

Original Article

Propofol inhibits neuronal apoptosis by decreasing p53 levels via upregulating miR-592-5p

Yunong Sun¹, Zhiya Chen², Chuxiong Pan³

¹Hendrix College, 1600 Washington Ave, Conway Arkansas 72032, The U.S.; ²State Key Laboratory of Membrane Biology, College of Life Sciences, PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing 100871, China; ³Department of Anesthesiology, Beijing Tongren Hospital, Capital Medical University, Beijing 100730, China

Received September 12, 2016; Accepted September 19, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Propofol is one of a commonly used intravenous anesthetics. Many studies have shown that propofol can protect the cells from apoptosis by regulating different miRNA. In this study, we found that propofol decreased p53 level via upregulating miR-592-5p. The technique used were real time PCR, western blots and luciferase reporter assay. Moreover, we also found that propofol prevented neurons against actinomycin D, extracellular amyloid β , TNF- α and H₂O₂ treatments. This study shown that miR-592-5p could be regulated by propofol on preventing cell death.

Keywords: Propofol, p53, apoptosis, microRNA, anesthetics, miR-592-5p

Introduction

MicroRNAs (miRNAs) are short non-coding version of RNAs [1]. Precursors of miRNAs forms a single-stranded miRNA first. And that single-stranded miRNA then interacts with the complementary mRNA sequences on either 3'UTR or 5'UTR area [2]. After series of translation repression or target degradation, as a result, miRNAs regulate the protein level of target genes [2]. Propofol, 2, 6-disopropylphenol, is a commonly used intravenous anesthetic agent [3]. Many miRNAs have been examined to have implications in general anesthesia [4-8]. Different anesthetic agents can also lead different effects on mRNA expression [9]. We have previously showed that various miRNAs are altered with propofol treatment in cultured primary mouse hippocampal neurons [10]. MiR-592-5p, the miRNA in mouse hippocampal neurons that increased after propofol treatment. MiR-592-5p was found to relate to aging and aging related disease in mice in previous studies [11].

In this study, we found that miR-592-5p which targets p53 mRNA 3'UTR, is remarkably upregulated with propofol treatment. Moreover, we showed that propofol is protective against

neuronal cell death induced by various drugs, such as actinomycin D (ActD), extracellular amyloid β ($A\beta$), TNF- α and H₂O₂. Our results provide a new cellular protective mechanism by microRNA regulation induced by propofol.

Experimental procedures

Cell culture and treatments

The source of mouse that we used in this experiment was supported and regulated by Peking University Animal Care and Use Committee [12]. We took the hippocampus of wild type C57 mouse which was born under 24 hours to culture the primary neuron. The fresh fetal mouse hippocampuses were taken from the brain in Dulbecco minimum essential medium (DMEM) at 2-3°C. Then we transferred the DMEM out and dissociated the fresh fetal mouse hippocampal tissues with 0.25% trypsin (Invitrogen, Carlsbad, CA). Cells were placed in 35 ml capsules in 37°C incubator with 5% circulating CO₂ for 20 minutes. We put coverslip in the 24-well plates and added 65 μ M poly-D-lysine on the cover slips to maximize the tension of cover slips. The plates were also placed in the same incubator for 1.5-2 hours. To inactivate the trypsin, DMEM+fetal bovine serum (FBS) was added

Propofol prevents neuronal apoptosis

to the cells, which was mixed by 5% decompartmented FBS (HyClone, Logan, UT) and DMEM. We mixed the cell with pipette for 10-12 times. After precipitation appeared, we transferred the clear upper cell layer to new culture mediums with obtaining 1.5 ml DMEM in each capsul and pipetted the cells to mix well. After settled down for 2 minutes, the clear upper cell layer was transferred to tubes. After centrifuging it, we collected the precipitation and added DMEM+FBS to resuspended the cells. For culture media, we washed out the poly-D-lysine from the prepared 24-well plates and 35 ml dishes with deionized water 3 times. Then we plated the cells on poly-L-lysine (Sigma, St. Louis, MO) glass coverslips or coated plates at the density of 3×10^6 cells/ml. We incubated the neurons at 37°C in DMEM+FBS with 5% circulating CO₂. After 4-6 hours, CNB (neurobasal+B27) was added to culture media to in with 200 μM on 24-well plates and 500 mM on 35 ml dishes. We added 10 μM Cytarabine 24 hours after plating to inhibit cell growth. We changed medium every 48 hours. Cells were treated at 7 days in culture. We added 100 nM propofol to the medium for 6 hours. The cells were then lysed.

Real-time PCR

The precudure of Real-time PCR was done similar as it was described before [13]. In this experiment, we made a mixture of forward primer, reverse primer, de-iodized water and supermix instead of adding them seperately. Based on the instruction (Life Technologies Corp.), TaqMan MicroRNA assay kit for mmu-miR-592-5p measured the miR-592-5p in the cerebrospinal fluid, serum and plasma. Working as the forward primer, TRIGene reagent (GenStar BioSolutions Co., Ltd., Beijing, China) collected the total RNA from the cell. TransScript II First-Strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China) was the reverse primer that used to reversely transcribe total RNA which was collected in 2 μg. TransStart Green q PCR SuperMix UDG (Beijing TransGen Biotech Co., Ltd., Beijing, China) was used for real-time PCRs. For each sample, Real-time PCR quantifications were 3 times replicated, and the average were determined. The house-keeping gene and amplification efficiency of target were kept in approximately equal, so that comparative Ct method for relative quantification can be used. The comparative Ct method was used for the quantificaion.

Expression levels for the target gene was normalized to the GAPDH of each sample [$2^{-\Delta Ct} = 2^{-(Ct(\text{target gene}) - Ct(\text{GAPDH}))}$]. Amplification ran 45 cycles at 95°C for 30 s, 59°C for 30 s, 72°C for 30 s, 95°C for 1 min, 59°C for 30 s and 95°C for 30 s.

Sources of reagents and sequences of miRNAs and 3'UTR

Mouse miR-592-5p (5'-AUUGUGUCAUAUGCG-AUGAUGU-3'), scramble microRNA control (5'-GTGTAACACGTCTATACGCCCA-3'), mimic miR-592-5p (5'-UUGUGUCGGUGTGUCGACGCCCA-3'), miR-592-5p inhibitor (5'-AACACAGCCGUAUGACUAAGCC-3') and mutant miR-592-5p (5'-UACCUUCCGCAUACUGAUUCGG-3'), complementary sequence (5'-AACACAG-3') were all purchased from Qiagen. Several altered mouse p53mRNA 3'UTR sequences used were as following: mutant p53 mRNA 3'UTR: 5'-TCTGTGTT-3'; MAP2-3'UTR: 5'-CATATTCATTCTTCAAACCATAG-3'. All WT and modified 3'UTR sequences were cloned into pLenti-Luc-UTR or pLenti-EGFP-UTR vector backbone (Abm). MicroRNAs were transfected into SH-Sy5y cells with HiPerFect Transfection Agent (Qiagen).

In this experiment, we diluted the propofol (Sigma), staurosporine (STS, Sigma), etoposide (Etop, Sigma) and glutamate (Sigma) before use, which were stored in 100x stock. Propofol was intraperietally injected (i.p.) to 1 month old C57 mice at the amount of 200 mg/kg. Before assays, 2% propofol was used to treat cultured cells for 6 hours.

Western blots and measurement of neuronal cell death

The procedure of western blots was as same as it was described in previous study [12]. For primary antibodies in western blots, we diluted Anti-p53 (Abcam) and actin (Sigma) antibodies at 1:1000. We calculated the relative density from the total absolute density of p53/actin control, Based on the manufacturer (Roche), we measured cell death with terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) staining performed using the *in situ* cell death detection kit I [12].

Luciferase reporter assay

To determine the effect of miR-592-5p on p53 expression, The Dual-Luciferase Reporter

Propofol prevents neuronal apoptosis

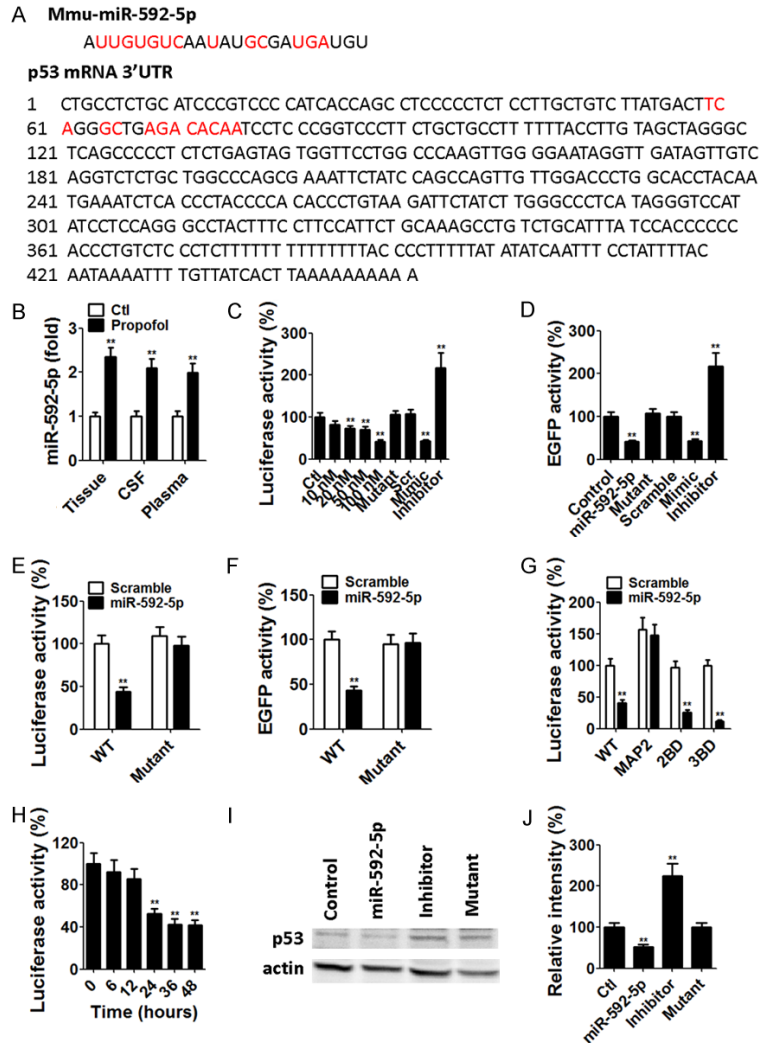


Figure 1. MiR-592-5p regulated p53 mRNA 3'UTR. A. The highlighted red was the matched sequence alignment of mouse p53 mRNA 3'UTR region and miR-592-5p. B. The amount (fold) of miR-592-5p significantly increased ($P < 0.01$) in the hippocampal tissues, CSF and plasma of mice before and after treating with propofol. C. Dose response of miR-592-5p on luciferase reporter assay in SH-Sy5y cells. D. Dose response of miR-592-5p on EGFP reporter assay in SH-Sy5y cells. E. The luciferase activity of miR-592-5p significantly decreased ($P < 0.01$) with WT p53 mRNA 3'UTR, while it slightly decreased with mutant p53 mRNA 3'UTR. F. The EGFP reporter activity of miR-592-5p significantly decreased with WT p53 mRNA 3'UTR. None changing with mutant p53 mRNA 3'UTR. G. The luciferase activity of miR-592-5p had no change with unrelated MAP2 mRNA 3'UTR. The luciferase activity of miR-592-5p significantly decreased ($P < 0.01$) with WT p53 mRNA 3'UTR, as well as p53 mRNA 3'UTR constructed with 2 times or 3 times of the putative binding domain (2BD, 3BD) of each binding site. H. Inhibition of miR-592-5p on p53 mRNA 3'UTR was decreasing by time, and it was significantly decreased after 12 hours. I. Western blot showed that p53 value in mimic miR-592-5p (lane 2) decreased, while p53 value increased in inhibitor miR-592-5p (lane 4). P53 value did not change in mutant miR-592-5p (lane 3). J. Statistics of I. Data represent Mean \pm SE ($n = 3$ for each group). **: changing significance at $P < 0.01$ comparing with control.

Assay System kit (E1910; Promega) was used in this experiment. The constructs with the fire-

fly (*Photinuspyralis*) luciferase gene downstream of the p53 3'UTR were co-transfected with the wild-type, mutant, scramble, mimic or inhibitor to miR-592-5p into SH-Sy5y cells. Firefly luminescence was detected as indicated by the manufacturer. The *Renilla reniformis* luciferase construct was co-transfected into the cells in order to control for transfection efficiency. The activity was corrected for the protein concentration of each sample and expressed as [(firefly/renilla luciferase) * 10,000]. The luminescence expression was shown as relative light unit [14].

Statistical evaluation

One-way analysis of variances (ANOVA) and Sheffé's test were used to analyze the Statistical significance. The statistic was significant at $P < 0.05$.

Results

p53 mRNA 3'UTR was a Target of miR-592-5p

By using the miRNA target prediction tool website (<http://www.microrna.org/microrna/home.do>), we found the matching complementary sequences for miR-592-5p and 3'UTR of p53 mRNA in mouse (Figure 1A, highlighted with red). In the hippocampal tissues of 1 month old mice treated with propofol, miR-592-5p was increased to about 4 folds (Figure 1B). According to previous study, miRNAs can diffuse into the plasma and CSF [15]. We compared the miR-592-5p levels with or without propofol treatment in the plasma and CSF of mice (Figure 1B).

Propofol prevents neuronal apoptosis

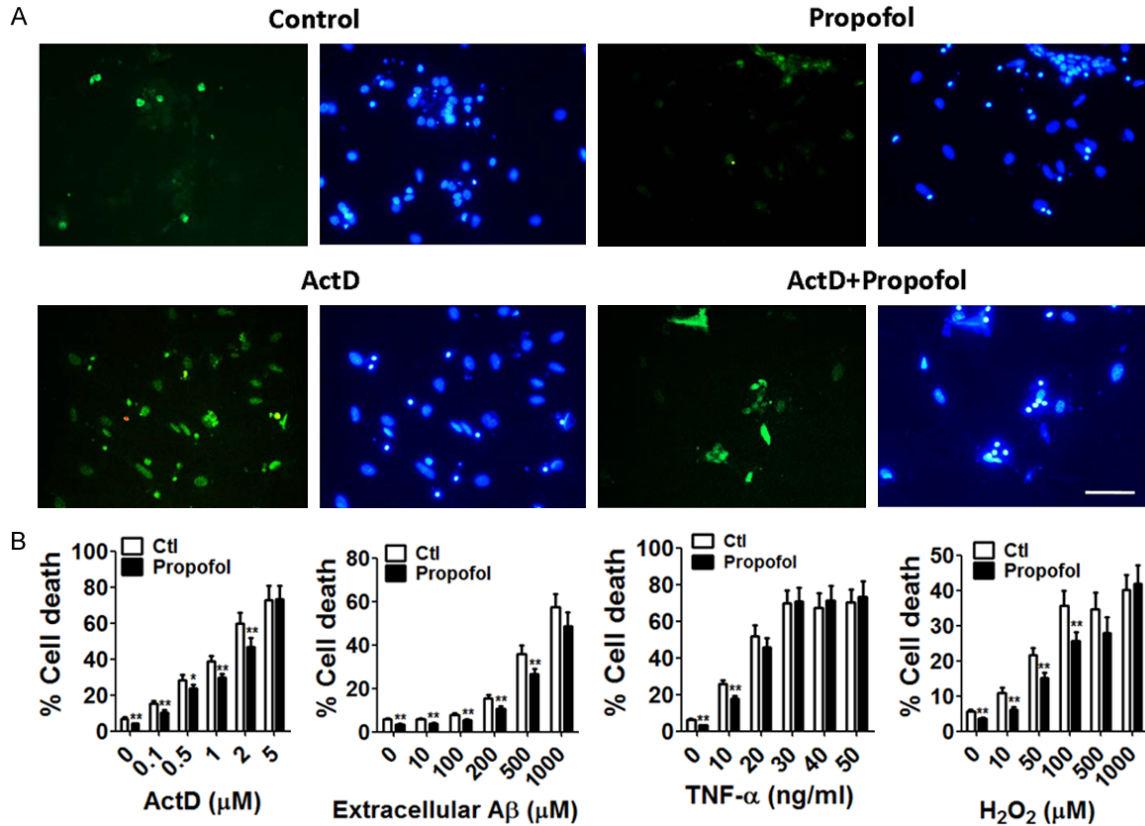


Figure 2. Propofol was protective against cell death. A. Representative images of cultured neurons treated with propofol or vehicle. Blue: Hoechst staining indicating nuclei; Green: TUNEL staining indicating apoptotic cells. Scale bar: 50 μm . B. In cultured neurons, ActD, extracellular A β , TNF- α or H₂O₂ decreased neuronal toxicity. With TNF- α treatment, the change of neuronal toxicity was not significant after 20 ng/ml. Propofol was used to treat neurons at 2% in the culture medium. Cell death was measured 6 hours after treatment. Data represent Mean + SE (n=3 for each group). **: changing significance at P<0.01 comparing with control. *: changing significance at P<0.05 comparing with control.

Regulating by p53 mRNA 3'UTR complementary region to miR-592-5p in SH-Sy5y cells, luciferase reporter assays were done with the expression of firefly. The renilla worked as an internal control for transfection efficiency. MiR-592-5p was found to inhibit luciferase activity dose-independently, while the mutant miR-592-5p and scramble control miRNA had no effect (**Figure 1C**). The mimic of miR-592-5p had a significant inhibition of the luciferase activity, while the inhibitor of miR-592-5p increased the luciferase activity (**Figure 1C**). Moreover, EGFP reporter assay also had the similar effect on inhibition of miR-592-5p to p53 mRNA 3'UTR in SH-Sy5y cells (**Figure 1D**).

We then mutated the p53 mRNA 3'UTR complementary sequence and performed luciferase assay and EGFP reporter assay with either scramble control miRNA or miR-592-5p. Our

results indicated that miR-592-5p only suppressed the luciferase activity or EGFP fluorescence with WT p53 mRNA 3'UTR, but not with mutant 3'UTR (**Figure 1E** and **1F**), suggesting the specificity of the interaction between miR-592-5p and p53 mRNA 3'UTR. To further validate that p53 mRNA 3'UTR was one of the targets for miR-592-5p, we made mutant p53 mRNA 3'UTR with 2 repeats of the putative complementary binding domain (BD) sequence (2BD) and 3 repeats of the BD sequence (3BD) for each putative binding site. Luciferase reporter system showed that miR-592-5p did not suppress the luciferase activity regulated by unrelated MAP2 mRNA 3'UTR (**Figure 1G**). Luciferase activity was greatly reduced in a BD dose-dependent manner with p53 mRNA 3'UTR (**Figure 1G**). Time course study showed that miR-592-5p inhibited luciferase activity from 12 hours after treatment and kept roughly the

same inhibition levels after 12 hours (**Figure 1H**). Western blotting of total p53 with treatments of miR-592-5p, inhibitor to miR-592-5p, and mutant miR-592-5p indicated that miR-592-5p indeed decreased p53 protein level (**Figure 1I** and **1J**), whereas inhibitor to miR-592-5p increased p53 level (**Figure 1I** and **1J**).

Propofol was protective against cell death

As indicated in the previous studies, p53 has an impact on cell apoptosis [16]. Therefore, the cell viability to different drugs in cultured neurons with or without propofol treatment was examined in this experiment. Our data showed that ActD, extracellular A β , TNF- α and H₂O₂ produced more severe cell death in neurons without treatment than neurons with propofol treatment (**Figure 2A**). Exogenous administration of propofol at 2% for 6 hours in the culture medium reduced the viability of neurons in mentioned 4 drugs (**Figure 2B**), showing that propofol may remarkably influence the neuronal viability.

Discussion

In this study, we found that with the treatment of propofol, miR-592-5p targeted to the 3'UTR of p53 mRNA and decreased p53 level in neurons. Under the insults of ActD, extracellular A β , TNF- α and H₂O₂, propofol still inhibited cell apoptosis. Although the mechanisms of the propofol protection were not yet unknown. One possible explanation could be that since the activation of p53 promotes cell death and apoptosis, decreasing p53 level could prevent cell death. Our current data are consistent with the previous observation that propofol may attenuate cell death through inhibition of p53 [17].

Propofol plays a protective role via various possible mechanisms [3]. Study showed that propofol can activate GABA_A receptor to protect against cell death which induced by acute mechanical injury and brain ischemia [18, 19]. Moreover, propofol could have an antioxidative role against various insults because propofol is structurally similar to some antioxidant, such as α -tocopherol [20]. Propofol also can decrease the development of halothane-induced injury by inhibiting the NF- κ B signal transduction pathway [21]. Other mechanisms suggested to be related to propofol protective role include

reduction of glutamate neurotoxicity, inhibition of aquaporin 4 over-expression, induction of heme oxygenase-1 expression [3, 22, 23]. Our study contributed one possible mechanism of the protectiveness of propofol, which is regulating p53 level through miRNA to prevent cell death.

Acknowledgements

This work was supported by the National Science Foundation of China (NSFC) Research Grant (81301131, 81571037 and 81671044).

Disclosure of conflict of interest

None.

Authors' contribution

Y.S., and Z.C. performed all the experiments and analyzed the data. C.P. conceptualized the study, performed analyses and drafted the manuscript.

Address correspondence to: Dr. Chuxiong Pan, Department of Anesthesiology, Beijing Tongren Hospital, Beijing 100730, China. E-mail: panded-ao@126.com

References

- [1] Lagos-Quintana M, Rauhut R, Lendeckel W and Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science* 2001; 294: 853-858.
- [2] Peters L and Meister G. Argonaute proteins: mediators of RNA silencing. *Mol Cell* 2007; 26: 611-623.
- [3] Fan W, Zhu X, Wu L, Wu Z, Li D, Huang F and He H. Propofol: an anesthetic possessing neuroprotective effects. *Eur Rev Med Pharmacol Sci* 2015; 19: 1520-1529.
- [4] Ishikawa M, Tanaka S, Arai M, Genda Y and Sakamoto A. Differences in microRNA changes of healthy rat liver between sevoflurane and propofol anesthesia. *Anesthesiology* 2012; 117: 1245-1252.
- [5] Tanaka S, Ishikawa M, Arai M, Genda Y and Sakamoto A. Changes in microRNA expression in rat lungs caused by sevoflurane anesthesia: a TaqMan(R) low-density array study. *Biomed Res* 2012; 33: 255-263.
- [6] Werner W, Sallmon H, Leder A, Lippert S, Reutzel-Selke A, Morgul MH, Jonas S, Dame C, Neuhaus P, Iacomini J, Tullius SG, Sauer IM and Raschzok N. Independent effects of sham laparotomy and anesthesia on hepatic microR-

Propofol prevents neuronal apoptosis

- NA expression in rats. *BMC Res Notes* 2014; 7: 702.
- [7] Jiang XL, Du BX, Chen J, Liu L, Shao WB and Song J. MicroRNA-34a negatively regulates anesthesia-induced hippocampal apoptosis and memory impairment through FGFR1. *Int J Clin Exp Pathol* 2014; 7: 6760-6767.
- [8] Lu Y, Jian MY, Ouyang YB and Han RQ. Changes in rat brain microRNA expression profiles following sevoflurane and propofol anesthesia. *Chin Med J (Engl)* 2015; 128: 1510-1515.
- [9] Goto G, Hori Y, Ishikawa M, Tanaka S and Sakamoto A. Changes in the gene expression levels of microRNAs in the rat hippocampus by sevoflurane and propofol anesthesia. *Mol Med Rep* 2014; 9: 1715-1722.
- [10] Luowa Shu XY, Xu Cui, Dingxi Zhou, Junfa Li, Tianzuo Li, Chuxiong Pan. Propofol treatment alters microRNA profiling in neurons. *Int J Clin Exp Med* 2016; 9: 11292-11298.
- [11] Dhahbi JM, Spindler SR, Atamna H, Yamakawa A, Guerrero N, Boffelli D, Mote P and Martin DI. Deep sequencing identifies circulating mouse miRNAs that are functionally implicated in manifestations of aging and responsive to calorie restriction. *Aging (Albany NY)* 2013; 5: 130-141.
- [12] Cui J, Wang Y, Dong Q, Wu S, Xiao X, Hu J, Chai Z and Zhang Y. Morphine protects against intracellular amyloid toxicity by inducing estradiol release and upregulation of Hsp70. *J Neurosci* 2011; 31: 16227-16240.
- [13] Wang Y, Cui J, Sun X and Zhang Y. Tunneling-nanotube development in astrocytes depends on p53 activation. *Cell Death Differ* 2011; 18: 732-742.
- [14] Zhang Y, Champagne N, Beitel LK, Goodyer CG, Trifiro M and LeBlanc A. Estrogen and androgen protection of human neurons against intracellular amyloid beta1-42 toxicity through heat shock protein 70. *J Neurosci* 2004; 24: 5315-5321.
- [15] Chan AW and Kocerha J. The path to microRNA therapeutics in psychiatric and neurodegenerative disorders. *Front Genet* 2012; 3: 82.
- [16] Tanikawa C, Matsuda K, Fukuda S, Nakamura Y and Arakawa H. p53RDL1 regulates p53-dependent apoptosis. *Nat Cell Biol* 2003; 5: 216-223.
- [17] Cui DR, Wang L, Jiang W, Qi AH, Zhou QH and Zhang XL. Propofol prevents cerebral ischemia-triggered autophagy activation and cell death in the rat hippocampus through the NF-kappaB/p53 signaling pathway. *Neuroscience* 2013; 246: 117-132.
- [18] Ito H, Watanabe Y, Isshiki A and Uchino H. Neuroprotective properties of propofol and midazolam, but not pentobarbital, on neuronal damage induced by forebrain ischemia, based on the GABAA receptors. *Acta Anaesthesiol Scand* 1999; 43: 153-162.
- [19] Hollrigel GS, Toth K and Soltesz I. Neuroprotection by propofol in acute mechanical injury: role of GABAergic inhibition. *J Neurophysiol* 1996; 76: 2412-2422.
- [20] Aarts L, van der Hee R, Dekker I, de Jong J, Langemeijer H and Bast A. The widely used anesthetic agent propofol can replace alpha-tocopherol as an antioxidant. *FEBS Lett* 1995; 357: 83-85.
- [21] Brasil LJ, San-Miguel B, Kretzmann NA, Amaral JL, Zettler CG, Marroni N, Gonzalez-Gallego J and Tunon MJ. Halothane induces oxidative stress and NF-kappaB activation in rat liver: protective effect of propofol. *Toxicology* 2006; 227: 53-61.
- [22] Grasshoff C and Gillessen T. Effects of propofol on N-methyl-D-aspartate receptor-mediated calcium increase in cultured rat cerebrocortical neurons. *Eur J Anaesthesiol* 2005; 22: 467-470.
- [23] Hans P, Bonhomme V, Collette J, Albert A and Moonen G. Propofol protects cultured rat hippocampal neurons against N-methyl-D-aspartate receptor-mediated glutamate toxicity. *J Neurosurg Anesthesiol* 1994; 6: 249-253.