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Protective effect of acetyl-L-carnitine on propofol-induced toxicity in embryonic neural stem cells

Fang Liu [a,*], Shuo W. Rainosek [b], Natalya Sadovova [c], Charles M. Fogle [a], Tucker A. Patterson [a], Joseph P. Hanig [d], Merle G. Paule [a], William Slikker Jr. [e], Cheng Wang [a,**]

[a] Division of Neurotoxicology, National Center for Toxicological Research/Food and Drug Administration, Jefferson, AR 72079, USA
[b] Department of Anesthesiology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA
[c] Toxicologic Pathology Associates, Jefferson, AR 72079, USA
[d] Center for Drug Evaluation and Research/Food and Drug Administration, Silver Spring, MD 20993, USA
[e] Office of the Director, National Center for Toxicological Research/Food and Drug Administration, Jefferson, AR 72079, USA

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ABSTRACT

Propofol is a widely used general anesthetic. A growing body of data suggests that perinatal exposure to general anesthetics can result in long-term deleterious effects on brain function. In the developing brain there is evidence that general anesthetics can cause cell death, synaptic remodeling, and altered brain cell morphology. Acetyl-L-carnitine (L-Ca), an anti-oxidant dietary supplement, has been reported to prevent neuronal damage from a variety of causes. To evaluate the ability of L-Ca to protect against propofol-induced neuronal toxicity, neural stem cells were isolated from gestational day 14 rat fetuses and on the eighth day in culture were exposed for 24 h to propofol at 10, 50, 100, 300 and 600 μM, with or without L-Ca (10 μM). Markers of cellular proliferation, mitochondrial health, cell death/damage and oxidative damage were monitored to determine: (1) the effects of propofol on neural stem cell proliferation; (2) the nature of propofol-induced neurotoxicity; (3) the degree of protection afforded by L-Ca; and (4) to provide information regarding possible mechanisms underlying protection. After propofol exposure at a clinically relevant concentration (50 μM), the number of dividing cells was significantly decreased, oxidative DNA damage was increased and a significant dose-dependent reduction in mitochondrial function/health was observed. No significant effect on lactase dehydrogenase (LDH) release was observed at propofol concentrations up to 100 μM. The oxidative damage at 50 μM propofol was blocked by L-Ca. Thus, clinically relevant concentrations of propofol induce dose-dependent adverse effects on rat embryonic neural stem cells by slowing or stopping cell division/proliferation and causing cellular damage. Elevated levels of 8-oxoguanine suggest enhanced oxidative damage [reactive oxygen species (ROS) generation] and L-Ca effectively blocks at least some of the toxicity of propofol, presumably by scavenging oxidative species and/or reducing their production.

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1. Introduction

Concerns have been raised that the effect of anesthetic drugs on the central nervous system (CNS) may result in long-term impairment after surgery or general anesthesia in humans and animals. Propofol (2,6-diisopropylphenol) has been widely used in clinical practice as an anesthetic and hypnotic agent because of its rapid induction and recovery with minimal side effects. Like other anesthetics, recent evidence has shown that propofol causes brain cell death in neonatal experimental animals (Vutskits et al., 2005; Cattano et al., 2008; Pesic et al., 2009; Bercker et al., 2009; Milanovic et al., 2010); a recent study reported that administration

Abbreviations: CNS, central nervous system; GABA, gamma-aminobutyric acid; L-Ca, acetyl-L-carnitine; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-uridine triphosphate (dUTP) nick end labeling; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase.

* Corresponding author at: National Center for Toxicological Research, HFT-132, Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079-0502, USA. Tel.: +1 870 543 7364; fax: +1 870 543 7745.
** Corresponding author at: National Center for Toxicological Research, HFT-132, Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079-0502, USA. Tel.: +1 870 543 7259; fax: +1 870 543 7745.
E-mail addresses: Fang.liu@fda.hhs.gov (F. Liu), Cheng.wang@fda.hhs.gov (C. Wang).

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of propofol to neonatal rats also affect the rats' neurocognitive function (Yu et al., 2013). These observations have raised the question of whether propofol has similar effects on the human brain. The adverse mechanisms of action of anesthetics are still uncertain, as well as their impact on development, and a recent cohort study suggest children under four years of age receiving general anesthesia have a higher incidence of developing learning disabilities in adolescence (Wilder et al., 2009). Therefore, the causal effects of early exposure to anesthetics merit further study.

Propofol is a popular and short acting general anesthetic. Propofol has been proposed to have several mechanisms of action, both through potentiation of $GABA_A$ (γ-aminobutyric acid type A) receptor activity (mainly), thereby slowing the channel-closing time, and also acting as a sodium channel blocker. Thus, activation of the $GABA_A$ receptor in adult brain results in a decrease of neuronal excitability by chloride influx and membrane hyperpolarization, while in the developing brain, activation of the $GABA_A$ receptor causes chloride efflux (Kahraman et al., 2008; Chen et al., 1996; Cherubini et al., 1991). Therefore, the immature neuronal membrane is depolarized. Subsequently, voltage dependent calcium channels are stimulated; and an increased calcium influx ensues, which could further activate caspase cascade, causing cell death (Pesic et al., 2009; Kahraman et al., 2008; Zou et al., 2013). In addition, other factors such as p75 neurotrophin receptor (Pearn et al., 2012) and nerve growth factor (Pesic et al., 2009) contribute to propofol-induced neuronal cell death. Recent research has also suggested that the endocannabinoid system may contribute significantly to propofol's anesthetic action and to its unique properties (Fowler, 2004; Haeseler et al., 2008).

Recent advances in our understanding of stem cell biology and neuroscience have opened up new avenues of research for detecting anesthetic-induced neurotoxicity and developing protection/prevention against anesthetic-induced neuronal injuries. Embryonic neural stem cells or precursor cells are multipotent cells in the CNS that can self-renew and differentiate into specialized cell types, such as astrocytes, oligodendrocytes and neurons. Proliferation and differentiation of neural stem cells are tightly regulated by multiple factors, including neurotransmitters, *e.g.*, GABA (Ge et al., 2007; Ben-Ari, 2002) which stimulates the $GABA_A$ receptor. Any alteration in neural stem cells/precursor cells has the potential to cause a deviation from normal brain development, therefore contributing to the morphological changes and behavioral dysfunction observed in pre-clinical and clinical studies (Lanigan et al., 1992; Trotter and Serpell, 1992).

L-carnitine, an antioxidant dietary supplement, plays an integral role in attenuating brain injury associated with mitochondrial neurodegenerative disorders (Liu et al., 2013; Zou et al., 2008). It has been reported that L-carnitine provides neuroprotective benefits in neurodegenerative and aging situations (Abdul and Butterfield, 2007; Abdul et al., 2006; Ishii et al., 2000; Zanelli et al., 2005). In this study, acetyl-L-carnitine (L-Ca; a more active agent in biological systems than carnitine itself) was utilized to evaluate the potential protective ability of antioxidants, associated with propofol administration.

In this study, an embryonic neural stem cell model was used to determine at a clinically relevant concentration of propofol: (1) how neural stem cells/precursor cells respond to stress, *e.g.*, prolonged propofol exposure; (2) how propofol exposure directs/signals the neural stem cells/precursor cells to undergo apoptosis or necrosis; (3) how their (neural stem cells) proliferation rate is affected by short-term or prolonged anesthetic exposures; (4) how anti-oxidant agents affect or alleviate the adverse effects of propofol; and (5) clinically relevant protective strategies against anesthetic-induced developmental neural damage.

2. Materials and methods

2.1. Drugs and materials

Propofol was purchased from Abbott Animal Health (Abbott Park, IL, USA) and diluted in Dulbecco's Modified Eagle's Medium (DMEM) from Invitrogen (Grand Island, NY, USA). Acetyl-L-carnitine was purchased from Sigma (St. Louis, MO, USA).

2.2. Harvest and culture of embryonic neural stem cells

All animal procedures were approved by the National Center for Toxicological Research Institutional Animal Care and Use Committee and conducted in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Embryonic neural stem cells were harvested from embryonic/gestational day 16 Sprague-Dawley rats. In brief, timed pregnant rats were euthanized by an overdose of isoflurane. After removal of the meningeal tissue, cortices from embryonic rat brain were mechanically dissociated in ice-cold Hank's calcium- and magnesium-free medium through a fire-polished Pasteur pipette and centrifuged for 10 min at 1000 rpm ($200 \times g$). The pellet was suspended and washed in Dulbecco's modified Eagle's medium (DMEM) by centrifuging for 10 min at 1000 rpm. The undifferentiated cells were evenly distributed and grown on polylysine-coated plates and Petri dishes (10 cm), with a seeding density of 3×10^4 cells/well (96-well plate). Cultures were maintained in DMEM/F12 supplemented with N2 (Gibco by Life Technologies, Grand Island, NY, USA) growth medium, complemented with EGF/BFGF (10 ng/ml) and PDGF/NT3 (5 ng/ml; Millipore, Billerica, MA, USA).

On the eighth day *in vitro* (DIV 8), when the neural stem cells were confluent, the cultures were exposed to 0 (control), 10, 50, 100, 300, or 600 μM propofol in the presence or absence of 10 μM L-Ca for 24 h.

2.3. MTT reduction cell viability assay

In viable cells, MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide], a tetrazolium dye, is reduced by mitochondria to formazan, a colored product that can be detected photometrically. Thus, the extent of MTT metabolism is an indicator of mitochondrial function/cell viability. The MTT assay was performed as previously described (Wang et al., 2000).

2.4. Lactate dehydrogenase (LDH) assay

LDH is a cytoplasmic enzyme sequestered inside viable cells with intact plasma membranes, and is released from cells with damaged membranes (*e.g.* necrotic cells). Therefore, the amount of LDH released from cells with damaged cell membranes into medium indicates the level of toxicity. After the neural stem cells were exposed to propofol for 24 h in growth medium, the medium was collected and assayed for LDH activity as described previously (Wang et al., 2005), using a cytotoxicity detection kit from Roche Applied Science (Indianapolis, IN, USA).

2.5. 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) ELISA

8-Oxo-dG and its analogs have been used as biomarkers of oxidative DNA damage and oxidative stress. To evaluate the effects of propofol on reactive oxygen species (ROS) generation and oxidative stress in neural stem cells, an 8-oxo-dG ELISA assay was conducted to measure 8-oxo-dG and its analogs in the culture media, according to the manufacturer's instructions (Trevigen, Gaithersburg, MD, USA) with slight modifications (Liu et al., 2013).