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Midazolam Attenuates Autophagy and Apoptosis Caused by Ketamine by Decreasing Reactive Oxygen Species in the Hippocampus of Fetal Rats

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Abstract—Every year between 0.75% and 2% of pregnant women require surgery that is related to either the pregnancy or other medical problems in USA. Therefore, the neurodegeneration following anesthesia in a variety of animal models has attracted our attention. Neurotoxic effects of ketamine cannot be ignored. In contrast, some anesthetics, including midazolam, protect neurons and increase dendritic spine density. However, the mechanism of neuroprotection by midazolam is not clear, and whether midazolam can relieve the damage caused by ketamine is unknown. Therefore, in this study, we explored the effects of midazolam on ketamine anesthesia. We measured protein levels of cleaved-caspase-3 (c-caspase-3), beclin-1, B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), autophagy-related gene 4 (ATG4), ATG5, p62 (SQSTM1), and the autophagy marker light chain 3 (LC3) in hippocampus by Western analysis. We also measured total antioxidant capacity (T-AOC), and levels of reactive oxygen species (ROS) and malondialdehyde (MDA) in hippocampus and PC12 cells. Results showed that ketamine induced apoptosis through activation of the mitochondrial pathway by increasing the expression of c-caspase-3 and Bax, and decreasing the expression of Bcl-2 at the protein level. Ketamine also increased the expression of LC3II and ATG5, proteins, decreased the expression of ATG4 and P62, and finally induced autophagy. Ketamine promoted the production of ROS and MDA, and reduced total antioxidant capacity (T-AOC); these effects were attenuated by midazolam. In conclusion, ketamine induces toxicity in human neurons through ROS-mediated activation of mitochondrial apoptotic pathway and autophagy. The harmful effects of ketamine can be ameliorated by midazolam. © 2018 Published by Elsevier Ltd on behalf of IBRO.

Key words: ketamine, midazolam, caspase-3, LC3, pregnancy.

INTRODUCTION

Every year many pregnant women are exposed to a variety of anesthetics for surgery or diagnosis, and some also undergo obstetrics-unrelated surgery during pregnancy. Anesthetics are generally considered safe, but some can cause neurotoxicity, even at normal doses. In clinical and surgical procedures, anesthetics or anticonvulsants are used to induce sedation. Most studies on anesthetics have focused on dose and adverse effects. In addition, nerve cells in the developing brain are very sensitive to anesthetics, which have been shown to induce neural dysfunction and neuronal apoptosis both *in vivo* and *in vitro* (Jevtovic-Todorovic et al., 2003). However, the impact of anesthesia on the developing brain is now attracting more attention. There is evidence that anesthesia can affect nerve development in children (Sun, 2010), an effect that is also observed in rodents and primates (Wang et al., 2005; Slikker et al., 2007; Zou et al., 2009). Every year, many pregnant women and children undergo surgery under anesthesia; therefore, the impact of anesthesia is a cause for concern. In fact, all general anesthetics can affect the fetus by crossing the placental barrier.

Ketamine is a noncompetitive N-methyl-D-aspartate receptor antagonist commonly used as an anesthetic. Ketamine can be used as an anesthetic, although its neurotoxic effects cannot be ignored (Bai et al., 2013; Ito et al., 2015). However, as early as 1999, it was reported that repeated administration of ketamine can induce neuronal apoptosis in the developing brain at postnatal day 7 (Ikonomidou et al., 1999). Intravenous ketamine for 24 h resulted in increased neuronal cell death in rhesus monkeys (Haley-Andrews, 2006; Slikker et al., 2007). Similarly, ketamine was shown to induce neuronal apoptosis *in vitro* (Soriano et al., 2010; Liu et al., 2013). According to previous reports, ketamine can affect learning and memory, and cause behavioral disorders in the offspring of rats anesthetized during pregnancy (Li et al.,

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Abbreviations: Atg4, autophagy-related gene 4; Bax, Bcl-2-associated X protein; Bcl-2, beclin-1, B-cell lymphoma-2; c-caspase-3, cleaved-caspase-3; LC3, autophagy marker light chain 3; MDA, malondialdehyde; ROS, reactive oxygen species; SQSTM1, Atg5, p62; T-AOC, total antioxidant capacity.

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2017a,b). Anesthetics can influence neural development by affecting the nerve synapses, and neuronal survival (Stratmann et al., 2009; Culley et al., 2011). Furthermore, anesthesia-induced neuronal death may be a cause of neurocognitive impairment. Due to the ethical problems associated with investigations of human tissue, *in vitro* models are used to assess the toxicity of various anesthetics in human neurons. In contrast, some anesthetics, including midazolam, protect neurons and increase dendritic spine density (De Roo et al., 2009; Briner et al., 2010). It has also been reported that midazolam protects neurons against degeneration and improves behavioral performance (Chong et al., 2012a). In this study, we investigated the effects of ketamine and midazolam on neurons.

Ketamine causes a compensatory increase in NMDA receptors expressed by excitatory neurons, and increased Ca^{2+} levels. Studies of nerve cell stimulation by ketamine have shown that the resulting Ca^{2+} accumulation leads to mitochondrial excitotoxic injury and production of reactive oxygen species (ROS) (Wang and Slikker, 2008; Liu et al., 2013; Wu et al., 2014; Yan et al., 2014; Roux et al., 2015). Autophagy can be activated by numerous extra- and intracellular stimuli, such as starvation, ROS, and hypoxia (Kim and Guan, 2015;Klionsky et al., 2016). Accumulation of ROS within a cell activates transcription factors, and upregulates the transcription of several proteins involved in autophagy (Li et al., 2015). A group of autophagy-related genes (ATG genes), including ATG4 and ATG5, are involved in autophagy processes such as initiation, maturation and fusion of autophagosomes and lysosomal degradation (He and Klionsky, 2009;Tan et al., 2012). Apoptosis can also be activated by ROS. Treatment with 2% isoflurane for 6 h increased ROS accumulation, facilitated cytochrome c release from the mitochondria into the cytosol, induced activation of caspase-9 and caspase-3, and finally cause apoptosis (Zhang et al., 2010). Ketamine-induced cell apoptosis is reported to involve changes in the permeability of the mitochondrial membrane induced by members of the B-cell lymphoma-2 (Bcl-2) family, resulting in the release of cytochrome c and subsequent activation of the mitochondrial pathway through the production of ROS and caspase-3 (Bosnjak et al., 2012; Bai et al., 2013).

Research into developmental neurotoxicity associated with human anesthesia is impeded by ethical issues. Moreover, ketamine anesthesia may have a greater impact on the fetus, whether midazolam can mitigate the effects of ketamine is unknown. Therefore, in this study, we used the rat fetus as a model to investigate the effect of ketamine on neurons in the fetal brain. In addition, we investigated the mechanism by which midazolam reduces the neuronal apoptosis and autophagy induced by ketamine.

EXPERIMENTAL PROCEDURES

Animals

Thirty-six Wistar rats (311–357 g) were housed in polypropylene cages in a temperature- and humidity-controlled chamber with a 12-h light/dark cycle. Rats had access to water and food *ad libitum*. All experimental procedures were performed according to the guidelines approved by the Northeast Agricultural University, Harbin, China. All efforts were made to minimize the total number of animals utilized in this study.

Copulation

One randomly selected male rat and two female rats were put into a cage to enable free mating. On the second day, observations were made for the presence of a vaginal embolus in the cage and sperm in a vaginal smear. If vaginal emboli or sperm were detected in a vaginal smear, the female rat was considered pregnant, and this day was designated gestational day 0 (G0).

Drug administration and sample collection

Twenty-four pregnant female rats were randomly divided into the following groups (n = 6 per group): control (C) group, ketamine (K) group, midazolam group, and ketamine and midazolam combined (K + M) group. At G19 (equivalent to the second trimester of human pregnancy) (Clancy et al., 2001), the C group were left undisturbed in their home cages; while the K group received a 200 mg/kg dose of ketamine (Gutian Pharmaceutical Co., Ltd. Fujian Province, China) via continuous intravenous infusion for 3 h with a pump (Sinomdt Co., Ltd, Shenzhen, China) via the tail vein and the M group received a 60 mg/kg dose of midazolam (Jiangsu Nhwa Pharmaceutical Co., Ltd., Jiangsu, China) via the tail vein. In the K + M group, midazolam was injected 20 min before the administration of ketamine via the tail vein. The total volume of ketamine was less than 2 mL/100 g. This ketamine infusion induced a sedative state between light anesthesia and deep sedation that was confirmed by a lack of voluntary movement, decreased muscle tone, and minimal reaction to pain but without compromising any cardiorespiratory functions. Rats were sacrificed by decapitating after drug administration and the fetuses were recovered for collection of the hippocampus on ice. Tissues were immediately frozen in liquid nitrogen. We use 9 fetal rats, which from 3 dams (3 fetal rats/dam). We took three hippocampals of fetal rats in same dam mixed extract protein count as one sample, and there is one data for each sample.

Cell culture and treatment

PC12 cells were obtained from the College of Pharmacy of the Sun Yat-Sen University (Guangzhou, China). Cells were seeded in 25 cm^2 flasks at a density of 1 × 10^5 cells and maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin (Beyotime Biotechnology, Shanghai, China), 100 U/ml streptomycin (Beyotime Biotechnology), and 10% fetal bovine serum (FBS, biological industries). Cells were cultured at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. Twenty-four hours prior to the start of experiments, NGF was added to PC12 cells induce synaptic production. The experimental groups

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