Propofol alleviates hypoxic neuronal injury by inhibiting high levels of mitochondrial fusion and fission

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Abstract. – OBJECTIVE: This study aimed to explore the mechanism of propofol in alleviating neuronal oxidative damage.

MATERIALS AND METHODS: The neuron cells were randomly assigned to normal group (NOR), model group (MOD), and propofol administration group (MED). A 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out to detect the viability of neuron cells, reverse transcription PCR (RT-PCR) assay to determine the gene expression of Fis and Mfn1, and Western blot assay to determine the protein expression of Caspase-3, Caspase-9, Bax, Bcl-2, and COX-2.

RESULTS: According to the results of cell proliferation rate, under normal circumstances, neuron cells would have some programmed death and weak apoptosis, while after hypoxia-reoxygenation, the apoptosis rate of neuron cells gradually increased with the increase of culture time, which was significantly higher than that of the NOR. After the addition of propofol, the overall apoptosis rate of neuron cells slowly increased, significantly lower than that in the MOD and close to that in the NOR. Compared with the NOR, the ROS content in the MOD was significantly reduced, and compared with the MOD, the ROS content in the MED significantly recovered. Furthermore, the RT-PCR results showed that compared with the NOR, the expression of mitochondrial fusion protein (Mfnl) in the MOD group declined significantly, and the expression of mitochondrial fission protein 1 (Fis1) increased significantly, while after the addition of propofol, the expression of Mfnl and Fis1 was closed to that in the NOR. WB results showed that compared with the NOR, the expression of apoptosis proteins (Caspase-3, Caspase-9, Bax, and COX-2) in the MOD increased significantly, and the expression of Bcl-2 reduced significantly (all $p<0.05$), and the addition of propofol improved the expression of corresponding proteins.

CONCLUSIONS: Propofol could alleviate hypoxic neuronal injury by inhibiting high levels of mitochondrial fusion and fission.

Key Words:
Propofol, Protein, Gene.

Introduction

Cardio-cerebrovascular diseases are currently recognized as common multiple diseases affecting human physical and mental health, with a high mortality rate[1,2]. Propofol (2, 6-diisopropyl phenol) protects against ischemia-reperfusion injury mainly by inhibiting cell apoptosis. Mitochondria, as the key organelle of cell function, are in constant balance of fission and fusion to maintain normal function[3,4]. Propofol is widely adopted for anesthesia, sedation and recovery after anesthesia[5]. There is evidence showing that propofol exerts protective effect on the heart, and its mechanism is similar to that of vitamin E and related to antioxidant effect[6].

However, the relationship of propofol between relieving hypoxic neuronal injury and regulating mitochondria remains unclear. In this experiment, we constructed a hypoxic cell injury model to explore the mechanism of propofol in intervening with neuronal injury, with the goal of providing data basis for future drug application.

Materials and Methods

Experimental Animals

Thirty Sprague Dawley (SD) rats were purchased from the Laboratory Animal Center of the

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Academy of Military Medical Sciences. The laboratory certification number was SCXK- (PLA) 2012-0004, and the feeding qualification number was: SYXK- (PLA) 2012-0004. The rats were 8 weeks old. They were fed with specially formulated pellet food and free access to water. The indoor temperature was kept at about 25°C, the relative humidity was 40%-70%, and the lighting time was 12 hours. The body mass of the rats was about (250.63 ± 3.83) g (Animal Ethics Committee approval number: 2018-1215A).

Experimental Animals and Grouping

The newborn SD rats were executed by cervical dislocation, and the whole brain tissue of each rat was removed and placed in a petri dish. Subsequently, the meninges and venation were carefully removed with curved microforceps, and the hippocampal tissues were taken out. The hippocampus tissues were divided into small pieces and added with mixed digestive solution containing ethylenediaminetetracetic acid (EDTA) and trypsin, and then the digestive solution was collected and placed in Eppendorf (EP; Hamburg, Germany) tubes, followed by centrifugation. The centrifuged solution was transferred to 25T culture flasks and cultured in a 5% CO_2 temperature-constant incubator at 37°C to obtain primary neuron cells. The cells were assigned to three groups: Normal group (NOR), model group (MOD), and propofol administration group (MED).

Culturing Conditions of Neuron Cells

The cell suspension was transferred to a 5% CO_2 cell incubator at 37°C, followed by 5 times additional complete culture medium, and centrifuged at 1500 rpm for 5 min. The adopted culture medium was Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and double antibody. During use, the medium was sucked out, washed with Hanks solution once, digested with 1 mL trypsin for 1 min, and then continuously sucked and centrifuged for cell counting.

Establishment of Hypoxia/Reoxygenation Injury Model

Cells in the NOR were cultured in DMEM during the experiment, and those in the MED were cultured with propofol for 48 h, and then subjected to hypoxia/reoxygenation incubation as follows: cells were cultured in anoxic solution in a closed container with 95% N_2 + 5% CO_2 for 2 h, and then cultured in sugar-containing reoxygenation liquid in the closed container with 95% N_2 + 5% CO_2 for 4 h to establish a hypoxia/reoxygenation injury model. Cells in the MOD cultured for 72 h were also subjected to hypoxia/reoxygenation incubation as follows: cells were cultured in anoxic solution in a closed container with 95% N_2 + 5% CO_2 for 2 h, and then cultured in sugar-containing reoxygenation liquid in the closed container for 4 h.

Determination of Cell Viability in Each Group

Cells in each group were seeded into 96-well plates at $5x10^5$ cells/well, and when they attached to the wall and sprawled out for 4 h, MTT reagent was added in a dark environment. Four hours later, the cells were added with dimethyl sulfoxide (DMSO), and then their optical density was measured at 570 nm.

Detection of Other Biochemical Indexes

RT-PCR assay was carried out to detect gene expression, and Western blot (WB) assay was used to determine protein expression.

Design of Gene Primers for Cells in Each Group

Primers of Mfnl, Fis1, and β-actin are shown in Table I.

Determination of Gene Expression by RT-PCR

Upstream and downstream primers of Mfnl, Fis1, and β-actin were designed, and fragments shorter than 150 bp were selected. RNA extraction: cells in each group were added into

Table I. Primer sequences for RT-PCR (n = 10).

Gene name	Forward primer	Backward primer	Length (bp)
Mfnl	CGCATCGTACGATCGGATA	CGCCTAGCCCTATATAAAGC	132
Fis1	CGATCGCTGATCCGTTAAA	CCGTAGACTTCGCTAGCGCT	125
β-actin	CGATGCATTCGGCCGTGG	CGCTAGCTTGCCGCTAGACA	119

qRT-PCR, quantitative Reverse-Transcription Polymerase Chain Reaction.

centrifuge tubes, and each tube was added with TRIzol reagent and chloroform, and then further centrifugated to obtain supernatant. Each tube was added with propofol to extract supernatant and precipitate. Finally, cells in each tube were dissolved with DEPC solution, and amplified with a PCR amplificatory. Loading: 50×TAE was diluted into 1× TAE, and the diluted 1× TAE was taken as solvent. Agarose (0.52 g) was added into 1× TAE solution, and then the solution was added with 4 μL nucleic acid dye after being boiled in microwave oven, shaken and mixed well. Finally, the agarose gel was horizontally put into the electrophoresis tank, and 6 μL DNA Maker and the PCR amplification product of the target gene were sequentially added.

Determination of Protein Content by Western Blot

Cells of each group were collected and washed with prepared phosphate-buffered saline (PBS) three times, then cell lysate was added, and ice bath was given after being mixed well. Subsequently, the cells were centrifuged at 12000 rpm and 4°C for 15 min to collect the supernatant. The optimal density of each well was determined using the Coomasie brilliant blue, and the protein concentration of each group was adjusted to the same level before protein loading. The nitrocellulose (NC) membrane transferred with protein after electrophoresis was placed into an incubation box, with the surface contacted by the gel upwards, and coated with phosphate-buffered saline and tween (PBST) buffer solution containing a proper amount of 5% degreased milk. Afterwards, the membrane was sealed in a horizontal shaking table for 1.5 h, and then washed with PBS three times and washed with PBST once. TBST buffer was adopted to dilute Caspase-3, Caspase-9, bax, Bcl-2 and COX-2 antibody by 1,000 times, and the primary antibody working fluid was fully contacted with the NC membrane, and incubated at 4°C overnight. The NC membrane incubated with primary antibody was taken out, washed with PBS three times and washed with PBST once. Subsequently, PBST buffer was used to dilute the secondary antibody by 1,000 times, and the secondary antibody working fluid was added into to the NC membrane drop by drop and incubated at room temperature for 1 h. The NC membrane incubated with secondary antibody was taken out and corresponding secondary antibody was recovered. Solution A and solution B in ECL chemiluminescent liquid were mixed at 1:1, and fully contacted with the NC membrane in a cassette. Finally, developing liquid and fixing liquid were used to develop the membrane, and photos were taken and recorded. In addition, the differential expression was calculated according to the gray value of the target band.

Statistical Analysis

Statistical analysis was carried out using SPSS 20.1 (IBM, Armonk, NY, USA), and all data were expressed by the mean ± standard deviation ($\bar{x} \pm s$). Data were analyzed using the homogeneity test, and compared between groups using the *t*-test. Pearson's correlation was carried out for correlation analysis. α=0.05 was taken as the detection criteria. Inter-group comparison was carried out using the χ^2. $p<0.05$ indicates a significant difference.

Results

Comparison of Neuronal Apoptosis Rate Among the NOR, MOD, and MED

The apoptosis rate of neuronal cells in the MED, NOR, and MOD are shown in Table II. It can be seen that under normal circumstances, neuron cells would have some programmed death and weak apoptosis. As shown in Figure 1, after hypoxia-reoxygenation, the apoptosis rate of neuron cells gradually increased with the increase of culture time, which increased

Table II. Comparison of cell apoptosis rate among groups (%).

Group	Total number of cells	24 h	48 h	72 h
NOR	5×10^5	7.65 ± 2.17	10.11 ± 1.66	14.36 ± 2.74
MOD	5×10^5	29.57 ± 3.75**	42.33 ± 3.56**	55.47 ± 2.75**
MED	5×10^5	15.12 ± 2.11*	18.76 ± 2.94*	20.77 ± 2.85
F-value	–	3.697	9.469	15.745
p-value	–	0.045	0.015	0.008

Note: *p < 0.05 *vs*. the normal group.

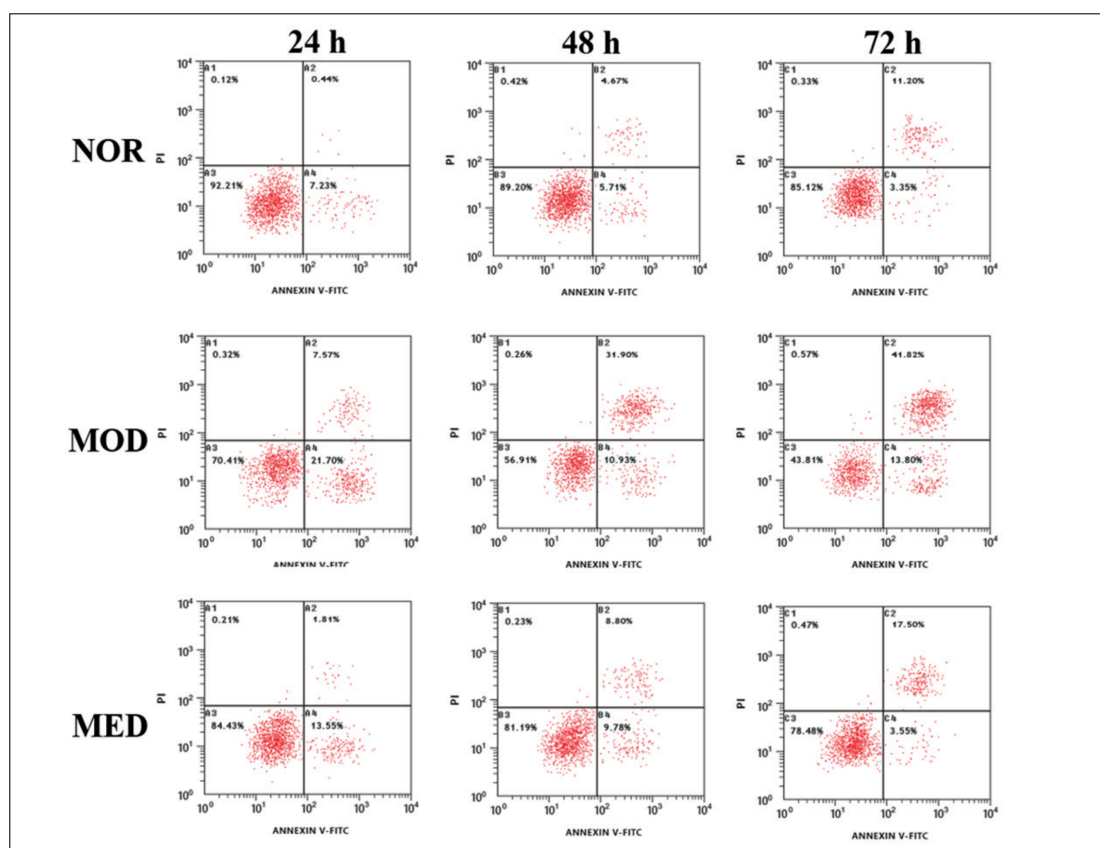

Figure 1. Neuronal apoptosis rate of each group determined by the flow cytometry.

to (29.57±3.75) after 24 h of hypoxia-reoxygenation, to (42.33±3.56) after 48 h of hypoxia-reoxygenation, and to (55.47±2.75) after 72 h of hypoxia-reoxygenation, significantly higher than that in the NOR at the same time point ((7.65±2.17), (10.11±1.66), and (14.36±2.74), respectively). In addition, after the addition of propofol, the overall apoptosis rate of neuron cells increased slowly, significantly lower than that in the MOD (55.47±2.75) and close to that in the NOR. Furthermore, there was a significant difference between the MOD and MED in cell proliferation after 24 h, 48 h, and 72 h of culturing. These results implied that propofol had a protective effect against neuronal cell injury.

Reactive Oxygen Species (ROS) Content Changes in Neuron Cells in the NOR, MOD, and MED

The results of ROS contents revealed that compared with the NOR, the MOD showed significantly decreased ROS content ($p<0.01$), and compared with the MOD, the ROS content in the MED significantly recovered, suggesting that propofol could strongly alleviate the oxidative damage of cells, but the mechanism required further investigation (Table III).

Table III. Comparison of ROS content in neuron cells among the NOR, MOD, and MED.

Group	ROS (nmol/mL)
NOR	1.01 ± 0.63
MOD	9.26 ± 1.15**
MED	4.32 ± 0.61**
F-value	13.347
p-value	0.010

Note: *Indicates that in comparison with the inflammatory factor content of the NOR, $p < 0.05$.

Expression of Mitochondrion Fusion and Fission Genes in the NOR, MOD, and MED

Mfnl is a mitochondrial fusion gene and Fis1 is a mitochondrial fission gene. In this experiment, cells in each group were collected

Table IV. Comparison of expression of mitochondrion fusion and fission genes in neuron cells among the NOR, MOD, and MED.

Group	Mfnl (gray value)	Fis1 (gray value)
NOR	1.00 ± 0.04	1.00 ± 0.05
MOD	0.23 ± 0.05**	3.74 ± 0.13**
MED	0.87 ± 0.06**	1.25 ± 0.07**
F-value	15.165	16.764
p-value	0.009	0.007

Note: *$p < 0.05$ *vs*. the NOR.

Table V. Comparison of the protein expression of Caspase-3 and Caspase-9 in neuron cells among the NOR, MOD, and MED.

Group	Caspase-3 (gray value)	Caspase-9 (gray value)
NOR	1.00 ± 0.02	1.00 ± 0.03
AMI	2.12 ± 0.08*	3.95 ± 0.08**
MED	1.57 ± 0.05	2.54 ± 0.10*
F-value	14.253	17.264
p-value	0.013	0.006

Note: *$p < 0.05$ *vs*. the NOR.

by centrifugation, and the RNA in the cells was extracted according to the kit instructions. The expression of genes in each group was determined by RT-PCR assay. The determination of Mfnl and Fis1 revealed that compared with the NOR, the expression of Mfnl in the MOD group declined significantly, while the expression of Fis1 in the group increased significantly, indicating abnormal mitochondrial fusion and fission. However, after the addition of propofol, the expression of each gene was close to that in the NOR, which indicated that propofol could inhibit neuronal cell damage by inhibiting mitochondrial fusion and fission abnormalities (Table IV).

Comparison of the Protein Expression of Caspase-3 and Caspase-9 in Neuron Cells Among the NOR, MOD, and MED

WB assay revealed that compared with the NOR, the expression of apoptosis-related proteins (Caspase-3 and Caspase-9) in the MOD significantly increased ($p<0.05$), and the expression of Caspase-3 and Caspase-9 protein in the MED group was significantly lower than that in the MOD ($p<0.05$), and close to that of the NOR, which may be caused by the inhibition on apoptosis genes and no response of apoptosis pathway (Table V and Figure 2).

Comparison of the Protein Expression of Bax and Bcl-2 in NEURON CELLS AMONG the NOR, MOD, and MED

Bax is an apoptosis-promoting protein, and Bcl-2 is an apoptosis-inhibiting protein. Their expressions are often used to balance the apoptosis of cells. WB results showed that compared with the NOR, the expression of Bax protein in the MOD increased significantly, and the expression of Bcl-2 decreased significantly (both $p<0.05$), and the addition of propofol improved the expression of corresponding proteins, which further confirmed that propofol could inhibit the apoptosis of neuron cells by inhibiting apoptosis pathway (Table VI and Figure 3).

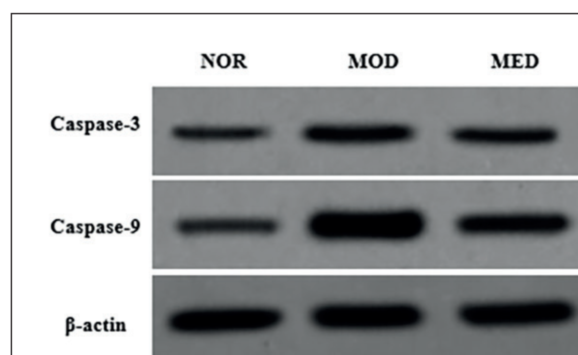

Figure 2. Expression of Caspase-3 and Caspase-9 protein in cells in each group determined by WB.

Table VI. Comparison of the protein expression of Bax and Bcl-2 in neuron cells among the NOR, MOD, and MED.

Group	Bax (gray value)	Bcl-2 (gray value)
NOR	1.00 ± 0.02	1.00 ± 0.03
MOD	3.01 ± 0.11*	0.53 ± 0.09**
MED	1.96 ± 0.14*	0.67 ± 0.07*
F-value	13.337	11.674
p-value	0.013	0.014

Note: $^*p < 0.05$ *vs*. the NOR.

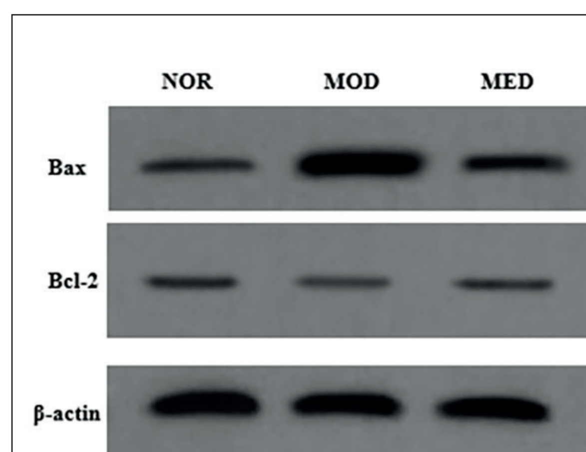

Figure 3. Protein expression of Bax and Bcl-2 in each group determined by WB.

Comparison of the Protein Expression of COX-2 in Neuron Cells Among the NOR, MOD, and MED

Cyclooxygenase is responsible for the synthesis of important biological hormones. The WB assay revealed that compared with the NOR, COX-2 protein expression in the MOD increased significantly (p<0.05), and the addition of isopropylphenol could inhibit the overexpression of COX-2 (Table VII and Figure 4).

Table VII. Comparison of the protein expression of COX-2 in neuron cells among the NOR, MOD, and MED.

Group	COX-2 (gray value)
NOR	1.00 ± 0.03
MOD	2.52 ± 0.10*
MED	1.86 ± 0.12*
F-value	14.231
p-value	0.011

Note: $^*p < 0.05$ *vs*. the NOR.

Discussion

With the progression and development of surgical technology, the number of infants receiving general anesthesia drugs among narcotic drugs is increasing. Propofol is a common drug for clinical intravenous anesthesia[7], which is widely used in intravenous anesthesia for infants. However, at a stage of rapid development, the infants' nerve is easily disturbed by external factors, so the influence of anesthetics on the nervous system of infants has captured a wide concern[8,9]. Mitochondria is a dynamically changing organelle, it constantly splits and fuses to ensure the normal function of mitochondria. This phenomenon of splitting and fusion is also called mitochondrial dynamics. There is evidence that mitochondrial dynamic dysfunction exists in many neurodegenerative diseases. Some studies have suggested that changes in mitochondrial dynamics may be the pathological cause of cognitive dysfunction, rather than a simple symptom. Mitochondrial dynamics are dynamically regulated by several GTPases. Mitochondrial fusion proteins 1 and 2 (Mfn1 and Mfn2) and Opa1 control the fusion elongation of mitochondria, while mitochondrial motility-related protein 1 (DRP1), fission protein

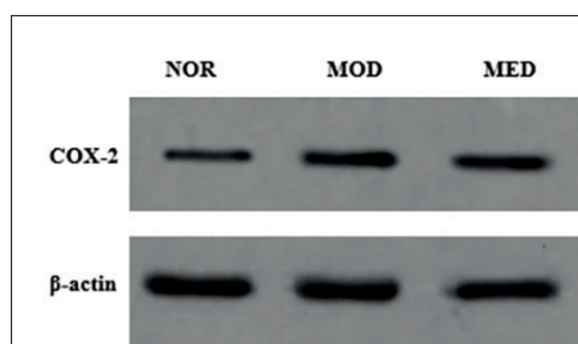

Figure 4. Protein expression of COX-2 in each group determined by WB.

(FIS1) and mitochondrial division factor (MFF) is the main functional protein in mitochondrial division. Mitochondrial dynamics are the basis for maintaining mitochondrial integrity and function, including mitochondrial energy metabolism, the production of oxidized free radicals and the regulation of apoptosis. This study explores the mechanism how propofol alleviates hypoxic damage to neurons by inhibiting the high-level fusion and division of mitochondria. We would like to clarify the mechanism of anesthetics on the nervous system of infants and young children from the perspective of the regulation of mitochondrial energy metabolism, and provide theoretical basis for clinical medication.

Changing mitochondrial morphology will affect cell apoptosis[10]. Guzun et al[11] have revealed that when HL-1 heart cells undergo simulated ischemia, mitochondria will undergo fission, resulting in fragmented mitochondria[11]. In our study, mitochondrial fission is evident during neuronal cell injury, and propofol can significantly inhibit this abnormality. Elevated oxidative stress level is the main pathogenesis of chronic fluorosis, and high ROS level can damage the body[12]. It has been reported that propofol increases the activity and protein expression of superoxide dismutase and catalase, alleviating cell oxidative damage[13]. In addition, it has been shown that mitochondrial fission induced by Fis1 can reduce the uncoupling of endoderm protons in other cell types, promoting cell generation of ROS and aggravating damage[14]. Mitochondrial fusion gene Mfnl and mitochondrial fission gene Fisl regulate mitochondrial fusion and fission through expression changes[15]. Abnormal expression of mitochondrial fusion and fission genes can change the mitochondrial morphology and affect mitochondrial function (e.g., membrane potential), and these abnormalities suggest apoptosis of initiating cells[16,17]. The results of our study showed that after oxidative damage, the expression of Fis1 in neuron cells increased significantly, while that of Mfn1 in neuron cells decreased significantly, and the ROS content in the MOD was significantly higher than that in the NOR, which were directly related to oxidative damage. However, the indexes of the MED were significantly decreased. Therefore, it is speculated that propofol may alleviate oxidative damage of cells by lowering the expression of Fis1 and Mfn1 during oxidative damage and relieving mitochondrial fusion and fission abnormalities.

Previous studies have also demonstrated that inhibiting Fis1 in heart cells HL-1 not only inhibits mitochondrial fission caused by acute I/R injury, but also reduces cell death in this case[18]. Fis1 and Mfn2 have been proved to be related in co-localization of Bax and Bcl-2 in outer mitochondrial membrane in response to apoptosis stimulation[19], and their internal pathway is mainly regulated by BCL-2 protein. Generally, apoptosis-controlling proteins are classified into 3 groups: 1) Anti-apoptosis proteins including BCL-2 and BCL-XL; 2) apoptosis-promoting effector proteins with multiple BH including BAX and BAK; 3) sensitizers/activators that promote apoptosis only, including BIM and BID[20]. Our data revealed that propofol could inhibit the high expression of OGD/R injury induced Mfn1 and Mfn2 proteins. Therefore, our research mainly focused on the effects of propofol on anti-apoptosis protein BCL-2 and pro-apoptosis protein Bax. These proteins were selected because of their importance in mitochondrial regulated apoptosis. It was found that compared with the NOR, the MOD showed significantly increased protein expression of Bax, and significantly decreased protein expression of Bcl-2, and with the addition of propofol, the expression of Bcl-2 was significantly up regulated, and the expression of Bax was significantly down regulated. In addition, the activation of apoptosis-related proteins Caspase-3,Caspase-9 and COX-2 was also detected during oxidative damage, and this activation can be reversed by propofol.

Conclusion

In summary, propofol can inhibit the apoptosis of neuronal cells by improving the abnormality of mitochondrial fusion and division, reducing the release of ROS, changing the expression of downstream proteins Caspase3, Caspase9, BCL-2, Bax and COX-2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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