetomidine on the expression of cytochrome c oxidase in primary hippocampal neurons isolated from neonatal mice. C57 neonatal mice were sacrificed within 24 h of birth and hippocampal tissue was collected. Primary hippocampal neurons were cultured *in vitro* and then randomly divided into a control group (0  $\mu$ M propofol and 0  $\mu$ M dexmedetomidine), propofol group (20  $\mu$ M propofol and 0  $\mu$ M dexmedetomidine) and propofol+dexmedetomidine group (20  $\mu$ M propofol and 10  $\mu$ M dexmedetomidine). The neurons were incubated for 1 h. Neuron morphology was observed under a light microscope. The expression of cytochrome c protein was detected semiquantitatively by Western blot and immunofluorescence staining. Compared with those in the control group, the primary hippocampal neurons in the propofol group were morphologically degenerated, with sparse axons and dendrites; the expression of cytochrome c protein was significantly higher in the propofol group ( $p < 0.0001$ ). The morphology of primary hippocampal neurons in the propofol+dexmedetomidine group was similar to that of primary hippocampal neurons in the control group, with plump and bright cell bodies and thickened neurites intertwined in a neuron network; additionally, cytochrome c protein expression was significantly lower in the hippocampal neurons in the propofol+dexmedetomidine group than in the hippocampal neurons in propofol group ( $p < 0.0001$ ). Dexmedetomidine reversed propofol induced injury and morphological abnormalities of primary hippocampal neurons. The mechanism may be related to the downregulation of cytochrome c protein expression.

**Key words:** Dexmedetomidine, propofol, primary hippocampal neurons, cytochrome C

Propofol is the most commonly used intravenous anesthesia drug in clinical practice and is widely used for the induction and maintenance of anesthesia in children and adults. Studies have shown that exposure of the developing brain to general anesthesia with propofol can cause damage to the central nervous system and inhibit the neurogenesis of the hippocampus, thereby affecting the learning and memory functions of infants and young children<sup>[1,2]</sup>. Dexmedetomidine is an alpha ( $\alpha$ )-adrenergic receptor agonist that acts on the presynaptic  $\alpha_2$  receptors of noradrenergic nerves, reducing the release of norepinephrine when the receptors are excited, resulting in sedation and analgesic effects<sup>[3]</sup>. Our preliminary animal studies<sup>[4]</sup> indicated that dexmedetomidine was able to prevent postoperative spatial memory deficits induced by surgery under propofol anesthesia through neuronal protection

and anti-inflammatory effects. The mechanism may be related to the protection of mitochondrial electron transport chain function. Cytochrome c is an important water-soluble mitochondrial protein that exists in the inner mitochondrial membrane and its main function is to transport electrons to the mitochondrial oxidative respiratory chain complex<sup>[5]</sup>. The aim of this study was to observe the effect of dexmedetomidine on mitochondrial protein cytochrome c in neonatal mouse primary hippocampal neurons incubated with propofol. The results of this study provide an experimental basis for the clinical application of

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dexmedetomidine for the prevention of the developmental neurotoxicity of propofol. C57 neonatal mice within 24 h of birth were used. The animals were clean grade, weighed 4.0 to 6.0 g and were provided by the Institute of Biophysics, Chinese Academy of Sciences. Propofol (500 mg/50 ml) was purchased from Xi'an Libang Pharmaceutical Co., Ltd. Dexmedetomidine (100 µg/1 ml) was purchased from Yangtze River Pharmaceutical Group Co., Ltd. Dulbecco's Modified Eagle's Medium (DMEM), Opti-Minimal Essential Medium (MEM), neurobasal medium and GlutaMAX medium additives were purchased from Gibco, USA. Fetal Bovine Serum (FBS) and anti-fluorescence quenching mounting medium were purchased from Invitrogen, Canada. Trypsin (0.125 %) and poly-L-lysine were purchased from Sigma Aldrich, USA. Anti-cytochrome c antibody and Alexa Fluor® 488 goat anti-rabbit reagent were purchased from Abcam Company. Normal goat serum (5 %) was purchased from Jackson Immuno Research Labs. The day before the material was collected; culture dishes or culture flasks were coated with 100 µg/ml poly-D-ornithine and placed in a 37° incubator overnight. After cleaning the dissection instruments, they were sterilized. H buffer was prepared. 24 h after birth, the external surface of the mouse was sterilized with 70 % ethanol and brain tissue was quickly harvested after decapitation with ophthalmic scissors. Hippocampal tissue was isolated under a stereo dissecting microscope, soaked in fresh H buffer, chopped with Venus scissors and then transferred to a petri dish. During digestion, the solution was gently shaken 3 times. After 30 min of digestion, 1 ml of precooled DMEM containing 10 % FBS was added to stop the digestion; after 2 min, the medium was removed and the process was repeated. After the digestion was terminated, 1 ml of prechilled DMEM containing 10 % FBS and 10 µl of 10 mg/ml DNase I (Roche) were added. The cells were subjected to repeat pipetting at a constant speed with a 1 ml pipette and then passed through a 200-mesh cell sieve; 0.9 ml of the cell suspension was collected and the cells were counted. Prewarmed DMEM containing 10 % FBS (1 ml) was used to resuspend and dilute the cells; the cells were seeded in a coated petri dish and then incubated for 1 h in an incubator at a constant temperature of 37° and 5 % CO<sub>2</sub>. The medium was replaced with prewarmed neurobasal medium

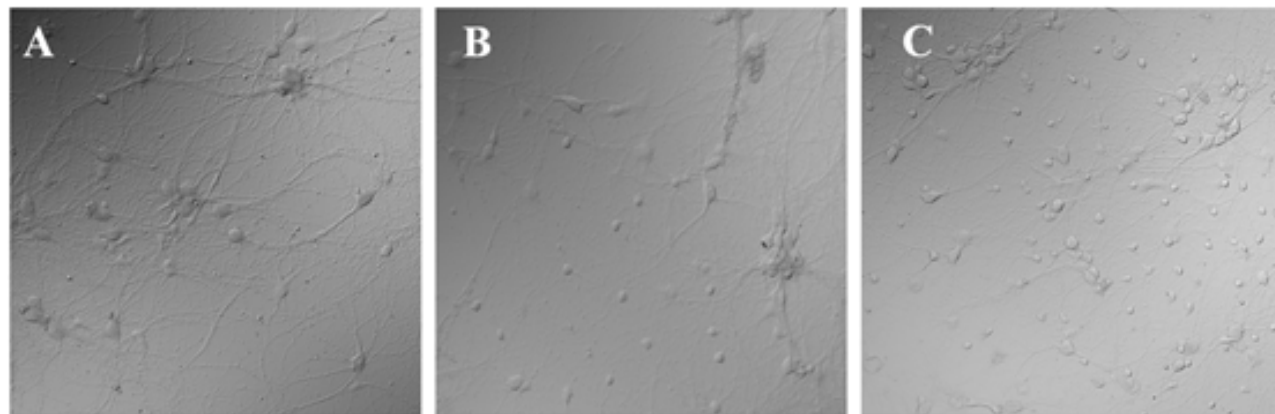
containing B27 and half the medium was changed every 3 d. The growth of neurons was observed under a microscope. Cultured primary hippocampal neurons were treated with different concentrations of propofol and dexmedetomidine based on the experimental groupings<sup>[6,7]</sup>; control group (0 µM propofol and 0 µM dexmedetomidine); propofol group (20 µM propofol and 0 µM dexmedetomidine) and propofol+dexmedetomidine group (20 µM propofol and 10 µM dexmedetomidine). All cells were incubated for 1 h. After centrifuging the cell suspension, the supernatant was discarded and the cells were washed 2-3 times with Phosphate-Buffered Saline (PBS). Then, 4 % paraformaldehyde was used to fix the cells for 10-15 min at room temperature. The cells were washed 3 times (5 min each) with PBS, after which 0.1 % Triton X-100 was added to permeabilize the cell membrane for 2 min at room temperature. Nonspecific binding sites were blocked with 5 % goat serum in PBS for 30 min at room temperature. After removing the blocking solution, the primary antibody (rabbit anti-cytochrome c) was added and the cells were incubated overnight at 4°. The cells were then washed 3 times, followed by the dropwise addition of the secondary antibody (Alexa Fluor® 488 goat anti-rabbit Immunoglobulin G (IgG)) and incubation at room temperature for 1 h. Then, the cells were washed and mounted with anti-fluorescence quenching mounting medium. The cells were excited with DyLight 488 at different excitation wavelengths and observed and photographed under an Olympus FluoView™ FV3000 confocal laser scanning microscope equipped with an oil lens. Hippocampal tissue was lysed in 10 times the volume of Radioimmunoprecipitation Assay (RIPA) lysis buffer, Phenylmethylsulfonyl Fluoride (PMSF), cytoplasmic protein extraction reagent A and cytoplasmic protein extraction reagent B mixture and ground with a grinder for 60 s. The tissue homogenate was centrifuged at low temperature (4°) at 13 000 rpm/min for 20 min. The supernatant was aspirated with a pipette and the protein concentration was measured using a Bicinchoninic acid (BCA) protein concentration assay kit. The 3 groups were subjected to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and the membranes were transferred to a Polyvinylidene Difluoride (PVDF) membrane using a wet transfer method. Then, the PVDF

membrane was immersed in 5 % nonfat milk blocking solution and slowly stirred on a shaker at room temperature for 1 h. The membrane was incubated with primary antibody, i.e., rabbit anti-cytochrome c antibody (1:5000, ab133504, Abcam, Cambridge, UK) and anti-beta actin rabbit pAb (1:1000, GB11001, Servicebio, Wuhan, China) overnight at 4°. After washing off the primary antibody with Tris Buffered Saline with Tween (TBST), the secondary antibody, i.e., goat anti-rabbit IgG (1:3000, GB23303, Servicebio, Wuhan, China) was added and the membrane was incubated at room temperature for 1 h. Then, the secondary antibody was recovered, and the membrane was washed with TBST 3 times on a shaker. Electrochemiluminescence (ECL) was used to visualize the bands. The content of the target protein was calculated as the ratio of the gray value of the target protein band to that of the internal reference band. Statistical Package for the Social Sciences (SPSS) 19.0 software was used for the statistical analyses. All data are expressed as the mean±Standard Deviation (mean±SD) and graphing was performed using GraphPad Prism 5.0. Analysis of Variance (ANOVA) or t test statistics are described in the legends.  $p < 0.05$  indicated that difference was statistically significant. In the control group, the primary hippocampal neurons of the neonatal mice were mature and the neurites were intertwined into a relatively dense neural network, with full cell bodies and rich cytoplasm. Neurons in the propofol group showed obvious degeneration, manifesting as shortened neurites, sparse axons and dendrites, and blank space in some areas. The morphology of neurons in the propofol+dexmedetomidine group was close to that of neurons in the control group. The cell body was full and translucent, and the neurites were thick and interconnected and formed a network, with little degeneration and sparse protrusions as shown in fig. 1. Clinical studies have shown that general anesthesia in infants and young children may lead to abnormal development of the central nervous system, manifesting as changes in neuronal toxicity, impaired synaptic plasticity and indirect effects on learning and memory functions<sup>[8]</sup>. Hippocampal neuronal synapse formation is the basis of learning and memory function<sup>[9]</sup>. Because the peak period of rodent neurodevelopment is from 2 d before birth to 1 w after birth, primary hippocampal neurons

were obtained from the hippocampal tissue of neonatal mice. When prepared as a single cell suspension for *in vitro* culture, the biological traits are closer to those in the *in vivo* state. Therefore, this study selected the hippocampal neurons of neonatal mice within 1 d of birth as the research object. Referring to the methods described by Ge *et al.*<sup>[6]</sup> and Lv *et al.*<sup>[7]</sup>, in this study hippocampal neurons were incubated with 20  $\mu$ M propofol to induce neuronal damage and the final concentration of dexmedetomidine was set to 10  $\mu$ M. The results of this study suggest that compared with those in the control group, the primary hippocampal neurons in the propofol group had an abnormal morphology, with shortened neurites and sparse axons and dendrites. Compared with that in the propofol group, the proportion of neuronal degeneration and sparse neurites in the propofol+dexmedetomidine group was significantly lower. These results suggest that dexmedetomidine can reverse propofol induced damage and morphological abnormalities in hippocampal neurons as shown in fig. 2 and fig. 3. The results of previous animal studies<sup>[4]</sup> showed that postoperative spatial memory deficits occurred in rats after propofol anesthesia and that dexmedetomidine ameliorated this cognitive impairment. The results of an investigation of the molecular mechanism by hippocampal transcriptome sequencing and differential gene enrichment analysis suggested that the differential expression of mitochondrial function-related genes may be involved in the process of dexmedetomidine improving spatial memory impairment after propofol anesthesia. Anesthesia and surgery can induce a systemic stress response, followed by the massive release of neuroendocrine hormones and inflammatory mediators. However, the ability of the central nervous system to secrete antioxidant substances is relatively weak, the overall level of antioxidative stress is low and neurons are vulnerable to free radicals and other substances<sup>[10]</sup>. Hippocampal neurons are rich in unsaturated fatty acids, making these neurons particularly sensitive to external oxidative stress stimuli<sup>[11]</sup>. Mitochondria are the energy metabolism core of neurons and generate Adenosine Triphosphate (ATP) to maintain cell life activities. Oxidative stress can cause mitochondrial dysfunction by disrupting the mitochondrial membrane potential, eventually leading to neuronal

damage and even apoptosis<sup>[12]</sup>. Cytochrome c oxidase (molecular weight, 12 kDa) is an important water-soluble mitochondrial protein encoded by nuclear genes. Cytochrome c is mainly involved in the mitochondrial electron transport chain, utilizing heme groups as redox intermediates to transport electrons to mitochondrial complex IV<sup>[15]</sup>. Cytochrome c is released from mitochondria to the cytoplasm when neurons are strongly stimulated (e.g., DNA damage, metabolic stress or the presence of unfolded proteins), acting as a key signaling molecule in neuroinflammation<sup>[13]</sup>. Studies have reported increased levels of cytochrome c during traumatic brain injury, cancer treatment and ischemic encephalopathy<sup>[14]</sup>. Cytochrome c can mediate glial cell activation by participating in Toll-like receptor interactions, causing neurotoxic inflammatory responses<sup>[15]</sup>. Zheng *et al.*<sup>[16]</sup> established a rat Postnatal Day (PND) model by performing heart bypass surgery on rats; the expression of cytochrome c in the hippocampus of the postoperative neurocognitive disorder rat model was abnormally increased compared with that in the control group. In this study, immunofluorescence staining and Western Blot (WB) were used to assess cytochrome c content and similar results were obtained; i.e., primary hippocampal neurons incubated with propofol expressed cytochrome c at high levels, but expression decreased to a level similar to that in the control group in cells coincubated with dexmedetomidine and propofol. The results suggest that dexmedetomidine significantly inhibits high cytochrome c expression in hippocampal neurons induced by propofol.

Dexmedetomidine is a highly selective  $\alpha_2$  adrenergic receptor agonist widely used in clinical anesthesia. Dexmedetomidine can reduce the stress response, maintain hemodynamic stability, and reduce or prevent the transmission of noxious stimuli and is also recognized to have anti-inflammatory and neuroprotective effects<sup>[17]</sup>. Scholars have created a hypoxic brain injury rat model and found that the learning and memory abilities of the rats significantly improved after dexmedetomidine treatment, an effect that may be due to the downregulation of cytochrome c, Apoptotic protease activating factor-1 (Apaf-1) and caspase-3 protein expression by dexmedetomidine, thus exerting neuroprotective effects<sup>[18]</sup>. Scholars have also reported that dexmedetomidine can prevent synaptic loss and cognitive dysfunction in neonatal rats with hypoxic-ischemic brain injury by reducing oxidative stress, neuroinflammation and excessive autophagy in the central nervous system<sup>[19,20]</sup>. However, there are also researchers who hold the opposite view, i.e., dexmedetomidine, when used in combination with other drugs, does not reduce the neurotoxicity to the neonatal brain caused by general anesthesia, a finding that may be related to the use of inhaled anesthetic rather than intravenous propofol in that study<sup>[21]</sup>. In conclusion, dexmedetomidine can reverse propofol-induced damage and morphological abnormalities in primary hippocampal neurons and the molecular mechanism may be related to inhibiting the overexpression of the mitochondrial protein cytochrome c in the hippocampus.



**Fig. 1:** Dexmedetomidine alleviates the impairment of primary hippocampal neurons caused by propofol, (A): The morphology of primary hippocampal neurons in the control group was normal; (B): Primary hippocampal neurons in the propofol group had degenerated, with sparse axons and dendrites and (C): The primary hippocampal neurons in the propofol+ dexmedetomidine group exhibited basically normal morphology. Neurites were thick and interconnected to form a network



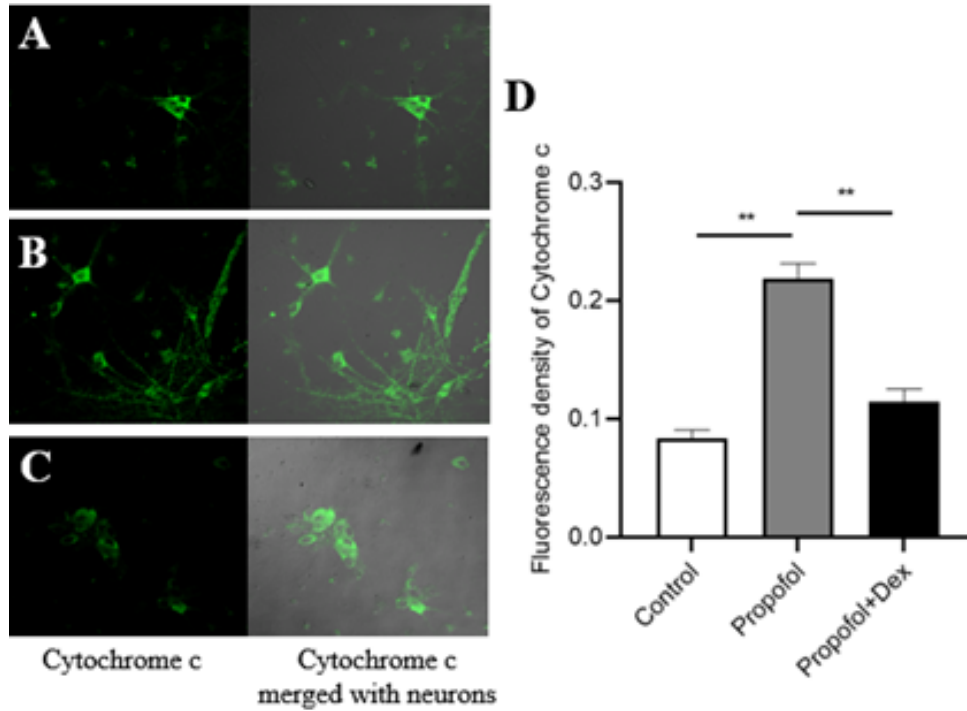


Fig. 2: Dexmedetomidine reverses the higher expression of cytochrome c in primary hippocampal neurons caused by propofol. Cytochrome c levels in isolated primary hippocampal neurons were examined by immunofluorescence. (A): Cytochrome c-like immunoreactivity was normal in the control group; (B): Cytochrome c-like immunoreactivity was significantly higher in the propofol group than in the control group; (C): Cytochrome c-like immunoreactivity was significantly decreased after treatment with 10  $\mu$ M dexmedetomidine and (D): Cytochrome c-like immunoreactivity was determined by computer-assisted image analysis Note: \*\* $p < 0.0001$

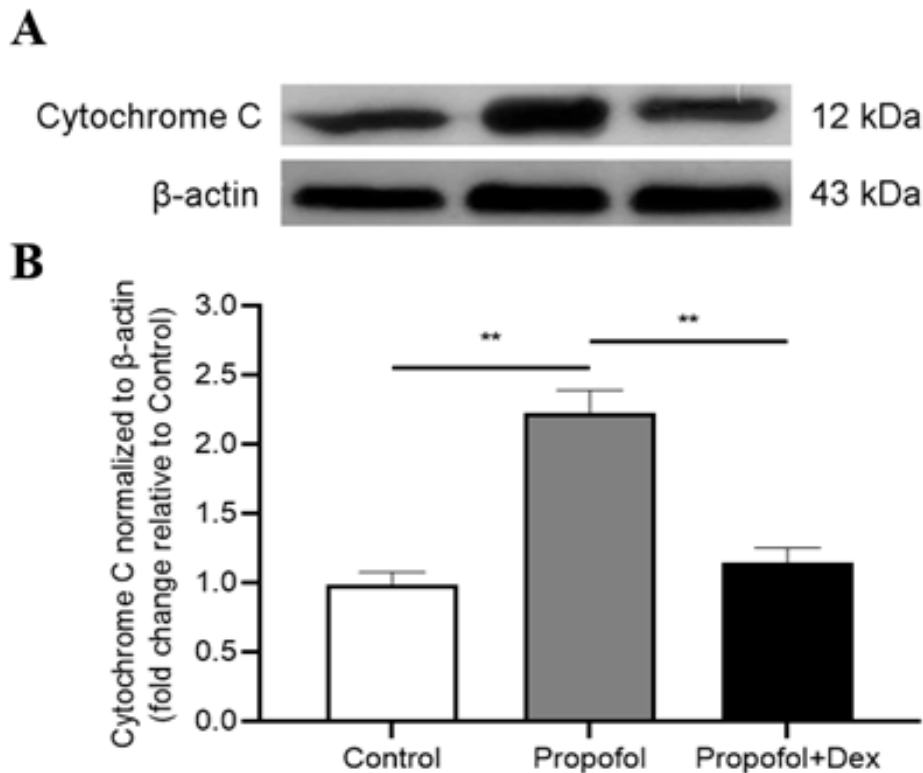


Fig. 3: Dexmedetomidine inhibits high cytochrome c expression in primary hippocampal tissue. WB was used to verify the expression of cytochrome c in hippocampal tissue, (A): Expression of cytochrome c in the isolated hippocampal tissue of neonatal mice after incubation with propofol and dexmedetomidine and (B): Hippocampal tissue incubated with propofol showed high expression of cytochrome and hippocampal tissue incubated with propofol and dexmedetomidine showed cytochrome c expression levels similar to that of the control group Note: \*\* $p < 0.0001$

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**Author's contributions:**

Xichun Yang and Lei Yang have contributed same to this work.

**Conflict of interests:**

The authors declared no conflict of interests.

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