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Neuroprotection of dexmedetomidine against propofol-induced neuroapoptosis partly mediated by PI3K/Akt pathway in hippocampal neurons of fetal rat*

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Abstract: The aim was to investigate how the PI3K/Akt pathway is involved in the protection of dexmedetomidine against propofol. The hippocampal neurons from fetal rats were separated and cultured in a neurobasal medium. Cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Then neurons were pretreated with different concentrations of dexmedetomidine before 100 μmol/L propofol was added. Akt, phospho-Akt (p-Akt), Bad, phospho-Bad (p-Bad), and Bcl-xL were detected by Western blot. Also, neurons were pretreated with dexmedetomidine alone or given the inhibitor LY294002 before dexmedetomidine pretreatment, and then propofol was added for 3 h. The results demonstrated that propofol decreased the cell viability and the expression of p-Akt and p-Bad proteins, increased the level of Bad, and reduced the ratio of Bcl-xL/Bad. Dexmedetomidine pretreatment could reverse these effects. The enhancement of p-Akt and p-Bad induced by dexmedetomidine was prevented by LY294002. These results showed that dexmedetomidine potently protected the developing neuron and this protection may be partly mediated by the PI3K/Akt pathway.

Key words: Dexmedetomidine; Propofol; Neuroapoptosis; PI3K/Akt
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1 Introduction

Previous experimental studies have shown that the exposure of the neonatal rodent brain to anesthetic drugs during a period of neurodevelopment can induce widespread neurodegeneration (Ikonomidou *et al.*, 1999; 2000; Jevtovic-Todorovic *et al.*, 2003). Many implicated anesthetics potentiate γ-aminobutyric acid (GABA) A receptors as agonists and/or inhibit *N*-methyl-D-aspartic acid (NMDA) receptors as antagonists (Ikonomidou *et al.*, 2001). Propofol is an intravenous anesthetic agent commonly used in pediatric anesthesia and intensive care practice, and has been shown as a GABA A receptor agonist and NMDA receptor antagonist (Irifune *et al.*, 2003; Nguyen *et al.*, 2009). Propofol can cross the placenta and it has been mediated by neuronal loss and disorders of neurotransmitter release (Yu *et al.*, 2013). Experimental investigations demonstrated that it can

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induce widespread neuroapoptosis of the fetal brain with just a single dose (Jauniaux *et al.*, 1998; Creeley *et al.*, 2013). Impairment of neurocognitive functions was found because repeated propofol investigations revealed that propofol might induce acute neurotrophic imbalance and behavioural changes in adolescent animals. These effects of propofol-induced neuroapoptosis have been attributed to transient increase of capase-3 and c-Fos, reduction of active mitogen-activated protein kinases (extracellular signal-regulated kinase (ERK), protein kinase B (Akt)), and downregulation of several observed neurotrophic factors (brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3)) (Yin *et al.*, 2011; Karen *et al.*, 2013).

Dexmedetomidine is a potent and highly selective 2-adrenergic receptor agonist with many actions on the central nervous system, including anesthetic-sparing effects, analgesia, and intensive care unit sedation (Bhana *et al.*, 2000). The respiratory depressant effect of dexmedetomidine is minimal and has little effect on the cardiovascular system, so the safety margin of this drug is favorable (Yuen, 2010).

Study has shown that dexmedetomidine can exert neuroprotective effect in in vitro and in vivo animal models (Sanders and Maze, 2007). Dexmedetomidine provides protection against anaesthetic-induced neuroapoptosis and neurocognitive impairment in the developing rat brain (Ramsay and Luterman, 2004). Dexmedetomidine also can reduce isoflurane-induced neuroapoptosis by preserving the PI3K/Akt pathway (Li *et al.*, 2014) or by increasing the expression of Bcl-2 and phosphorylated ERK1/2 (Sanders *et al.*, 2010). In Liao *et al*. (2014)'s study, c-Jun NH_2-terminal kinase (JNK) and the p38 pathway were involved in dexmedetomidine-induced neuroprotection against isoflurane effects.

The concomitant use of dexmedetomidine in adolescents undergoing spinal fusion reduced propofol infusion requirements (Ngwenyama *et al.*, 2008). This may reduce the side effects and risks associated with prolonged propofol infusion in children (Yuen, 2010). Whether there are other mechanisms underlying dexmedetomidine-caused neuroprotection against propofol-induced apoptosis in immature brain is still undetermined. This study investigated whether dexmedetomidine reversed these propofol-induced protein changes in the fetal brain and provided neuroprotection. We hypothesized that dexmedetomidine pretreatment attenuates propofol-induced neurodegeneration in the fetal brain through PI3K/Akt activity. Hippocampal neurons were isolated to study the expression of Akt, phospho-Akt (p-Akt), Bad, phospho-Bad (p-Bad), and Bcl-xL.

2 Materials and methods

2.1 Hippocampal neuron culture and identification

Sprague-Dawley rats on 16–18 d of pregnancy were sacrificed and fetal rats were taken from the abdominal cavity. The hippocampus of the fetal rats was separated and hippocampal neuron cells were seeded in a culture plate for 7 d. NeuN monoclonal antibody was used to identify whether hippocampal neuron cells were successfully acquired by the immunohistochemistry method.

2.2 MTT assay

Neuron cells were seeded in a 96-well culture plate at 1×10^4 cells per well. Cells were pretreated with saline, 0.1, 1, 10, and 100 μmol/L dexmedetomidine. After 30 min, 100 μmol/L propofol was added to each cell and incubated for 3 h. Then 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated at 37 °C for 4 h. At the end of the incubation period, the media were removed and 150 μl dimethyl sulfoxide (DMSO) was added to lyse cells to dissolve dye. Absorbance of the converted dye was measured using a microtiter plate reader (iMark, Bio-Rad, USA) at 492 nm.

2.3 Western blot assay

Neuron cells were cultured for 7 d. Cells were divided into 6 groups and pretreated with saline, 0.1, 1, 10, and 100 μmol/L dexmedetomidine, respectively. After 30 min 100 μmol/L propofol was added to each cell and incubated for 3 h.

The cells were harvested and washed twice with ice-cold phosphate buffer saline (PBS). The whole cell extracts were obtained by lysing the cells with radioimmunoprecipitation assay (RIPA) lysis buffer (1 mmol/L phenylmethanesulfonyl fluoride (PMSF) was added before use; Beyotime, Jiangsu, China). Protein concentrations of samples were determined

using the bicinchoninic acid (BCA) protein assay. Protein from each group (50 μg) was subjected to 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4 °C with blocking buffer (Beyotime, China). Then the membrane was incubated with primary antibodies: antiphospho-Akt at 1:1000 dilution (Millipore, USA), anti-Akt at 1:2000 dilution (Millipore, USA), antiphospho-Bad at 1:1000 dilution (Abcam, UK), anti-Bad at 1:1000 dilution (Millipore, USA), anti-Bcl-xL at 1:2000 dilution (Millipore, USA), and anti-β-actin at 1:2000 dilution (Santa Cruz, USA). After washing three times with Tris-buffered saline with Tween 20 (TBST) for 15 min, the membrane was incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) for 1 h at room temperature (Zhongshan Jinqiao, China). The results were visualized by enhanced chemiluminescence (ECL). The quantitative protein band density was detected and assayed by the Quantity One system (Universal Hood II, Bio-Rad, USA).

In experiment two, cells were incubated with 20 μmol LY294002 20 min before the pretreatment with dexmedetomidine and propofol. Then the cells were harvested and protein was detected by Western blot.

2.4 Statistical analysis

Data are expressed as mean±standard deviation (SD) of at least three independent experiments for statistical analysis. Comparisons of the protein expression within groups were performed using the paired *t*-test. A *P*-value of <0.05 was considered statistically significant.

3 Results

3.1 Identification of hippocampal neurons by NeuN antibody

The neurons were characterized by immunohistochemistry with NeuN antibody. Many brown positive particles existed in cultured cells as examined under the microscope. The positive cells were 90% at the 8th day. The neuron cells were cultured successfully and could be used in protein detection (Fig. 1).

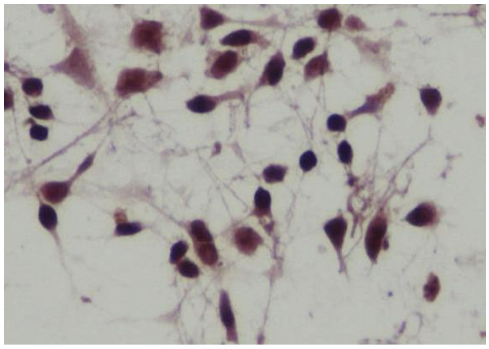

Fig. 1 Hippocampal neurons from Sprague-Dawley rats on 16–18 d of pregnancy identified by immunochemistry with NeuN antibody

A lot of brown positive particles existed in cultured cells under the microscope (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

3.2 Effect of propofol on the cell viability of hippocampal neurons

Propofol can induce widespread neuroapoptosis in the fetal brain. In order to determine whether propofol influences cell viability, MTT assay was performed. Propofol significantly decreased the cell viability compared to the control group (P<0.01). Pretreated with 100 μmol/L dexmedetomidine significantly increased the cell viability (P<0.01), while 0.1, 1, and 10 μmol/L dexmedetomidine did not. The results are shown in Fig. 2.

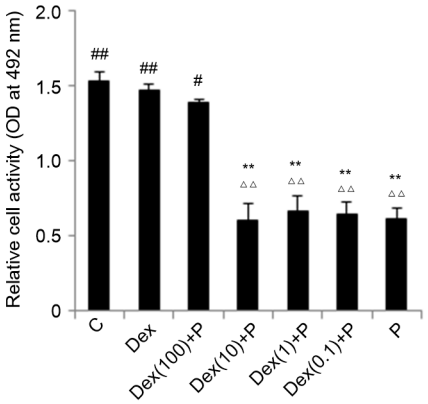

Fig. 2 Cell viability measured by MTT assay

Cells were seeded into a 96-well plate with the density of 1×10^4 cells per well with 100 μmol/L propofol or pretreated with 0.1, 1, 10, and 100 μmol/L dexmedetomidine. MTT assay was carried out after 3 h. The data are expressed as mean±SD of three independent experiments. C: normal cells; Dex: cells treated with dexmedetomidine only; Dex(100): pretreatment with 100 μmol/L dexmedetomidine; Dex(10): pretreatment with 10 μmol/L dexmedetomidine; Dex(1): pretreatment with 1 μmol/L dexmedetomidine; Dex(0.1): pretreatment with 0.1 μmol/L dexmedetomidine; P: propofol. * P<0.05, ** P<0.01 versus C; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01 versus P; $^{\Delta}$ P<0.05, $^{\Delta\Delta}$ P<0.01 versus Dex

3.3 Reversion of propofol-induced protein changes by dexmedetomidine

Treatment with 100 μmol/L propofol significantly decreased the protein expression of p-Akt and p-Bad. However, pretreatment with dexmedetomidine (0.1, 1, 10, and 100 μmol/L) increased the expression of p-Akt and p-Bad compared to the propofol-treated cells. Dexmedetomidine (0.1, 1, and 10 μmol/L) and propofol did not significantly increase the protein expression of p-Akt or p-Bad (P>0.05), while 100 μmol/L dexmedetomidine did (P<0.05). The expression of p-Akt and p-Bad in cells treated with dexmedetomidine alone was similar to that in the control group.

The increase of Bad protein (P<0.01) was also reversed with dexmedetomidine. Treatment with 100 μmol/L propofol significantly increased the protein expression of Bad. However, pretreatment with dexmedetomidine (0.1, 1, 10, and 100 μmol/L) decreased the expression of Bad compared with the propofol-treated cells. In groups treated with dexmedetomidine (0.1, 1, and 10 μmol/L) and propofol, the expression of Bad was increased compared to the control group (P<0.05), while there was no difference between the 100 μmol/L dexmedetomidine-treated group and the control group. The Bcl-xL expression was similar in all groups. Propofol significantly reduced the ratio of Bcl-xL/Bad, while 100 μmol/L dexmedetomidine recovered the ratio (P<0.01). The results are shown in Fig. 3.

3.4 Neuroprotection of dexmedetomidine partly mediated by PI3K/Akt pathway

The PI3K inhibitor LY294002 was used to investigate whether the PI3K pathway participated in the neuroprotection of dexmedetomidine against propofol. Pretreatment with 0.1, 1, 10, and 100 μmol/L dexmedetomidine not only reversed propofol-induced decreases of p-Akt and p-Bad, but also reduced propofol-induced increase of Bad. LY294002 can reverse dexmedetomidine pretreatment-induced

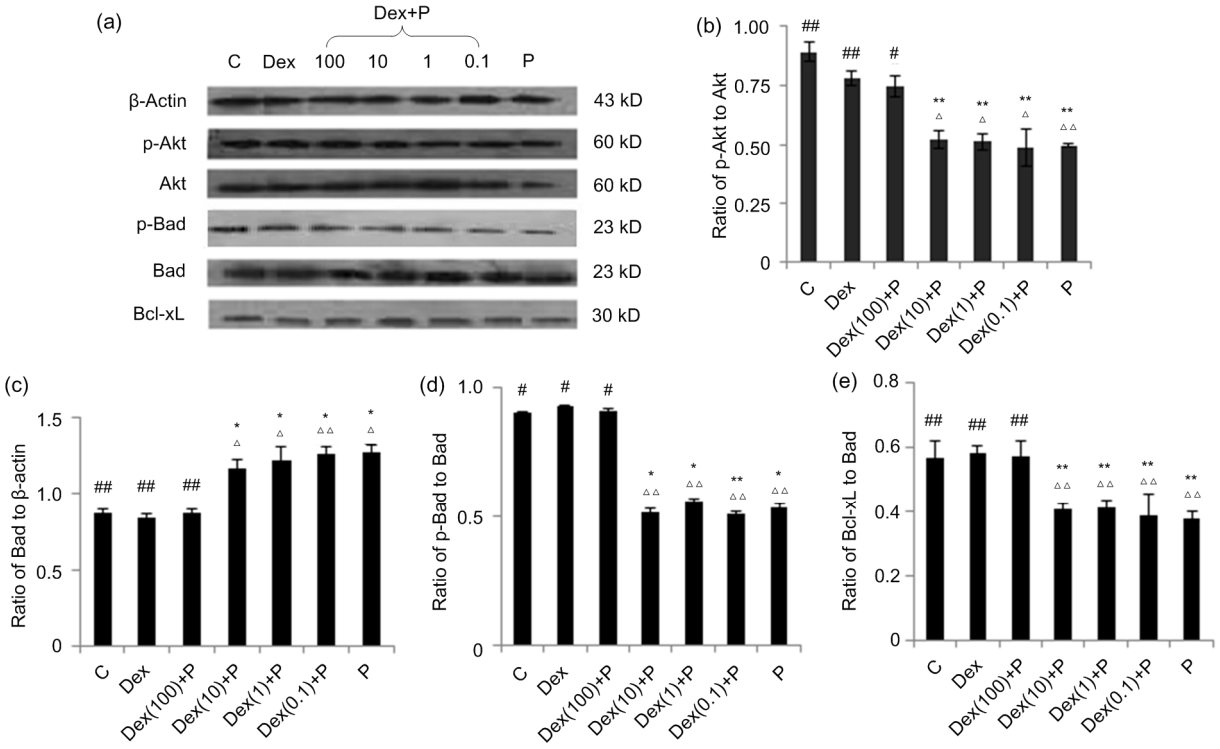

Fig. 3 Dexmedetomidine reversed propofol-induced protein changes in p-Akt, p-Bad, and the ratio of Bcl-xL/Bad

(a) Representative Western blot of p-Akt, Akt, p-Bad, Bad, and Bcl-xL; (b–e) Quantitative analyses of p-Akt/Akt (b), Bad/β-actin (c), p-Bad/Bad (d), and Bcl-xL/Bad (e) by Student's t-test. Results are expressed as mean±SD of three independent experiments. C: Normal cells; Dex: cells treated with dexmedetomidine only; Dex(100): pretreatment with 100 μmol/L dexmedetomidine; Dex(10): pretreatment with 10 μmol/L dexmedetomidine; Dex(1): pretreatment with 1 μm/L dexmedetomidine; Dex(0.1): pretreatment with 0.1 μmol/L dexmedetomidine; P: propofol. * P<0.05, ** P<0.01 versus C; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01 versus P; $^{\Delta}$ P<0.05, $^{\Delta\Delta}$ P<0.01 versus Dex

neuroprotection by reducing the expression of p-Akt and p-Bad, and increasing Bad protein expression. The expression of p-Akt and p-Bad was inhibited by LY294002 alone, while the total Bad increased compared to the control group (Fig. 4).

4 Discussion

Propofol is widely used in numerous surgical procedures because of its rapid onset of action and short duration. It can cross the placenta and may depress the metabolism of the fetus (Jauniaux *et al.*, 1998). Previous studies have indicated that propofol induces apoptotic neurodegeneration when administered to rodent or nonhuman primates during early brain development (Orrei *et al.*, 1986; Cattano *et al.*, 2008; Creeley *et al.*, 2013; Yu *et al.*, 2013). Short propofol anesthesia can induce a decrease in NGF expression and an increase in tumor necrosis factor α (TNFα) expression in the cortex and in the thalamus of P7 rats. Also a decrease in phosphorylated Akt expression, caspase-3 activation, and cell death has been found (Pesic *et al.*, 2009).

Li *et al*. (2016) reported that dexmedetomidine could attenuate neuronal injury induced by maternal propofol anesthesia in the fetal brain, providing neurocognitive protection in the offspring rats. However, the mechanism by which dexmedetomidine produces neuroprotective effects on the fetal brain has not been reported. We used hippocampal neurons from fetal rats to study the possible mechanism by which dexmedetomidine exerts neuroprotective effects. Fetal rats from pregnant rats on G18 were used because this age approximately correlates to the later first trimester in humans, according to the developmental time of mammalian species (Clancy *et al.*, 2001; Workman *et al.*, 2013). The present study demonstrated that when dexmedetomidine was used as a pretreatment it provided neuroprotection against

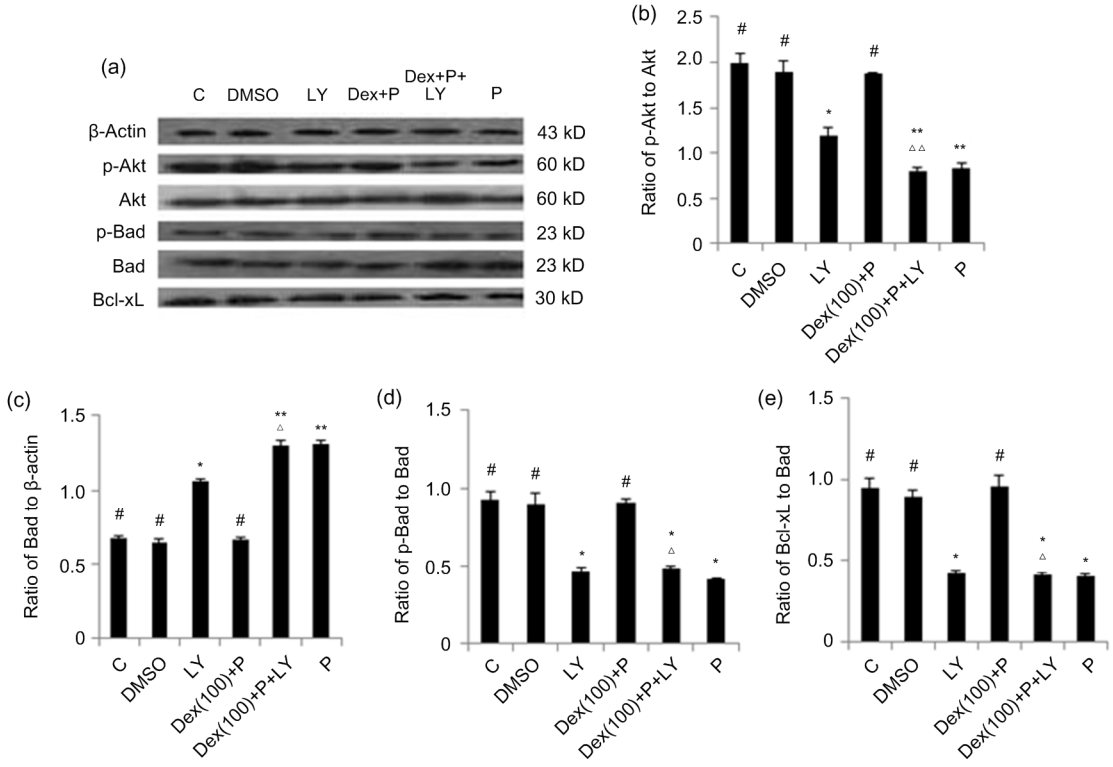

Fig. 4 LY294002 partly inhibited protective effect of dexmedetomidine

(a) Representative Western blot of p-Akt, Akt, p-Bad, Bad, and Bcl-xL; (b–e) Quantitative analyses of p-Akt/Akt (b), Bad/β-actin (c), p-Bad/Bad (d), and Bcl-xL/Bad (e) by Student's *t*-test. Results are expressed as mean±SD of three independent experiments. C: Normal cells; DMSO: dimethyl sulfoxide; LY: LY294002; Dex(100): pretreatment with 100 μmol/L dexmedetomidine; Dex(100)+LY: pretreatment with 100 μmol/L dexmedetomidine and LY294002; P: propofol. * P<0.05, ** P<0.01 versus C; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01 versus P; $^{\Delta}$ P<0.05, $^{\Delta\Delta}$ P<0.01 versus Dex(100)+P

propofol-induced neuroapoptosis in a dose-dependent manner in vitro. Moreover, the phosphorylation of Akt and Bad was inhibited, the Bcl-xL/Bad ratio was downregulated and the level of Bad was increased by propofol, while dexmedetomidine pretreatment could reverse these effects. The enhancement of p-Akt and p-Bad induced by dexmedetomidine was prevented by treatment with inhibitors of LY294002. These results showed that dexmedetomidine potently protected the developing neuron and this protection may be mediated by the PI3K/Akt pathway, which plays an important role in cell growth, proliferation, and survival (Cantley, 2002).

Dexmedetomidine mediates its neuroprotection by α2-adrenergic receptors, especially the α2A-adrenergic receptor (Paris *et al.*, 2006). Since the antagonist atipamezole of the α2-adrenoceptor only partly reversed the neuroprotective effects of dexmedetomidine on neurotoxicity in rats induced by isoflurane, there may be other mechanisms. Several pathways have been reportedly involved in the neuroprotection of dexmedetomidine (Cai *et al.*, 2014; Duan *et al.*, 2014; Liao *et al.*, 2014; Xiong *et al.*, 2014). Li *et al.* (2014) found that dexmedetomidine pretreatment dose-dependently inhibited isoflurane-induced neuroapoptosis by preserving the PI3K/Akt pathway in the hippocampus in neonatal rats. These findings are consistent with our results. Liao *et al.* (2014) reported that both JNK and P38 MAPK pathways participate in the protection by dexmedetomidine against isoflurane-induced neuroapoptosis in the hippocampus of neonatal rats. There have been studies reporting that dexmedetomidine renders brain protection in several brain injury models (Clancy *et al.*, 2001; Schoeler *et al.*, 2012; Degos *et al.*, 2013; Xiong *et al.*, 2014; Pan *et al.*, 2016). Dexmedetomidine has also shown beneficial effects in other experimental models, for example by decreasing inflammatory mediators in endotoxin-induced shock in rats (Taniguchi *et al.*, 2004) or lipopolysaccha-ride-stimulated astrocytes (Zhang *et al.*, 2014).

In conclusion, dexmedetomidine reduced propofol-induced neuroapoptosis by preserving the PI3K/Akt pathway. Hippocampal neuron cells from fetal rats were used in this study, and more studies in vivo and more pathways are needed to determine the mechanisms of how dexmedetomidine exerts neuroprotection, because it is likely to be multifactorial. These results suggest that the combination regimen of propofol and dexmedetomidine may be preferable to the use of propofol as a single agent.

5 Conclusions

In conclusion, this study used hippocampal neurons from fetal rats to investigate how the PI3K/Akt pathway may be involved in the protection of dexmedetomidine against propofol. The results showed that cell viability decreased when treated by propofol. Propofol also decreased the expression of p-Akt and p-Bad proteins, increased the level of Bad, and reduced the ratio of Bcl-xL/Bad. Dexmedetomidine pretreatment could reverse these effects. The protection was partly mediated by the PI3K/Akt pathway. More pathways should be studied both in vivo and in vitro.

Compliance with ethics guidelines

Ning ZHANG, Quan-ping SU, Wei-xia ZHANG, Nian-jun SHI, Hao ZHANG, Ling-ping WANG, Zhong-kai LIU, and Ke-zhong LI declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：PI3K/Akt 信号通路部分参与了右美托咪定对异丙酚诱导的胎鼠海马神经元凋亡的保护作用

目　的：研究 PI3K/Akt 信号通路是否参与了右美托咪定对异丙酚诱导的胎鼠海马神经元凋亡的保护作用，并初步探讨可能的作用机制。

创新点：首次利用胎鼠海马神经元研究发现右美托咪定对异丙酚诱导的神经元凋亡作用部分是由 PI3K/Akt 信号通路介导的。

方　法：首先分离胎鼠海马神经元并鉴定。使用 MTT 法检测异丙酚对神经元活性的影响。然后将神经元分为不同的组，分别用 0.1、1、10 和 100 μmol/L 右美托咪定预处理细胞，然后加入 100 μmol/L 的异丙酚继续培养，同时设异丙酚组和正常对照组。使用蛋白质印迹（Western blot）方法检测 Akt、p-Akt、Bad、p-Bad 和 Bcl-xL 的表达变化。在 100 μmol/L 右美托咪定预处理前加入 LY294002，进一步研究 PI3K/Akt 途径是否参与了右美托咪定对异丙酚诱导的胎鼠海马神经元凋亡的保护作用。

结　论：实验结果显示，异丙酚明显降低了神经元的细胞活性及 p-Akt 和 p-Bad 的表达水平，增加了 Bad 的表达，从而 Bcl-xL/Bad 的比率升高。100 μmol/L 右美托咪定预处理可以逆转这种效果。LY294002 可以抑制右美托咪定的保护作用，说明右美托咪定对异丙酚诱导的胎鼠海马神经元凋亡的保护作用部分是由 PI3K/Akt 信号通路介导的。

关键词：右美托咪定；异丙酚；神经凋亡；PI3K/Akt