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*Research Article*

# The Utility of a Nonhuman Primate Model for Assessing Anesthetic-Induced Developmental Neurotoxicity

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**Abstract** *Background.* In studies on anesthetic-induced developmental neurotoxicity, the animals' physiological status and the depth of anesthesia during anesthesia need to be monitored. In addition, blood borne indicators of prolonged sevoflurane exposure have yet to be explored. The nonhuman primate model could have more advantages over rodent model in these aspects. *Methods.* Twenty-seven rhesus monkeys [postnatal day (PND) 5 or 6] were monitored for their physiological status during the eight-hour exposures to 2.5% sevoflurane or room air. Plasma samples collected during the exposures were analyzed for proinflammatory mediators. Tissue from the frontal cortex was examined via electron microscopy (EM) four hours following the exposure in a subgroup of animals. Micro-positron emission tomography (microPET) using the radiolabeled ligand [$^{18}$F]FEPPA, which binds to activated microglia and astrocytes in the central nervous system (CNS), was performed one day following the exposure. *Results.* Key physiological metrics observed in PND 5/6 monkeys undergoing anesthesia were not altered significantly in comparison with those for control animals. Sevoflurane-induced neural damage was confirmed by EM observations. Increased production of interleukin (IL)-6 and CCL-2 was detected in plasma at the end of the eight-hour sevoflurane exposure, and enhanced uptake of [$^{18}$F]FEPPA was observed in the frontal cortical region the day following anesthesia. The coincident increase in proinflammatory mediators in the peripheral circulation suggested that this sevoflurane-induced response may be used as an indicator of anesthetic-induced developmental neurotoxicity. *Conclusions.* The valuable information obtained in these studies demonstrates the usefulness of the nonhuman primate model in the evaluation of anesthetic-induced developmental neurotoxicity. The elevation of IL-6 and CCL-2 may be the useful indicator of anesthetic-induced developmental neurotoxicity.

**Keywords** nonhuman primate; volatile anesthetic; developmental neurotoxicity

## 1. Introduction

Prolonged exposure to commonly administrated general anesthetics at clinically relevant concentrations during the brain growth spurt has been shown to cause a spectrum of neurodegenerative alterations, including widespread apoptotic neuronal cell death and neurobehavioral deficits in later life in several animal models including rodents [1] and nonhuman primates [2]. To date, however, there have been no confirmative prospective human studies regarding general anesthetic induced developmental neurotoxicity [3, 4]. Animal models have proven useful in demonstrating that anesthetic-induced apoptotic neurodegeneration is associated with mitochondrial impairment [5,6], disturbances in the release of, and signaling by, neurotrophic factors [7,8, 9] and upregulated neuroinflammatory markers [10,11].

Due to the lack of continuous monitoring of expired $CO_2$, blood glucose levels, and acid-base status in much of the rodent work, the roles of hypercarbia, respiratory acidosis, and hypoglycemia on the increases in apoptotic neuronal cell death have not been well studied and have been suspected by some to be causative [12,13]. Nonhuman primate models are thought to be more relevant than rodent models because of their similarities to human beings in regards to the length of pregnancy, brain neuroanatomical organization, and pharmacodynamics following the administration of anesthetics [14] and these models have been used in previous studies [15,16,17]. In the current study, minimally invasive monitoring of physiological parameters in neonatal monkeys was utilized throughout the exposures such that it was possible to evaluate whether physiological homeostasis in the exposed animals was significantly affected during anesthesia.

We have previously reported on the pathogenic role of oxidative stress in anesthetic-induced developmental neurotoxicity and the neuroprotection conferred by L-carnitines,

**Table 1:** General characteristics for animals in the four treatment groups.

| Group | Number (male/female) | Gestational age (d)[#] | Age (d)[#] | Body weight (kg)[#] |
|---|---|---|---|---|
| Control | 6 (4/2) | $168.2 \pm 2.7$ | $5.5 \pm 0.2$ | $0.50 \pm 0.02$ |
| Control + ALC | 6 (3/3) | $163.5 \pm 3.9$ | $5.2 \pm 0.2$ | $0.47 \pm 0.03$ |
| Sevoflurane | 8 (5/3) | $166.8 \pm 2.1$ | $5.4 \pm 0.2$ | $0.56 \pm 0.03$ |
| Sevoflurane + ALC | 7 (4/3) | $171.5 \pm 2.7$ | $5.3 \pm 0.2$ | $0.47 \pm 0.02$ |
| Total | 27 (16/11) | $167.9 \pm 1.4$ | $5.3 \pm 0.1$ | $0.50 \pm 0.01$ |

[#] = mean $\pm$ standard error.

including acetyl-L-carnitine (ALC) [6,18,19,20]. The supplement of ALC has been shown to attenuate the neuronal injury by presumably normalizing mitochondrial energy metabolites, stabilizing mitochondrial membranes, and enhancing antioxidant activities [21]. In the current study, morphological alterations in mitochondria in frontal cortical neurons were demonstrated four hours following eight-hour sevoflurane exposures. In addition to neuronal apoptosis, an upregulation of proinflammatory mediators in the forebrain and hippocampal regions had been previously demonstrated [10,11]. Therefore, neuroinflammatory changes in the central nervous system (CNS) can be regarded as measures of anesthetic effects as well. Micro-positron emission tomography (microPET) imaging using a radiolabeled ligand specific to activated microglia and astrocytes to detect the occurrence of active inflammatory processes has been used to monitor such processes [16]. Nevertheless, markers of neurotoxicity that can be observed in readily accessible bodily fluids have long been sought [22,23]. As it seems likely that damage to the nervous system would be associated with the production of chemical markers and that such markers might appear in peripheral blood [24, 25], it was thought important to determine whether levels of proinflammatory mediators, likely markers of toxicity, could be detected in plasma following a bout of general anesthesia. Hence, in the current study, we evaluated the levels of a series of cytokines and chemokines in blood samples collected during sevoflurane exposure. Concomitantly, microPET scans using [$^{18}$F]-labeled fluoroethoxybenzyl-N-(4-phenoxypyridin-3-yl) acetamide ([$^{18}$F]FEPPA) were performed to quantify activated microglia and astrocytes in the brain following anesthetic exposure.

## 2. Materials and methods

### 2.1. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the National Center for Toxicological Research (NCTR) and conducted in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals. Neonatal rhesus monkeys (*Macaca mulatta*) were obtained from the NCTR breeding colony as described previously [15]. Briefly, monkey breeders were housed individually under a 12:12-hour light/dark cycle, provided with water ad lib and fed with high protein jumbo monkey diet supplemented routinely with fresh fruit. Female breeders were monitored daily using vaginal swabs to identify the first day of menstruation. Female breeders in the midst of their menstrual cycles were placed with a male breeder for three days. The female breeder was then monitored via ultrasound for detection of pregnancy and the first day of gestation was considered to be the first day of placement with the breeder male. All births occurred via natural delivery and the day of birth was counted as postnatal day (PND) 0. Neonatal monkeys stayed with their mothers except for the anesthetic exposure or control sequestrations, until they were weaned at six months of age.

### 2.2. Experimental groups

Twenty-seven neonatal monkeys [16 males (59.3%) and 11 females (40.7%)], born between May 2012 and June 2014, were included in the study. Monkeys on PND 5 ($n = 18$) or 6 ($n = 9$) were assigned randomly to four treatment groups: control ($n = 6$); control with coadministration of ALC (ALC, $n = 6$); sevoflurane exposed ($n = 8$); and sevoflurane exposed with coadministration of ALC ($n = 7$). The general characteristics of these animals are summarized by group in Table 1.

### 2.3. Sevoflurane exposure and control sequestration

Neonatal monkeys were exposed on PND 5 or 6 to sevoflurane (Webster Veterinary Supply, Sterling, MA, USA) within a transparent anesthesia induction chamber ($18 \times 9 \times 8$ in, E-Z Anesthesia; E-Z-Systems, Palmer, PA, USA), or to room air within an incubator (Ohio Care Plus; Ohmeda Medical, Laurel, MD, USA), respectively. Thirty minutes prior to the experiment, neonatal monkeys were separated from their mothers by briefly immobilizing the mothers with physical restraint. The neonate was then transferred to and kept in the incubator at 32 °C for 30 min before the start of experiment to allow for gastric emptying. The induction chamber was warmed from the bottom by a heat therapy pump (T/PUMP; Gaymar Industries, NY, USA). Sevoflurane was delivered at the concentration of 2.5% (v/v) through a sevoflurane specific vaporizer (Tec 7, Baxter, Dallas, TX, USA) along with USP grade oxygen (nexAir) into the chamber at a rate of about 0.5–1.0 L/min. Anesthetized monkeys were maintained in a laterally recumbent position. A charcoal filter canister was used to

absorb the waste anesthetic from the chamber. During the exposures, all neonates were given two IM doses of the anticholinergic glycopyrrolate (0.01 mg/kg): one just prior to the start of the exposure and the other six hours after in order to decrease secretions in the airway. All subjects received 5 mL of 5% dextrose and 0.45% sodium chloride (Baxter) by lavage through gastric tube four times at two-hour intervals during the eight-hour exposure procedure. In the ALC-treated groups, monkeys were administrated with ALC (Sigma-Aldrich, MO, USA) dissolved in normal saline by IP injection at 100 mg/kg body weight 1 h before and 4 h following the start of exposure either to sevoflurane or air. Following the eight-hour exposures to sevoflurane or room air, neonatal monkeys were retained in the incubator for two hours before they were taken back to their dams.

### 2.4. Monitoring of physiological parameters

During the eight-hour exposures, the monkeys were monitored noninvasively using pulse oximetry (Mouse OX Plus; STARR Life Sciences, Oakmont, PA, USA), capnography (Tidal Wave capnograph; Novametrix Medical Systems, Wallingford, CT, USA), sphygmomanometry (Cardell Veterinary Monitor; Midmark, Dayton, OH, USA), and a digital thermometer. The pulse rate, peripheral oxygen saturation of hemoglobin, concentration of expired $CO_2$, heart rate, respiration rate, rectal body temperature, systemic and diastolic blood pressures were recorded at two-hour intervals. In addition, venipuncture of an arm vessel using heparinized syringes (Terumo) was performed to collect blood (0.3 mL) at hours 0, 2, 4, 6, and 8 during the exposures. Using an automated blood gas analyzer (GEM Premier 4000; Instrumentation Laboratory, Bedford, MA, USA), values for pH, $O_2$, and $CO_2$ partial pressure; hematocrit; electrolytes (including $Na^+$, $K^+$, $Ca^{++}$, $Cl^-$); glucose; lactate; and the percentages of oxyhemoglobin ($O_2Hb$), deoxyhemoglobin (HHb), carboxyhemoglobin (COHb), and methemoglobin (MetHb) were analyzed using simultaneous CO-oximetry.

### 2.5. Analysis of monkey plasma cytokines and chemokines

Approximately 0.2 mL of each blood sample collected at hours 0, 2, 4, 6, and 8 were centrifuged in plasma separator tubes containing lithium heparin (Microtainer; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at about $2,147 \times g$ (i.e., 4,000 rpm with a radius of 12 cm from centrifuge center to the sample) at 4 °C for 10 min (Eppendorf Centrifuge 5810R, Germany). Plasma (around 0.1 mL) was then decanted using a Pasteur pipette and stored at −20 °C until further analysis. Cytokine Monkey Magnetic 29 Plex bead panel kits were purchased from Life Technologies (Novex; Frederick, MD, USA). The kits are designed to assess 29 analytes including cytokines [IL-1$\beta$ (interleukin-1$\beta$), IL-1RA (IL-1 receptor antagonist), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), IFN-$\gamma$ (interferon-$\gamma$), IP-10 (IFN-$\gamma$-induced protein-10), TNF-$\alpha$ (tumor necrosis factor-$\alpha$)], chemokines [eotaxin, IL-8, MCP-1/CCL2 (monocyte chemoattractant protein-1 or C-C motif ligand 2), MDC (macrophage-derived chemokine), MIF (macrophage migration inhibitory factor), MIG (monokine induced by gamma interferon), MIP-1$\alpha$ (macrophage inflammatory protein-1$\alpha$), MIP-1$\beta$, I-TAC (interferon-inducible T-cell alpha chemoattractant), RANTES (regulated on activation, normal T cell expressed and secreted)], and growth factors [EGF (epidermal growth factor), FGF-basic (basic fibroblast growth factor), HGF (hepatocyte growth factor), and VEGF (vascular endothelial growth factor)]. The entire assay was performed according to the instructions supplied with the kit. First, plasma samples were thawed in ice and centrifuged at $13,226 \times g$ (i.e., 13,000 rpm with a radius of 7 cm from centrifuge center to the sample) for 10 min to remove particulates. Manufacturer supplied polystyrene beads conjugated to protein-specific capture antibodies were then added to the wells of a microplate. Eight working standards were prepared in duplicate by serially diluting the reconstituted standards. The kits are designed to assess 29 analytes including fifteen cytokines, ten chemokines, and four growth factors. The plasma samples were diluted 1:2 with supplied assay buffer. The beads were washed with the wash buffer followed by addition of incubation buffer. After reconstitution, 100 mL of standard and samples were added to the wells of the microplate containing the beads. These reaction mixtures were allowed to incubate with agitation for a period of two hours during which the proteins bound to the capture antibodies. After the two-hour incubation, the beads were washed with the wash buffer followed by addition of protein specific biotinylated detector antibodies and incubated with the beads for one hour. After this incubation, the bead mixture was washed thoroughly again to remove excess antibodies. This was followed by the addition of streptavidin conjugated to fluorescent protein R phycoerythrin (Streptavidin RPE) and a 30-minute incubation during which a solid phase sandwich was formed. After washing to remove unbound streptavidin, the beads were analyzed using the luminex detection system.

### 2.6. Electron microscopy (EM)

To examine the severity and the nature of volatile anesthetic-induced neuronal damage, an additional six monkey infants (exposed on either PND 5 or 6) were randomly assigned to a sevoflurane alone group ($n = 3$) and a control, room air exposure group ($n = 3$). Brain tissues were collected from these subjects four hours after the end of the

exposures for EM observations. Samples were taken from the frontal cortex (left hemisphere) and fixed in ice-cold (4 °C) 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The tissue was washed ($3 \times 30$ min) in 0.1 M phosphate buffer (pH 7.4), postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, then washed with 25% and 50% ethanol plus 5% uranyl acetate, followed by dehydration in an ascending ethanol series and embedded in Epon. The semisections (1 $\mu$m) were counter-stained with Toluidine Blue dye and then examined under a Nikon light microscope. The thin sections were then counter-stained with uranyl acetate and lead citrate and randomly selected fields were examined at 100 kV using a Jeol-2100 electron microscope.

### 2.7. MicroPET scans and data analysis

On the day following the exposure, neonatal monkeys underwent microPET scanning (Focus 220; Siemens Preclinical Solutions, Knoxville, TN, USA) to image the radiolabeled ligand [$^{18}$F]fluoroethoxybenzyl-N-(4-phenoxypyridin-3-yl) acetamide ([$^{18}$F]FEPPA, 3D Imaging LLC, Little Rock, AR, USA). [$^{18}$F]FEPPA was used as a ligand specific for the mitochondrial translocator protein (TSPO), formerly referred to as the peripheral benzodiazepine receptor (PBR) that is expressed primarily on activated microglia and astrocytes which are thought to emerge in the presence of neuronal cell damage. A more detailed description of the [$^{18}$F]FEPPA preparation and the imaging procedures have been described elsewhere [16]. Briefly, anesthetized neonates were placed in the prone position and maintained under general anesthesia via inhalation of 1.5% isoflurane plus oxygen through a customized mask throughout the two-hour microPET scans. [$^{18}$F]FEPPA [radioactivity of 56 megabecquerel (MBq)] was administrated via intravenous injection immediately prior to the start of the brain scan. The radioactivity in the frontal and temporal cortices was captured for specific regions of interest (ROIs) that were outlined using the medical image analysis software, ASIPro (Concorde Microsystems, Knoxville, TN, USA). The radioactivity in the ROIs was converted to standard uptake values (SUVs) for statistical analysis.

### 2.8. Statistical analysis

Descriptive summary statistics are expressed as means $\pm$ standard errors of the mean (SEMs) for continuous data and as frequency (percent) for categorical/count data. A linear mixed model was used to evaluate the effect of treatment on physiological parameters, blood analytes, and proinflammatory mediators. A compound symmetry covariance structure in the context of repeated measures ANOVA was used to model covariance patterns of repeated measurements on the same animal assuming homogeneous variance/covariance. The Bonferroni correction was used for pairwise comparisons to control the Type I error rate at the nominal level. Dunnett's multiplicity adjustment was used for comparisons relative to the control group. Student's $t$-test was used for the SUV comparison between the sevoflurane-exposed group and control group without ALC. Statistical significance was assessed at the 5% level for two sided tests.

## 3. Results

*No significant physiological disturbances were observed during eight-hour sevoflurane exposures in the neonatal monkey.*

Throughout the eight-hour exposures or isolations in room air, vital signs, including heart rate, blood pressure, respiration rate, end-tidal $CO_2$ ($ETCO_2$), rectal temperature, and blood oxygen saturation, were monitored closely. Analysis of venous blood every two hours also demonstrated that there were no significant alterations in pH, glucose, hematocrit, electrolytes, $CO_2$ or $O_2$ partial pressures, or hemoglobin oxygenation throughout the exposures (Figure 1).

The respiration rate was moderately lower in the sevoflurane-exposed animals in comparison with the control animals without statistical significance ($P = .100$). Most of the time during the exposure, the differences in respiration rates between these two groups were not statistically significant ($P > .05$), with an exception at hour four ($P = .011$). In addition, the $ETCO_2$ in the sevoflurane-exposed animals was moderately increased compared with that in the control animals, yet without statistical significance ($P = .07$). Throughout the exposure time, the differences in $ETCO_2$ were not statistically significant, except at hour eight when it was significantly higher in the anesthetized animals ($25.6 \pm 2.3$) than in the control group ($14.3 \pm 2.6$) ($P = .005$). Furthermore, a combination of venous blood parameters, including oxygen saturation, $CO_2$ and $O_2$ partial pressures, $O_2$Hb, HHb, blood glucose and pH, did not differ between the sevoflurane-exposed monkeys and controls. Taken together, these data demonstrate that neither compromised ventilation, respiratory acidosis, nor hypoglycemia occurred in the sevoflurane-exposed animals. Volatile and other general anesthetics are well known to cause myocardial suppression. In the present study, the heart rate of sevoflurane-exposed neonatal monkeys was moderately reduced in comparison with that of control animals ($P = .022$) but there were no significant effects on blood pressure ($P > .05$). The decrease in HR in the sevoflurane-exposed animals occurred as early as hour two following the start of exposure ($P < .001$) and remained steady thereafter. The sevoflurane-exposed animals also experienced a slight yet significant ($P < .001$) drop in core body temperature. As five blood samples were taken during the eight hours of observation, the hematocrit in all

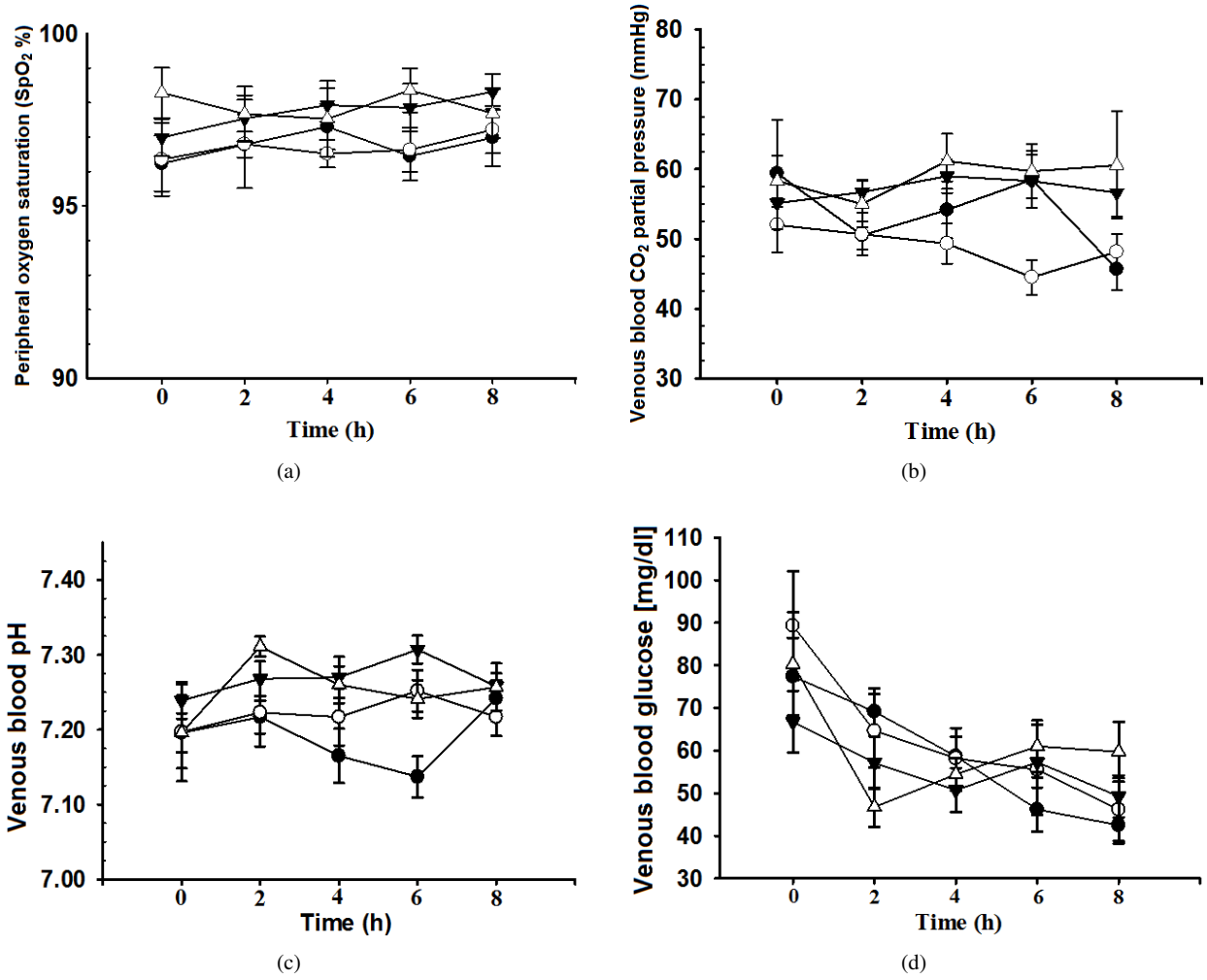


**Figure 1:** Physiological parameters monitored throughout the eight-hour exposures to 2.5% sevoflurane were not changed significantly. PND 5/6 monkeys were exposed to 2.5% sevoflurane mixed with oxygen for 8 h and control monkeys were exposed to room air only. Physiological parameters were monitored every 30 min and venous blood samples were collected at hours 0, 2, 4, 6, and 8. The level of peripheral oxygen saturation by pulse oximetry (a), venous blood $CO_2$ partial pressure (b), pH (c), and glucose (d) are presented as means $\pm$SEMs ($n = 6$ to 8). The statistical significance of treatments was assessed in comparison with the room air only control group at hour 8 (linear mixed model).

groups declined slightly over time ($P < .05$). There were no changes in plasma electrolytes ($Na^+$, $K^+$, $Ca^{++}$, and $Cl^-$) between the sevoflurane-exposed animals and controls ($P > .05$). The coadministration of ALC alone did not result in any significant effects on any of the above-mentioned parameters ($P > .05$).

*Neuronal damage typified by mitochondrial alterations following eight-hour sevoflurane-exposures.*

Sevoflurane-induced neurotoxicity was also examined at the cellular level via EM. Consistent with the noted increase in [$^{18}$F]-FEPPA uptake, direct evidence of increased neuronal damage after sevoflurane exposure was confirmed in frontal cortical tissue. Figure 2(a) shows a representative cortical pyramidal neuron from the frontal cortex of a control animal showing intact cytoplasm and mitochondrial and nuclear membranes. In contrast, sevoflurane exposure was associated with frontal cortical neurons that were characterized by the typical nuclear damage, autophagic vacuoles, mitochondrial enlargement/swelling and cytoplasmic swelling thought to represent the advanced morphological changes of necrosis, apoptosis or their combination [26] (Figure 2(b)).

*Expression of circulating proinflammatory mediators increased selectively following eight-hour sevoflurane-exposure.*

To investigate the modulatory effects of sevoflurane-exposure on the levels of cytokines in circulating blood, protein levels were assessed using a monkey-specific Luminex

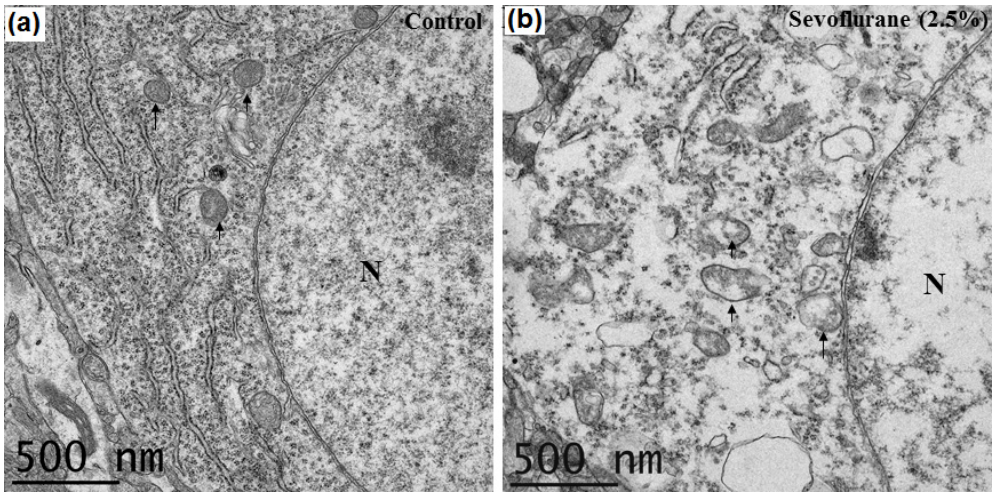


**Figure 2:** The morphology of mitochondria in a frontal cortical pyramidal neuron was altered following the eight-hour sevoflurane (2.5%) exposure as seen in this electron micrograph. PND 5/6 monkeys were exposed to 2.5% sevoflurane mixed with oxygen for 8 h. Control monkeys were exposed to room air only. EMs presented here depict the intact morphology of cytoplasmic mitochondria (arrows) in a neuron from a control animal (a) and the mitochondrial swelling (arrows) in a neuron from a sevoflurane-exposed neonatal monkey (b).

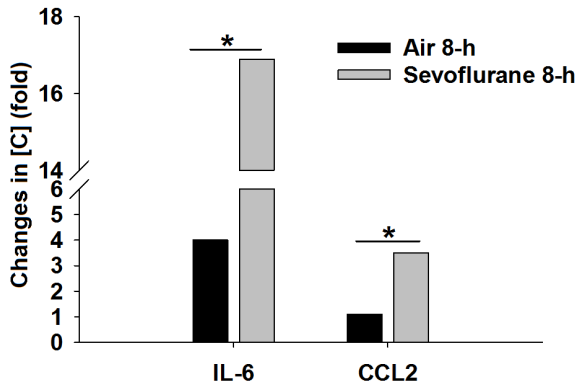


**Figure 3:** Blood levels of proinflammatory mediators IL-6 and CCL2 were increased following an eight-hour sevoflurane exposure. PND 5/6 monkeys were exposed to 2.5% sevoflurane mixed with oxygen for 8 h. Control monkeys were exposed to room air only. Plasma collected at hours 0 and 8 were analyzed for mediator levels using multianalyte magnetic bead arrays. The levels of cytokines or chemokines are presented as means ±SEMs ($n = 3$ or 4) of fold changes in plasma concentrations. The statistical significance of treatments in comparison with the air only control group at hour 8 is indicated by an asterisk $*$ ($P < .05$, linear mixed model).

assay panel designed for 29 analytes. The levels of these 29 mediators immediately before (0 h) and 8 h after exposure are presented inclusively in the supplementary table (see the supplementary table). Among the proinflammatory cytokines and chemokines analyzed, IL-6 and MCP-1 are the two mediators that were increased significantly in peripheral plasma after eight hours of sevoflurane exposure. Sevoflurane exposure was accompanied by significant increases ($\sim$ 17-fold) in IL-6 (from $9.7 \pm 3.0$ at 0 h to $163.9 \pm 24.2$ pg/mL at 8 h, $P = .06$; Figure 3) whereas in air only controls these levels only increased 4-fold (not significant; $13.8 \pm 8.5$ at 0 h vs. $55.9 \pm 28.3$ pg/mL at 8 h, $P = .57$; Table 2). There was a significant difference (2.9 fold, $P = .005$) in the levels of IL-6 between the sevoflurane-treated monkeys and the control monkeys at the eight-hour exposure time. The administration of ALC in air-only exposed monkeys also elicited a significant increase ($\sim$ 17-fold) in IL-6 production (from $12.2 \pm 3.4$ at 0 h to $206.3 \pm 80.3$ pg/mL at 8 h, $P = .012$; Table 2) and the coadministration of ALC did not attenuate the sevoflurane exposure-associated increase in IL-6 production ($252.2 \pm 50.0$ vs. $163.9 \pm 50.0$ pg/mL, in sevoflurane plus ALC group versus sevoflurane alone group, resp.; $P = .56$). The expression of monocyte chemotactic protein-1 (MCP-1, or C-C motif ligand 2, CCL2) was increased significantly ($\sim$ 3.5-fold) in the sevoflurane-treated monkeys following the exposure (from $354.1 \pm 52.4$ at 0 h to $1,229.0 \pm 232.8$ pg/mL, $P = .002$, Figure 3). This is in contrast with the insignificant change in CCL2 seen in control monkeys ($464.7 \pm 75.4$ at 0 h vs. $515.3 \pm 127.3$ pg/mL at 8 h, $P = .81$). There was a significant difference (2.4 fold, $P = .005$) in the level of CCL2 between the sevoflurane-treated monkeys and the control monkeys following the eight-hour exposure. Again, the administration of ALC in the control monkeys induced a moderate ($\sim$ 2-fold) yet significant increase in CCL-2 at the end of the eight-hour exposure ($460.8 \pm 40.5$

**Table 2:** Sevoflurane-exposure for eight hours increased levels of proinflammatory mediators [#] in peripheral blood of neonatal monkeys.

| Proinflammatory mediators | IL-6 | | CCL2 | |
|---|---|---|---|---|
| | 0 h | 8 h | 0 h | 8 h |
| Control | $13.8 \pm 8.5$ | $55.9 \pm 28.3$ | $464.7 \pm 75.4$ | $515.3 \pm 127.3$ |
| Control + ALC | $12.2 \pm 3.4$ | $206.3 \pm 80.3^*$ | $460.8 \pm 40.5$ | $987.5 \pm 185.2^*$ |
| Sevoflurane | $9.7 \pm 3.0$ | $163.9 \pm 24.2^*$ | $354.1 \pm 52.4$ | $1,229.0 \pm 232.8^*$ |
| Sevoflurane + ALC | $21.4 \pm 5.5$ | $252.2 \pm 96.9^*$ | $533.1 \pm 159.0$ | $1,243.3 \pm 308.1^*$ |

[#] = mean $\pm$ standard error.

[*] = statistically significance at $P < .05$ for 0 h versus 8 h comparisons within the same group.

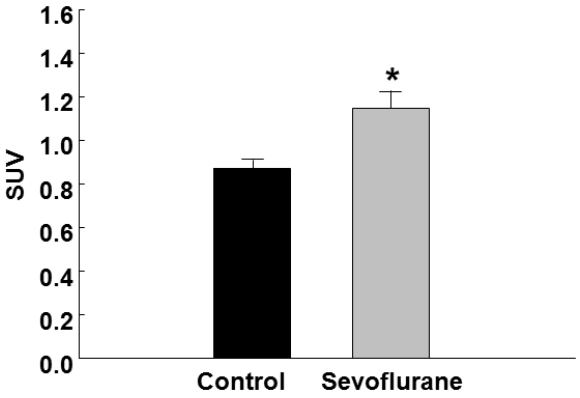


**Figure 4:** Sevoflurane-induced neurotoxic injury was monitored using microPET imaging of a ligand thought to be specific for reactive gliosis one day following the eight-hour sevoflurane exposure or room air only. The radioactivity was measured in frontal cortical regions and then converted to SUVs (presented as means $\pm$SEMs). There was a significantly greater FEPPA signal in the sevoflurane-exposed group ($n = 4$) compared to controls ($n = 4$). ($* = P < .05$, $t$-test).

at 0 h vs. $987.5 \pm 185.2$ pg/mL at 8 h, $P = .014$). Here also the coadministration of ALC to sevoflurane-exposed monkeys did not attenuate the increase in CCL-2 production associated with exposure to the anesthetic ($P = .97$).

The expression of the other proinflammatory mediators, including IL-1$\beta$, TNF-$\alpha$, G-CSF, GM-CSF, and the rest of the chemokines, was not significantly affected following exposure to sevoflurane. The expression of cytokines associated either with the activation of T helper 1 (Th1) cells (including IL-12, IL-2 and IFN-$\gamma$) or driving Th2 cell formation (including IL-4, IL-5, and IL-10) was not affected by exposure to sevoflurane. In addition to the cytokines and chemokines, expression levels for a group of growth factors were also determined. Among them, the expression of vascular endothelial growth factor was marginally increased following sevoflurane exposure (from $2.3 \pm 0.7$ at 0 h to $4.7 \pm 1.7$ pg/mL at 8 h, $P = .063$), and levels for all other growth factors were not affected (see the supplementary table).

*microPET imaging: Uptake of [$^{18}$F]FEPPA in frontal cortex increased one day following sevoflurane exposure.*

To assess reactive neuroinflammatory processes in the CNS after an eight-hour bout of sevoflurane exposure, microPET imaging using [$^{18}$F]FEPPA as a specific ligand for the 18 kDa translocator protein (TSPO), a marker of activated microglia and astrocytes, was performed the day following the exposure. The uptakes of radiolabeled ligand in frontal cortical regions of interest (ROIs) were recorded in 24 images acquired over the two-hour scans. SUVs were then determined from the accumulated radioactivity from within the frontal cortical ROIs and compared between the experimental and control groups. The mean SUV for the sevoflurane exposed group was significantly higher than that for the control group ($P < .05$, $t$-test) (Figure 4).

## 4. Discussion

Animal models in the developmental neurotoxicity research are critical to the improvement of our understanding about the impairment inflicted by general anesthetic exposure. Considering the resemblance shared by nonhuman primate and human in neurodevelopment, the neonatal monkey model provided a valuable means for preclinical assessment of damages to the CNS by prolonged general anesthetic exposure [27].

In the current study, it was demonstrated that an eight-hour exposure of neonatal monkeys to sevoflurane at a clinically relevant concentration did not significantly alter critical physiological parameters compared with those in control animals, demonstrating that the sevoflurane-induced neurotoxicity observed here was not attributed to compromised homeostatic mechanisms. Furthermore, indicators of active inflammatory responses were observed in both peripheral blood (increased expression of proinflammatory mediators) and in the CNS (enhanced uptake of a PET ligand thought to be specific for activated microglia and astrocytes).

Sevoflurane is commonly used as a pediatric general anesthetic [28, 29, 30] and the concentration of 2.5% used in the present study is consistent with those used for the maintenance of general anesthesia in clinical practice [31]. Sevoflurane exposure can also depress respiration by inhibition of neurons in the brainstem respiratory center [32]. In

the present study, however, the concentration of sevoflurane administered did not significantly affect respiration rate throughout most of the eight hours of exposure. Spontaneous respiration, supportive maintenance of the airway to prevent aspiration, pre-exposure food restriction, administration of glycopyrrolate to reduce pulmonary and gastric secretions, and maintenance of subjects in lateral recumbency helped to insure the animals' well-being throughout the sevoflurane exposures. Importantly, $ETCO_2$, partial pressures of $CO_2$ and $O_2$, percent $O_2$Hb and HHb, and arterial oxygen saturation levels in sevoflurane-exposed neonatal monkeys were maintained within ranges which were not significantly different from those of the control animals.

The eight-hour duration of the sevoflurane-exposure, while longer than the majority of procedures requiring general anesthesia in most pediatric clinical settings, is still clinically relevant. The observed sevoflurane-induced developmental neurotoxicity has yet to be associated with subsequent functional effects in the nonhuman primate as it has with other species [33] but studies looking into this phenomenon are underway [34]. The sevoflurane-induced developmental neurotoxicity that has been demonstrated in previous animal studies [33,35] and in the present work should lead to increased vigilance regarding its use in patients of an age most likely to be susceptible to the adverse effects of general anesthetics [36].

In previous studies on the anti-inflammatory effects of ALC, it was shown in various models that ALC can attenuate the production of proinflammatory mediators [37, 38]. In the current study, however, ALC did not reduce the presence of sevoflurane associated proinflammatory mediators in plasma. Moreover, ALC, when given to control animals was itself associated with moderate yet significant increases in levels of IL-6 and CCL2. It has previously been reported that L-carnitine congeners were capable of dose-dependently eliciting the production of proinflammatory mediators by peripheral blood mononuclear cells in normal control subjects [39]. As ALC modulation of immune responses was shown to be dose dependent, it seems clear that the dose of ALC used in the present study was above the threshold for stimulating proinflammatory mediator production. It will be necessary to conduct additional studies to determine an appropriate dosing regimen for ALC that minimizes the induction of proinflammatory mediators yet still affords protection against the adverse effects of sevoflurane exposure.

In the present study, we found an increased production of proinflammatory mediators in the peripheral circulation following an eight-hour exposure to sevoflurane. These included IL-6 and CCL2. Furthermore, indicators of enhanced inflammation in the frontal cortex were demonstrated the day following the exposure. The origin of the increase in proinflammatory mediators in the peripheral blood is not clear. It is possible that they might come from inflamed cells in the CNS via leakage through a compromised blood-brain barrier (BBB) or directly from peripheral immune cells sensitive to sevoflurane. It has been postulated that the immunomodulatory effects of volatile anesthetics on peripheral immune cells can be mediated by trifluorocarbon ($CF_3$) groups found on halogenated molecules [40].

Since the CNS is protected by the BBB and the exchange of molecules between the CNS and systemic circulation is under strict regulation, specific indicators of CNS injuries have been rarely found in peripheral blood. However, it has been shown that cytokines can affect communication between cells within and outside the CNS [41,42]. Plasma markers of CNS damage would be extremely valuable if their presence was temporally associated with CNS injuries, in contrast to functional neurobehavioral defects that often do not manifest until long after the insult [2,22]. The present results indicate that increases in proinflammatory mediators are detectable in peripheral blood very soon after or during a toxic exposure to sevoflurane. It is presumed that such increases will be dependent upon a variety of factors including exposure duration and concentration of agent. Should that bear out, then plasma levels of specific cytokines and/or chemokines could be used as early indicators of the onset and perhaps severity of neuroinflammation elicited by sevoflurane exposure or other toxic insults. Unfortunately, the presence of cytokines and chemokines in peripheral blood can be modulated by various disease states and, thus, confound interpretation. The immunomodulatory effects of volatile anesthetics on peripheral immune cells should be studied more closely in future studies.

In summary, it has been demonstrated that a single eight-hour exposure to sevoflurane did not cause significant alterations in critical homeostatic mechanisms. The coadministration of ALC did not result in any significant effect on depth of anesthesia. Additional studies will be needed to further characterize the proinflammatory responses seen in the circulation following sevoflurane exposure.

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**Conflict of interest** The authors declare that they have no conflict of interest.

## References

[1] V. Jevtovic-Todorovic, R. E. Hartman, Y. Izumi, N. D. Benshoff, K. Dikranian, C. F. Zorumski, et al., *Early exposure to common anesthetic agents causes widespread neurodegeneration in the*

*developing rat brain and persistent learning deficits*, J Neurosci, 23 (2003), 876–882.

[2] M. G. Paule, M. Li, R. R. Allen, F. Liu, X. Zou, C. Hotchkiss, et al., *Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys*, Neurotoxicol Teratol, 33 (2011), 220–230.

[3] T. G. Hansen, *Anesthesia-related neurotoxicity and the developing animal brain is not a significant problem in children*, Paediatr Anaesth, 25 (2015), 65–72.

[4] M. E. McCann and S. G. Soriano, *Is anesthesia bad for the newborn brain?*, Anesthesiol Clin, 27 (2009), 269–284.

[5] J. H. Yon, J. Daniel-Johnson, L. B. Carter, and V. Jevtovic-Todorovic, *Anesthesia induces neuronal cell death in the developing rat brain via the intrinsic and extrinsic apoptotic pathways*, Neuroscience, 135 (2005), 815–827.

[6] X. Zou, N. Sadovova, T. A. Patterson, R. L. Divine, C. E. Hotchkiss, S. F. Ali, et al., *The effects of L-carnitine on the combination of, inhalation anesthetic-induced developmental, neuronal apoptosis in the rat frontal cortex*, Neuroscience, 151 (2008), 1053–1065.

[7] B. Head, H. Patel, I. Niesman, J. Drummond, D. Roth, and P. Patel, *Inhibition of p75 neurotrophin receptor attenuates isoflurane-mediated neuronal apoptosis in the neonatal central nervous system*, Anesthesiology, 110 (2009), 813–825.

[8] M. L. Pearn, Y. Hu, I. R. Niesman, H. H. Patel, J. C. Drummond, D. M. Roth, et al., *Propofol neurotoxicity is mediated by p75 neurotrophin receptor activation*, Anesthesiology, 116 (2012), 352–361.

[9] J. Popic, V. Pesic, D. Milanovic, S. Todorovic, S. Kanazir, V. Jevtovic-Todorovic, et al., *Propofol-induced changes in neurotrophic signaling in the developing nervous system in vivo*, PLoS One, 7 (2012), e34396.

[10] X. Shen, Y. Dong, Z. Xu, H. Wang, C. Miao, S. G. Soriano, et al., *Selective anesthesia-induced neuroinflammation in developing mouse brain and cognitive impairment*, Anesthesiology, 118 (2013), 502–515.

[11] P. Kargaran, S. Lenglet, F. Montecucco, F. Mach, J. C. Copin, and L. Vutskits, *Impact of propofol anaesthesia on cytokine expression profiles in the developing rat brain: A randomised placebo-controlled experimental in-vivo study*, Eur J Anaesthesiol, 32 (2015), 336–345.

[12] B. Wu, Z. Yu, S. You, Y. Zheng, J. Liu, Y. Gao, et al., *Physiological disturbance may contribute to neurodegeneration induced by isoflurane or sevoflurane in 14 day old rats*, PLoS One, 9 (2014), e84622.

[13] G. Stratmann, L. D. May, J. W. Sall, R. S. Alvi, J. S. Bell, B. K. Ormerod, et al., *Effect of hypercarbia and isoflurane on brain cell death and neurocognitive dysfunction in 7-day-old rats*, Anesthesiology, 110 (2009), 849–861.

[14] C. Wang, *Advanced pre-clinical research approaches and models to studying pediatric anesthetic neurotoxicity*, Front Neurol, 3 (2012), 142.

[15] W. Slikker Jr., X. Zou, C. Hotchkiss, R. Divine, N. Sadovova, N. Twaddle, et al., *Ketamine-induced neuronal cell death in the perinatal rhesus monkey*, Toxicol Sci, 98 (2007), 145–158.

[16] X. Zhang, M. G. Paule, G. D. Newport, F. Liu, R. Callicott, S. Liu, et al., *MicroPET/CT imaging of [$^{18}$F]-FEPPA in the nonhuman primate: A potential biomarker of pathogenic processes associated with anesthetic-induced neurotoxicity*, ISRN Anesthesiol, 2012 (2012), 261640.

[17] C. E. Hotchkiss, C. Wang, and W. Slikker Jr., *Effect of prolonged ketamine exposure on cardiovascular physiology in pregnant and infant rhesus monkeys (Macaca mulatta)*, J Am Assoc Lab Anim Sci, 46 (2007), 21–28.

[18] F. Liu, T. A. Patterson, N. Sadovova, X. Zhang, S. Liu, X. Zou, et al., *Ketamine-induced neuronal damage and altered N-methyl-D-aspartate receptor function in rat primary forebrain culture*, Toxicol Sci, 131 (2013), 548–557.

[19] F. Liu, S. W. Rainosek, N. Sadovova, C. M. Fogle, T. A. Patterson, J. P. Hanig, et al., *Protective effect of acetyl-L-carnitine on propofol-induced toxicity in embryonic neural stem cells*, Neurotoxicology, 42 (2014), 49–57.

[20] C. Wang, N. Sadovova, H. K. Ali, H. M. Duhart, X. Fu, X. Zou, et al., *L-carnitine protects neurons from 1-methyl-4-phenylpyridinium-induced neuronal apoptosis in rat forebrain culture*, Neuroscience, 144 (2007), 46–55.

[21] L. L. Jones, D. A. McDonald, and P. R. Borum, *Acylcarnitines: role in brain*, Prog Lipid Res, 49 (2010), 61–75.

[22] H. Evans, *Markers of neurotoxicity: from behavior to autoantibodies against brain proteins*, Clin Chem, 41 (1995), 1874–1881.

[23] C. E. Osuna, P. Grandjean, P. Weihe, and H. A. El-Fawal, *Autoantibodies associated with prenatal and childhood exposure to environmental chemicals in Faroese children*, Toxicol Sci, 142 (2014), 158–166.

[24] A. De Luigi, S. Pizzimenti, P. Quadri, U. Lucca, M. Tettamanti, C. Fragiacomo, et al., *Peripheral inflammatory response in Alzheimer's disease and multiinfarct dementia*, Neurobiol Dis, 11 (2002), 308–314.

[25] M. G. De Simoni, P. Milia, M. Barba, A. De Luigi, L. Parnetti, and V. Gallai, *The inflammatory response in cerebral ischemia: focus on cytokines in stroke patients*, Clin Exp Hypertens, 24 (2002), 535–542.

[26] X. Zou, F. Liu, X. Zhang, T. A. Patterson, R. Callicott, S. Liu, et al., *Inhalation anesthetic-induced neuronal damage in the developing rhesus monkey*, Neurotoxicol Teratol, 33 (2011), 592–597.

[27] C. Wang and W. Slikker Jr., *Strategies and experimental models for evaluating anesthetics: effects on the developing nervous system*, Anesth Analg, 106 (2008), 1643–1658.

[28] J. Lerman, *Sevoflurane in pediatric anesthesia*, Anesth Analg, 81 (1995), S4–S10.

[29] F. Michel, R. Vialet, S. Hassid, C. Nicaise, A. Garbi, L. Thomachot, et al., *Sevoflurane for central catheter placement in neonatal intensive care: a randomized trial*, Paediatr Anaesth, 20 (2010), 712–719.

[30] P. F. White, J. Tang, R. H. Wender, R. Yumul, O. J. Stokes, A. Sloninsky, et al., *Desflurane versus sevoflurane for maintenance of outpatient anesthesia: the effect on early versus late recovery and perioperative coughing*, Anesth Analg, 109 (2009), 387–393.

[31] F. Michel and J. M. Constantin, *Sevoflurane inside and outside the operating room*, Expert Opin Pharmacother, 10 (2009), 861–873.

[32] J. Campagna, K. Miller, and S. Forman, *Mechanisms of actions of inhaled anesthetics*, N Engl J Med, 348 (2003), 2110–2124.

[33] X. Shen, Y. Liu, S. Xu, Q. Zhao, X. Guo, R. Shen, et al., *Early life exposure to sevoflurane impairs adulthood spatial memory in the rat*, Neurotoxicology, 39 (2013), 45–56.

[34] F. Liu, S. W. Rainosek, J. L. Frisch-Daiello, T. A. Patterson, M. G. Paule, W. Slikker Jr., et al., *Potential adverse effects of prolonged sevoflurane exposure on developing monkey brain: From abnormal lipid metabolism to neuronal damage*, Toxicol Sci, 147 (2015), 562–572.

[35] X. Zhang, S. Liu, G. D. Newport, M. G. Paule, R. Callicott, J. Thompson, et al., *In vivo monitoring of sevoflurane-induced adverse effects in neonatal nonhuman primates using small-animal positron emission tomography*, Anesthesiology, 125 (2016), 133–146.

[36] R. D. Mellon, A. F. Simone, and B. A. Rappaport, *Use of anesthetic agents in neonates and young children*, Anesth Analg, 104 (2007), 509–520.

[37] Z. Altun, Y. Olgun, P. Ercetin, S. Aktas, G. Kirkim, B. Serbetcioglu, et al., *Protective effect of acetyl-l-carnitine against cisplatin ototoxicity: role of apoptosis-related genes and pro-inflammatory cytokines*, Cell Prolif, 47 (2014), 72–80.

[38] G. Fortin, K. Yurchenko, C. Collette, M. Rubio, A. C. Villani, A. Bitton, et al., *L-carnitine, a diet component and organic cation transporter OCTN ligand, displays immunosuppressive properties and abrogates intestinal inflammation*, Clin Exp Immunol, 156 (2009), 161–171.

[39] N. M. Kouttab and C. De Simone, *Modulation of cytokine production by carnitine*, Mediators Inflamm, 2 (1993), S25–S28.

[40] M. Urner, L. K. Limbach, I. K. Herrmann, B. Müller-Edenborn, B. Roth-Z'Graggen, A. Schlicker, et al., *Fluorinated groups mediate the immunomodulatory effects of volatile anesthetics in acute cell injury*, Am J Respir Cell Mol Biol, 45 (2011), 617–624.

[41] J. P. Konsman, P. Parnet, and R. Dantzer, *Cytokine-induced sickness behaviour: mechanisms and implications*, Trends Neurosci, 25 (2002), 154–159.

[42] A. K. Patanella, M. Zinno, D. Quaranta, V. Nociti, G. Frisullo, G. Gainotti, et al., *Correlations between peripheral blood mononuclear cell production of BDNF, TNF-alpha, IL-6, IL-10 and cognitive performances in multiple sclerosis patients*, J Neurosci Res, 88 (2010), 1106–1112.