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Research Article

The Evaluation of Sevoflurane-Induced Apoptotic Neurodegeneration with MicroPET Using [^{18}F]-DFNSH in the Developing Rat Brain

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Abstract General anesthetic-induced apoptotic neurodegeneration confirmed in animal models caused substantial concerns over the safety of pediatric patients undergoing general anesthesia. Therefore, studies in search of translational molecular probes for labeling apoptotic neurons are imperative. The purpose of the current study is to examine the utility of [^{18}F]-DFNSH as an apoptosis probe for positron emission tomography (PET) in evaluating sevoflurane-induced neuronal apoptosis in the developing rat brain. Neonatal rats were exposed to 2.5% sevoflurane for 3, 6, or 9 h on postnatal day (PND) 7. MicroPET scans using [^{18}F]-DFNSH as radiotracer were performed in weeks 1, 3, or 8 after sevoflurane exposure, respectively. In week 1 or 3 after sevoflurane exposure, standard uptake values (SUVs) in frontal cortex in the 9-h-exposed rats, but not in 3- or 6-h-exposed rats, are significantly higher than those of the controls, suggesting increased uptake and retention of [^{18}F]-DFNSH. Interestingly, the uptake of this tracer was attenuated in 9-h-exposed rats when co-administered with L-carnitine. Collectively, the results suggest that [^{18}F]-DFNSH-PET studies can help define the temporal course of sevoflurane-induced apoptosis in the developing rat brain.

Keywords [^{18}F]-DFNSH; microPET; sevoflurane; apoptosis

1. Introduction

For almost two centuries, general anesthetics have been used to benefit countless numbers of patients by eliminating their surgical pain [36]. In the last couple of decades, the inhalation anesthetics have been used frequently to anesthetize infants and young children for surgery and other invasive procedures, or imaging studies [12,15,24]. For example, sevoflurane, a colorless, nonpungent, liquid halogenated ether inhalation anesthetic, is used to induce (at around 7% to 8%) and maintain (at around 0.5% to 3%, with or without N_2O) general anesthesia [15]. Since it was approved by the FDA in 1995, sevoflurane has been administrated frequently in pediatric patients probably because of its low solubility that facilitates precise control over the depth of anesthesia and rapid and smooth induction [16,24]. Until recently, the general anesthetics had been considered completely safe for pediatric patients. However, the demonstration of anesthetic-induced apoptotic neurodegeneration in the developing brain, identified by histological evaluations in studies using animal models [21,39,44,45,48], has raised concern about their use in children, particularly neonates. In studies using small rodents or nonhuman primates [21, 39,48], the exposure of animals to anesthetics at clinically relevant concentrations during their brain spurt growth period elicited increased apoptotic neurodegeneration in a dose- and exposure duration-dependent manner. Anesthetic-induced neuroapoptosis in the immature brain has been attributed to their actions either to antagonize the N-methyl-D-aspartate type glutamate receptor (NMDAR) or to agonize the γ-aminobutyric acid type A receptor ($GABA_AR$) [8,14,17,19,21,30,39,45]. For example, histological evaluations have confirmed that prolonged exposure of neonatal mice to sevoflurane, isoflurane, or desflurane at equipotent anesthetic concentrations induces comparable neuronal apoptosis [20]. Exposure of young animals to general anesthetics during the period of peak synaptogenesis activates both the intrinsic (mitochondrial) and extrinsic (death receptor) apoptotic pathways [48]. Although it is not yet known exactly how general anesthetics induce apoptosis, impairment of mitochondrial function has been generally recognized as a pathology central to the processes leading to apoptosis [47]. The L-carnitine system, including L-carnitine, acyl-carnitines, and relevant cellular enzymes is known to be involved in facilitating mitochondrial function via several mechanisms [3,52]. Supplementary L-carnitine or its esters have been demonstrated to be protective in a broad spectrum of neurological disorders in which the mitochondria are compromised, including anesthetic-induced neuronal apoptosis [1,3,31,32,37,38,43,52]. Whether the anesthetic-induced neurotoxicity that occurs in animal models also occurs in pediatric patients undergoing

general anesthesia has not been confirmed [28], and the histological approaches used to evaluate neuronal apoptosis in animal studies are typically not applicable in clinical settings. Therefore, in order to be able to address the issue of whether general anesthetics administered to pediatric patients are capable of inducing neuronal apoptosis, it will be desirable to identify minimally-invasive procedures that will provide the opportunity to make such determinations.

Positron emission tomography (PET) using radiolabeled tracers represents the modality of choice for non-invasive molecular imaging having high sensitivity for detecting trace amounts of the molecule of interest and the capability of quantifying biological processes *in vivo* [34]. The high-spatial-resolution microPET has been developed to provide imaging capabilities for small animals [7,9,23,35,41]. Our research group employs microPET as a minimally invasive research tool for investigating anesthetic-induced apoptotic neurodegeneration in animals [50,51]. Several classes of radiolabeled probes targeting different molecular events in the apoptosis pathways have been developed for PET imaging [7,29,35,42]. Specifically, members of a family of small-molecule biomarkers, known as the Aposense family (Aposense Ltd., Petach Tikva, Israel), that were designed to selectively label the apoptotic membrane imprint and accumulate within the cytoplasm, have been under intensive investigation recently [11,35,42]. In a previous microPET study by Zhang et al., [^{18}F]-DFNSH [5-(dimethylamino)-N′-(4-fluorobenzylidene) naphthalene-1-sulfonohydrazide], a dansylhydrazone derivative of the Aposense compound, NST-732 (developed by Zeng et al. independently [49]), was used to detect ketamine-induced neuronal apoptosis in rats. On postnatal day (PND) 35, the uptake of [^{18}F]-DFNSH in the frontal cortex was elevated, and the retention was prolonged in rats exposed to ketamine on PND 7, suggesting a prolonged increase in neuronal apoptosis [50].

In the present study, we utilized microPET with [^{18}F]-DFNSH to evaluate the exposure duration-dependent effect and time course of sevoflurane-induced apoptotic neurodegeneration in young rats. In addition, we sought to determine if a neuroprotective effect of L-carnitine could be demonstrated using PET imaging with [^{18}F]-DFNSH.

2. Materials and methods

2.1. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the National Center for Toxicological Research (NCTR) and conducted in full accordance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals.

Sprague-Dawley rat pups obtained from the NCTR breeding colony were used. All rat pups were maintained with their dam in the animal facility at NCTR with room temperature maintained at 22 ± 2 °C. The animals were provided ad lib standard rat chow and tap water under a light/dark cycle of 12 h/12 h. Rat pups of the same gender were weaned into one cage (two to three rats per cage) at three weeks of age. A total of 68 rats were randomly assigned to experimental groups as follows: sevoflurane treated (3 h, $n = 13$; 6 h, $n = 13$; 9 h, $n = 9$), 9-h-sevoflurane-exposed with L-carnitine pretreatment ($n = 6$); control ($n = 22$), and controls pretreated treated with L-carnitine ($n = 5$).

2.2. Experimental treatment

Seven-day-old rat pups in were placed in an anesthesia induction chamber (E-Z Anesthesia®, Palmer, PA, USA) into which 2.5% sevoflurane (Webster Veterinary Supply, Sterling, MA, USA) blended with oxygen was delivered from a sevoflurane-specific vaporizer (Tec 7, Baxter, Dallas, TX, USA) and an oxygen tank at 1–2 liters/min. The body temperature and blood oxygen saturation levels of anesthetized animals were monitored every hour via pulse oximetery (Mouse OX Plus, STARR Life Sciences, Oakmont, PA, USA). Sevoflurane exposures lasted 3, 6, or 9 h, after which the rat pups were returned to their dam. Control rat pups remained with their dam and received room air. L-carnitine (Sigma-Aldrich), dissolved in normal saline and sterile filtered (0.45 μm), was given by *i.p.* injection at 300 mg/kg body weight 1 h prior to and 6 h following the start of the 9-h-exposure to either sevoflurane or room air. All rat pups were returned to and kept in the animal facility for subsequent microPET studies.

2.3. Radiotracer preparation

[^{18}F]-DFNSH was prepared by 3D Imaging LLC (Little Rock, AR, USA) following procedures published previously [50]. Briefly, ^{18}F fluoride was reacted with p-trimethylammonium benzaldehyde triflate to produce ^{18}F-labeled p-fluorobenzaldehyde. The DMSO reaction solution was diluted at least tenfold with water and passed over a reverse phase cartridge which retained fluorobenzaldehyde. After washing with water, it was eluted with 1.0 mL 10% ethyl acetate in hexane. After separation of the water phase from the eluent, dansylhydrazine in methanol, 100 mL, was added and reacted. Final purification was accomplished by reverse-phase HPLC eluted with 50% ethanol in water. The collected product was evaporated to dryness, taken up in saline, and sterile-filtered for i.v. administration. Typically, about 5.5 gigabecquerel (GBq) (150 mCi) of [^{18}F]-DFNSH was produced at the end of the synthesis, 90 min end of bombardment (EOB), from 75 GBq (2 Ci) of fluoride. The typical chemical yield of fluorobenzaldehyde from fluoride was 75%, and of DFNSH from fluorobenzaldehyde was 20%. The DFNSH had specific activity at 90 min EOB of 1.1–2.2 terabecquerel (TBq) (30–60 Ci) per micromole.

2.4. MicroPET

All images of the rat brain were acquired quantitatively utilizing a Focus 220, high-resolution small animal PET scanner (Siemens Preclinical Solution, Knoxville, USA). The scanner has 96 lutetium oxyortho-silicate (LOS) detectors and provides a transaxial resolution of 1.35 mm full-width at half-maximum (FWHM) at the center of field of view. Data were collected in a $128 \times 128 \times 95$ matrix with a pixel width of 0.475 mm and a slice thickness of 0.815 mm.

2.5. MicroPET image acquisition

For all microPET scans, animals were induced and maintained under anesthesia with isoflurane (1.5%) blended with oxygen and delivered via a homemade face mask. [^{18}F]-DFNSH was administrated on 1, 3 (18.5 megabecquerel (MBq)/dose, *i.p.*), and 8 weeks (37 MBq/dose, *i.v.*) after sevoflurane or room air exposure. MicroPET scans were performed for 90 min, beginning 30 min after *i.p.* injection or immediately after *i.v.* injection of the radiolabeled DFNSH. A set of serial microPET images (18 frames, 5 min per frame) was used to assess the uptake of the tracer over the 90-min scanning period.

2.6. MicroPET data analysis

Medical image analysis software, ASIPro™ (Concorde Microsystems, Inc., Knoxville, TN, USA) was used for the quantitative analyses of the imaging data. The forebrain was selected as the location for the region of interest (ROI) because it has been shown to be one of the most vulnerable regions to the toxic effects of anesthetic exposure [44, 51]. Three-dimensional ROIs, 5 pixels in diameter, were drawn in the coronal plane with reference to transverse and sagittal planes displayed simultaneously. The radioactivity following [^{18}F]-DFNSH injection was measured using the software provided by ASIPro™. [^{18}F]-labeled DFNSH tracer accumulation in the ROIs was converted into standard uptake values [SUV = average concentration of radioactivity in ROI (mCi/mL)/injected dose (mCi)/body weight (g)].

2.7. Statistics

SUVs are expressed as means ± standard errors. The difference between the experimental and control groups was assessed using one way ANOVA (SigmaPlot for windows 11.0). A p-value $< .05$ was considered statistically significant.

3. Results

3.1. The [^{18}F]-DFNSH uptake in frontal cortices was increased following sevoflurane treatment in an exposure duration-dependent manner

Since previous animal studies have indicated that anesthetic-induced neurodegenerative effects are dependent on the anesthetic dose and exposure duration, it was important to determine in the present study whether dose- and exposure time-dependent neurotoxic effects would also be detected using a microPET approach. Here, rat pups were exposed to sevoflurane on PND 7 at a fixed concentration for 3, 6, or 9 h. To compare the effects of exposure duration on the uptake of DFNSH, the SUVs for the ROIs in the frontal cortices were plotted against exposure duration (Figure 1). One week after exposure to sevoflurane (on PND 14), the SUVs in all sevoflurane-exposed groups were elevated to various extents over all 18 time frames, compared to controls. However, only those in the group exposed to sevoflurane for 9 h were significantly different controls, at least from 2.5 to 62.5 min during the 90-min imaging session ($p < .05$, one way ANOVA). Thus, the exposure duration effects noted with sevoflurane-induced apoptotic neurodegeneration was also evident using microPET imaging with [^{18}F]-DFNSH.

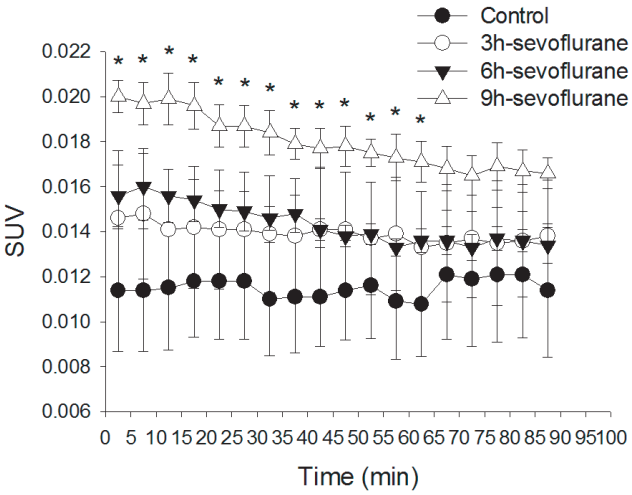

Figure 1: *Prolonged exposure of sevoflurane on PND 7 leads to increased [^{18}F]-DFNSH uptake in frontal cortices one week later.* On postnatal day (PND) 7, the rats were exposed to sevoflurane (2.5%) for 3 h ($n = 4$), 6 h ($n = 4$), or 9 h ($n = 6$); control rats ($n = 9$) were exposed to room air for 9 h. One week after sevoflurane exposure (on PND 14), standard uptake values (SUVs) for [^{18}F]-DFNSH were obtained from frontal cortical areas (shown are means ± standard errors). SUVs from rats exposed to sevoflurane for 9 h were significantly higher than those in control rats over the first 60 min of the scan ("$*$" = $p < .05$, one-way ANOVA). SUVs from the 3- or 6-h exposure groups were not significantly different from those in control animals ($p > .05$, one-way ANOVA).

3.2. The time course of [^{18}F]-DFNSH uptake in frontal cortices following sevoflurane exposure

In contrast to cross-sectional studies that employ traditional, terminal histological approaches, microPET imaging using

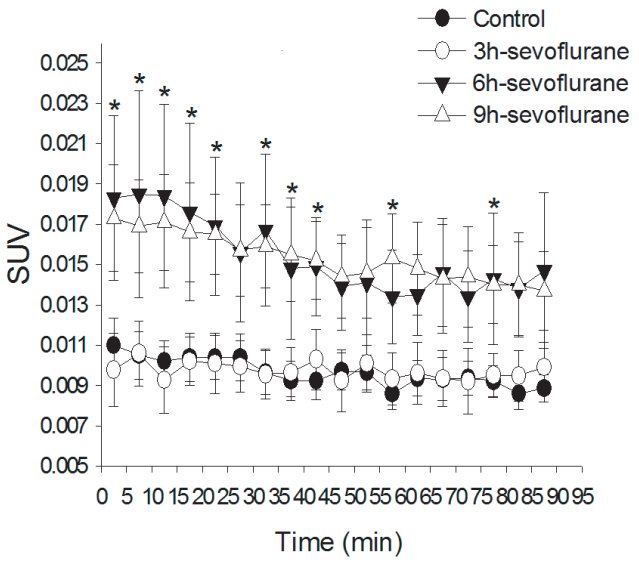

Figure 2: *The uptake of [^{18}F]-DFNSH in the frontal cortices of sevoflurane-exposed rats remained significantly higher than controls 3 weeks after exposure (PND* 28*).* Following the administration of [^{18}F]-DFNSH, SUVs (means $\pm$ standard errors) in the frontal cortices remained significantly higher in animals exposed to sevoflurane for 6 or 9 h ($n = 5$ per group) than in controls ($n = 9$) ("$*$" = $p < .05$, one-way ANOVA).

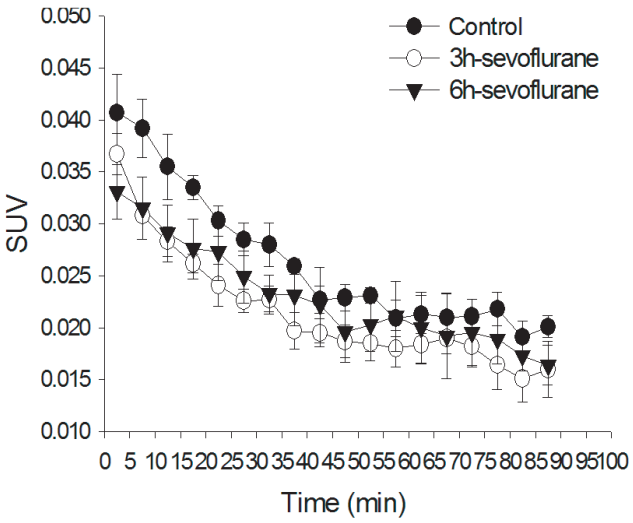

Figure 3: *Eight weeks after exposure, there were no significant differences in uptake of [^{18}F]-DFNSH in frontal cortices among sevoflurane-exposed rats and control rats.* Following the administration of [^{18}F]-DFNSH, *i.v.*, SUVs in the frontal cortices (means $\pm$ standard errors) were not significantly different between groups ($p > .05$, one-way ANOVA; $n = 4$ per group).

radiolabeled tracers can be carried out longitudinally in the same subjects relatively non-invasively. In this manner, the time course of anesthetic-induced apoptotic neurodegeneration, as depicted by the levels of radiolabeled DFNSH uptake *in vivo* can be determined with relatively few subjects. In a previous study, ketamine-induced apoptotic neurodegeneration was detectable in rats for up to four weeks (on PND 35) following exposure using microPET imaging with [^{18}F]-DFNSH [50]. The time-course over which [^{18}F]-DFNSH uptake will be detectable following sevoflurane exposure has not yet been determined.

Three weeks after the experimental exposure (on PND 28), the DFNSH SUVs for both the 6- and 9- h exposure groups remained significantly elevated in comparison with those for control subjects (Figure 2). At multiple, specific times during SUV determination, there were significant differences between the 6- and 9-h exposure groups and controls ($p < .05$, one way ANOVA, see Figure 2). The SUVs seen in the 9-h-exposed subjects at around 3 weeks after exposure to sevoflurane (on PND 28) were slightly less than those observed at around 1 week after exposure to sevoflurane (on PND 14), whereas those in the 6-h-exposed subjects remained at similar levels. The SUVs for the 3-h-exposed subjects decreased in comparison with those observed at around 1 week after sevoflurane exposure (on PND 14), remaining close to those for the control group throughout the imaging period. Eight weeks after the experimental exposures (PND 63), SUVs for the 3- and 6-h exposure groups were not significantly different from those for the control group ($p > .05$, one-way ANOVA; Figure 3).

3.3. Sevoflurane-induced [^{18}F]-DFNSH uptake in frontal cortices was attenuated by L-carnitine pretreatment

There are continuing efforts to find compounds that can protect cells in the developing brain from the adverse effects of anesthetic exposure [26,27,46,52]. It would be useful to determine whether microPET imaging using [^{18}F]-DFNSH is capable of monitoring the response of subjects to the effects of putative protective agents. Exogenous L-carnitine has been shown to protect neurons when it is co-administrated with anesthetics [52]. Therefore, in the current study, we sought to determine the effects of L-carnitine on the uptake of the radiolabeled apoptosis probe following sevoflurane exposure. Here, L-carnitine (300 mg/kg, *i.p.*) was given twice in an effort to prolong the period over which it would be present with sevoflurane: 1 h prior to and 6 h following the beginning of sevoflurane or room air exposure. Because the SUVs for the control group injected with L-carnitine alone ($n = 5$) were very close to those of the uninjected control group (without L-carnitine; $n = 5$), the SUVs from these two groups were pooled together for the statistical analysis. Eight days after the experimental exposures (on PND 14), the SUVs in the L-carnitine plus sevoflurane group were reduced notably in comparison to

those of sevoflurane group without L-carnitine (Figure 4). In contrast to the significant differences in SUVs between the 9-h-exposed group and the control group ($p < .05$, one-way ANOVA), there were no significant differences in SUVs between the group with 9-h-sevoflurane exposure co-administered with L-carnitine and the control group.

4. Discussion

In current study, it was found that the uptake of [^{18}F]-DFNSH in frontal cortex was increased in an exposure-duration dependent manner following sevoflurane-exposure on PND 7 for at least 3 weeks, and the neuroprotective effect of L-carnitine was demonstrated with remarkable attenuation of radioactivity uptake induced by exposure to sevoflurane. It can be suggested that microPET in combination with [^{18}F]-DFNSH could be used to monitor sevoflurane-induced apoptotic neurodegeneration in the developing rat brain with regard to the evolvement over time and response to co-administered protective agent.

Despite the clear evidence of massive anesthetic-induced apoptotic neurodegeneration in animal studies, the major differences between the animal models of developing neurotoxicity and general anesthesia administered in clinical settings continue to hamper the clear translation of the pre-clinical findings to clinical practice [2,10,19,25]. In addition, since apoptosis is the major physiological mechanism utilized for eliminating superfluous cells during development, some have questioned whether the anesthetic-induced increase in apoptosis was inappropriately eliminating neurons or simply accelerating a normal physiological process [25]. On the other hand, neuronal signaling via NMDARs and $GABA_A$Rs are of critical importance to synapse formation, neuron maturation, and neuronal circuit development during development [4,5, 13]. Disrupting the NMDAR- and $GABA_A$R-mediated neuronal transduction by prolonged exposure of infants and young children to general anesthetics could, thus, logically be viewed detrimental to the normal development of the central nervous system (CNS) and potentially result in neurocognitive dysfunction later in life [18,22,28,33, 40]. It is, therefore, prudent to explore approaches for detecting apoptotic neurodegeneration *in vivo* with a view toward developing markers of such that could be useful both pre-clinically and clinically.

A few classes of radiolabeled tracers have been developed that target several molecular events that occur during apoptosis including: annexin-V and its modified forms that bind to externalized phosphatidylserine (PS); isatin sulfonamide analogs which inhibit the executioner caspases which are activated during apoptosis; phosphonium cations which help visualize the collapse of mitochondrial membrane potentials in apoptotic cells; and a set of small-molecule compounds which bind

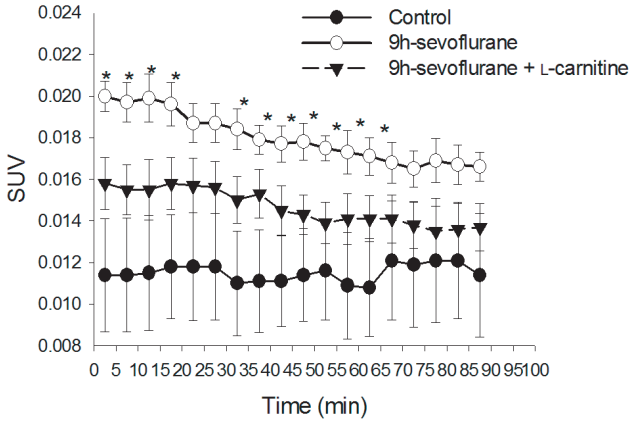

Figure 4: *Sevoflurane-induced [^{18}F]-DFNSH uptake in frontal cortices was attenuated by L-carnitine co-administration.* Rats ($n = 6$) were co-administered with L-carnitine at 300 mg/kg of body weight at 1 h prior to and 6 h following the beginning of a 9-h sevoflurane exposure in comparison with those with 9-h sevoflurane exposure alone ($n = 4$) or control rats that were injected with L-carnitine and exposed to room air only for 9 h ($n = 6$). About one week later (on PND 14), SUVs from the frontal cortices (means $\pm$ standard errors) demonstrated that L-carnitine was able to remarkably attenuate the effects of sevoflurane alone: the asterisks denote the significant difference in radiotracer uptake between the 9-h-sevoflurane exposed rats and control rats ("$*$" = $p < 0.05$, one-way ANOVA).

to cell membranes and accumulate in the cytoplasm of apoptotic cells, a process probably mediated by apoptosis-induced phospholipid scramblase activation and cellular acidification [6,29,42]. DFNSH has been shown using fluorescence microscopy to accumulate selectively *in vitro* in the cytoplasm of paclitaxel-induced apoptotic cells [49]. In a previous microPET study, ketamine-induced apoptotic neurodegeneration in rats was detected using [^{18}F]-DFNSH as evidenced by an increased uptake of the radiotracer in the frontal cortices [50].

In the current microPET study, it was found that, severity of exposure-induced apoptosis was dependent upon exposure duration with longer exposures leading to higher levels of uptake of [^{18}F]-DFNSH. MicroPET imaging using [^{18}F]-DFNSH appears to provide adequate sensitivity for detecting anesthetic-induced apoptotic neurodegeneration and assessing the severity of the pathology in a quantitative manner. With minimal invasiveness, it is possible to repeatedly collect microPET images and, thus, monitor the temporal aspects of anesthetic-induced neurodegeneration. It has been confirmed using caspase-3 immunocytochemistry and cell degeneration-selective silver stain that a wave of neuronal apoptosis is initiated within one day or so following anesthetic exposure [19,21].

The time course over which anesthetic-induced apoptosis continues has not yet been clarified, but the data from the present study indicate that, if the uptake of [^{18}F]-DFNSH is indicative of the presence of apoptotic neurons, then the apoptotic neurodegeneration that occurs in the frontal cortices is detectable for at least three weeks following exposure to sevoflurane.

In animals co-administered L-carnitine, there was clear attenuation of the uptake of radiolabeled DFNSH, suggesting an ability of L-carnitine to ameliorate at least some of the adverse effects of sevoflurane exposure. MicroPET imaging, thus, is also likely to be useful for monitoring the effectiveness of treatments protective against anesthetic-induced neurotoxicity. Behavioral assessments could be carried out in parallel with microPET imaging studies since the subjects remain intact and this combination should prove invaluable for correlating microPET signals with functional outcomes.

In summary, the neural apoptosis induced by prolonged exposure to sevoflurane in neonatal rats as indicated by [^{18}F]-DFNSH uptake appears to be long lasting: the signal is detectable for up to three weeks after anesthetic exposure. The neuroprotective effect of L-carnitine against sevoflurane-induced neural apoptosis was evidenced by significant decreases in DFNSH SUVs in animals pretreated with L-carnitine. Histological examinations are needed to corroborate the imaging findings present here.

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