# Sevoflurane in combination with propofol, not thiopental, induces a more robust neuroapoptosis than sevoflurane alone in the neonatal mouse brain

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**Abstract**

*Purpose* Sevoflurane is the most widely used volatile anesthetic of general anesthesia. In children and neonates, it is commonly used alone or in combination with thiopental or propofol. A few recent studies reported that sevoflurane induced neuronal death in the developing rodent brain. We measured the neurotoxicity of these anesthetics at clinical doses, alone and in combination, in the developing mouse brain.

*Methods* Seven-day-old C57BL/6 mice were randomly assigned to 6 treatment groups. Three groups were exposed to 3% sevoflurane for 6 h after injection of saline, thiopental (5 mg/kg), or propofol (10 mg/kg), whereas three groups were exposed to room air for 6 h after injection of equal doses of saline, thiopental, or propofol. Apoptosis in the hippocampal CA1 region (CA1) and retrosplenial cortex (RC) was assessed using caspase-3 immunostaining.

*Results* Sevoflurane alone caused significantly higher apoptosis in the CA1 compared with saline plus air ($P$ = 0.04). Sevoflurane in combination with propofol resulted in significantly greater numbers of apoptotic neurons than svoflurane alone in both the CA1 and the RC ($P$ = 0.04). However, there was no significant difference in apoptotic neuron density in both the regions between the groups treated with sevoflurane alone and in combination with thiopental ($P$ = 0.683).

*Conclusion* Sevoflurane alone can induce neuronal apoptosis, and this effect is enhanced by propofol. Thiopental did not exacerbate the neurotoxicity of sevoflurane. There is the possibility that the combination of sevoflurane and propofol is more harmful anesthetic technique than sevoflulane alone in pediatric patients.

## Introduction

Anesthesic exposure, even during critical stages of neurodevelopment, has been considered safe and without any adverse long-term consequences. However, many recent animal studies suggest that exposure to certain anesthetics during periods of rapid synaptogenesis can evoke neuronal apoptosis and inhibit neurogenesis, leading to lasting cognitive impairments [1].

Many clinical anesthetics potentiate inhibitory transmission mediated by gamma aminobutyric acid type A ($GABA_A$) receptors and/or inhibit excitatory glutamateric transmission mediated by N-methyl-D-aspartate (NMDA) receptors [2]. Simultaneous administration of a $GABA_A$ agonist and NMDA receptor antagonist in particular can evoke widespread neuronal apoptosis in the developing brain, suggesting that anesthetics targeting these ionotropic receptors may have deleterious effects on the infant brian [3, 4]. Exposure of infant rats to midazolam, nitrous oxide, and isoflurane for 6 h (each at clinically relevant doses) caused widespread neurodegeneration and cognitive deficits that persisted to adulthood [3]. The NMDA antagonist ketamine and a $GABA_A$ agonist (diazepam, thiopental, or propfol), partcularly in combination, induced neuronal apoptosis and long-term motor and cognitive dysfunction in young mice [4-6].

However, these particular anesthesia cocktails are no longer used for most pediatric anesthetic procedures.

Sevoflurane (2,2,2-trifluoro-1-[trifluoromethyl]ethyl fluoromethyl ether) is currently the most widely used inhalation anesthetic for pediatric surgery and cesarean delivery because of its properties of rapid inhalational induction, tolerable odor, quick emergence and relative cardiovascular stability for infants and children [7]. In a survey conducted at the Society for Pediatric Anesthesia, 95% of anesthesiologists reported the use of sevoflurane [8]. Sevoflurane enhances $GABA_A$ receptor currents [9] and likely also blocks NMDA receptors [10]. For induction of routine pediatric anesthesia, sevoflurane is usually used alone or in combination with thiopental or propofol. A few recently published studies reported neurotoxic effects of sevoflurane alone [11-13] but no enhancement when used in combination with other substances.

In light of evidence demonstrating apoptosis in the neonatal brain following administration of sevoflurane, thiopental, and/or propofol, and the frequent use of these agents in current pediatric anesthesia practice, we examined the neurotoxic effects of these agents, alone and in combination, in neonatal mice during the brain development period.

## Materials and methods

### Animals

All experiments were conducted according to the institutional ethical guidelines for animal experiments of Mie University and were approved by the Committee for Animal Research at Mie University School of Medicine, Tsu, Mie, Japan. The C57BL/6 mice were purchased from SLC (SLC Japan Inc., Shizuoka, Japan). The animals were maintained on a 12-h light-dark cycle (lights on from 07:00 to 19:00) in a temperature controlled room (21 ± 1 °C). The mice had *ad libitum* access to water and food. Both male and female mice were used in the experimental and control groups. Control and experimental pups were drawn randomly from the same litters. Efforts were made to minimize the number of animals used and their suffering.

### Anesthesia Treatment

Postnatal day 7 (P7) mice were randomly assigned to one of six treatment groups (n = 8 per group): (1) a vehicle (Veh) group administered a single subcutaneous injection of saline followed by 6 h exposure with air, (2) a Thio group given a single subcutaneous injection of thiopental (5 mg/kg) followed by 6 h exposure to air, (3) a Prop group given a single subcutaneous injection of propofol (10 mg/kg) followed by 6 h exposure to air,

(4) a Sevo group administered a single subcutaneous injection of saline followed by 6 h exposure to 3% sevoflurane, (5) a Thio+Sevo group was given a single subcutaneous injection of thiopental (5 mg/kg) followed by 6 h exposure to 3% sevoflurane, and (6) a Prop+Sevo group was given a single subcutaneous injection of propofol (10 mg/kg) followed by 6 h exposure to 3% sevoflurane. Thiopental and propofol were disolved in saline and all mice were injected with an equal volume of solution per unit weight (20 ml/kg). For sevoflurane or air exposure, mice were placed in an acrylic box connected to an anesthetic machine. The total gas flow was 2 l/min. Air was used as the carrier for sevoflurane. During anesthetic exposure, the mice were maintained at 38°C using a heating pad. The concentration of sevoflurane (3%) was based on previously published work [13], and fell within the range used for pediatric anesthesia [7, 14]. At 3% sevoflurane, the 6 h exposure had no detrimental effect on cerebral perfusion, arterial blood gas concentrations, or blood glucose concentration in neonatal C57BL/6 mice [13]. The doses of thiopental (5 mg/kg) and propofol (10 mg/kg) administered alone are low and do not increase neonatal neurodegeneration [4]. These low doses were chosen to avoid suppression of respiration when administered with 3% sevoflurane because the infant mice are too small to permit adequate airway management.

$SpO_2$ Analysis

To avoid any potential complications from invasive manipulations and to reduce the number of experimental animals, blood gases and end tidal $CO_2$ were not measured. However, Percutaneous oxygen saturation ($SpO_2$) was measured using a $SpO_2$ monitoring system (Mouse STAT; Kent Scientific Co., Torrington, CT). A monitoring sensor was attached to the leg of each mouse.

Laser Doppler

Cerebral blood flow (CBF) was measured by a laser-doppler blood flowmeter (ALF21N; ADVANCE, Tokyo, Japan). Mice were taken out of the chamber before and every hour during anesthetic treatment and were placed face down on the floor while being continuously exposed to sevoflurane *via* a tube with its opening positioned at the nose of the animals. Veh group mice were exposed to air for corresponding period of the anesthetized mice. Their head skins were peeled for scanning CBF, and data were captured using appropriate software (LabChart version 8; ADInstruments, Nagoya, Japan). The perfusion response is presented in arbitrary perfusion units. Arbitrary perfusion unit values were compared among groups at baseline and at 1 h intervals for 6 h.

Histopathological Studies

Immediately following experimental treatments, mice were perfused transcardially with 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde. Brain sections were prepared and processed for activated caspase-3 immunostaining, a well-established procedure to measure neonatal apoptosis in the developing brain [12, 15, 16]. In brief, brains were removed and post-fixed overnight in the same fixative at 4°C. Serial coronal sections of 80 μm thickness were cut on a vibratome, blocked for 1 h in blocking solution (PBS with 2% BSA, 0.2% skim milk powder, and 0.1% triton X-100), followed by incubation in rabbit anti-activated caspase-3 polyclonal antibody (1:250; Cell Signaling, Danvers, MA; 9661). Immunolabeled sections were washed and incubated in a goat anti-rabbit Ig conjugated to Alexa Fluor 488 (1:250, A11034; Invitrogen, Carlsbad, CA) or goat anti-mouse antibodies conjugated to Alexa Fluor 594 (1:250, A11032; Invitrogen) for 4 h at room temperature.

Caspase-3-positive cells were counted in the hippocampal CA1 region (CA1) and retrosplenial cortex (RC). These two regions were chosen because they are particularly vulnerable to anesthesia-mediated neurodegeneration [3, 11, 13, 17] and are integral components of the extrahippocampal circuit, which have been identified as important for mediating learning and memory functions [18]. Images were acquired using an Olympus FV1000 confocal microscope controlled by FV10-ASW 1.7 software

(Olympus Corporation, Tokyo, Japan). The images were then printed on paper, and an investigator blind to the treatment history counted the number of caspase-3-positive cells in the CA1 and RC. These regions were defined by plate 48–52 in the Paxinos and Franklin mouse brain atlas [19]. The density of caspase-3 positive cells in a particular brain region was calculated by dividing the number of caspase 3 positive cells by the area of that brain region.

Statistical Analysis

Previous experiments in neonatal rodents and nonhuman primates demonstrated extensive neurotoxicity in response to anesthetics and other toxins, allowing for the use of relatively small numbers of experimental animals (3−8) per treatment group [3, 4, 11, 13, 15-17, 20, 21]. Statistical comparisons between the groups were performed by using Kruskal-Wallis test. Two-way ANOVA for repeated measures was performed to examine differences of the $SpO_2$ and CBF. All calculations were performed using the SPSS program (IBM Co., Tokyo, Japan). Statistical significance was accepted at $P < 0.05$.

**Results**

To test the potential neurotoxicity of sevoflurane (Sevo) alone and in combination with thiopental (Thio) or propofol (Prop), P7 mice were divided into 6 treatment groups and exposed to air or 3% Sevo for 6 h following injection with saline (Veh and Sevo groups), 5 mg/kg Thio (Thio and Thio+Sevo groups), or 10 mg/kg Prop (Prop and Prop+Sevo groups). All 48 animals survived the anesthetic exposures. Mice subjected to Sevo, Thio+Sevo, and Prop+Sevo reached deep anesthesia as indicated by absent or only minor reactions to pain stimuli. Administration of Thio and Prop alone with air exposure resulted in transient sluggishness but no deep anesthesia. $SpO_2$ did not differ significantly among groups (Table 1; two-way repeated measures ANOVA, groups: $F$ = 2.22, $P$ = 0.07, time: $F$ = 0.72, $P$ = 0.64, interaction between groups and time: $F$ = 0.41, $P$ = 0.99), suggesting that all anesthesia treatments did not induce significant disturbances in oxygenation. Moreover, to assess adequacy of cerebral perfusion, we measured CBF during anesthesia using a laser-doppler blood flowmeter. There were no significant differences in CBF at any point during the 6 h of anesthesia among groups (Table 2; two-way repeated measures ANOVA, groups: $F$ = 0.51, $P$ = 0.77, time: $F$ = 1.12, $P$ = 0.31, interaction between groups and time: $F$ = 0.91, $P$ = 0.61).

Caspase-3-positive cells were observed sporadically in both the CA1 and the RC of Veh group mice (Fig. 1). Neither Thio nor Prop injection alone induced a significant

increase in the number of caspase-3-positive cells (Thio and Prop groups) compared with the Veh group (Fig. 2). The density of apoptotic neurons was significantly higher in the Sevo group compared with the Veh group in the CA1. In both the CA1 and the RC, Prop+Sevo induced a significant increase in the number of caspase-3-positive cells compared with Sevo ($P$ = 0.04). However, there were no significant differences in caspase-3-positive cell density between the Sevo and Thio+Sevo groups ($P$ = 0.683).

**Discussion**

General anesthetics can induce widespread neuronal cell death and persistent behavioral sequelae, including motor and cognitive deficits, in both the neonatal rodent and primate brains [1]. These results have led to a re-evaluation of the safety of many anesthetics used for pediatric patients. Sevoflurane has a more favorable neurotoxicity profile than the other commonly used inhalational anesthetics isoflurane and desflurane [11, 22] and therefore has become the preferred choice for pediatric anesthesia. To examine the potential neurotoxicity of sevoflurane, both alone and when combined with thiopental or propofol, we measured apoptotic cell numbers in the CA1 and RC following anesthetic treatment.

In the present study, sevoflurane in combination with propofol was more neurotoxic than sevoflurane alone. However, thipental did not exacerbate servofluorane-induced neurotoxicity. Propofol, like sevoflurane, is acting mainly via $GABA_A$ receptor agonism and to a lesser extent via NMDA receptor antagonism [23], whle thiopental only potentiates $GABA_A$ receptor functioning. Neonatal co-administration of an NMDA antagonist and a $GABA_A$ agonist even at doses that do not cause apoptosis alone led to significant deleterious effect compared with when these were administered alone. Furthermore, the effects seem to be synergistic [3, 4].

One limitatoion of this study is that the blood gases and end tidal $CO_2$ of individual animals were not measured. To assess oxygenation levels, we examined $SpO_2$ in mice every hour during anesthetic treatment. $SpO_2$ exceeded 90% in all groups before and every hour during anesthetic treatment. In additon, hypercarbia does not significantly increase brain cell death in the hippocampus and cortex of neonatal rats, compared with control animals exposed to room air [24]. These facts led us to presume the unlikelihood of apoptosis in this study being caused by hypoxia/hypercarbia.

Consistent with previous studies [11-13], we demonstrated that even sevoflurane alone caused significant neuronal apoptosis in the CA1. However, no commonly used anesthetic has a perfect safety record in animals, and withholding anesthesia during

surgery or other invasive procedures is unethical. Therefore, there appears to be no current alternative to sevoflurane. Furthermore, painful stimulation in unanesthetized newborn humans and animals can also result in neurological and behavioral abnormalities [25-30]. Most pediatric surgical procedures are not elective and cannot be postponed without incurring some degree of risk. If surgery is required, anesthesia with a small risk of limited neurotoxicity is superior to no anesthesia. Alleviation of pain and stress during treatment should remain the primary goal.

Based on our results, there is the possibility that the combination of sevoflurane and propofol is more prone to induce apoptosis in brain than sevoflulane alone in pediatric patients, although there are the difficulties transferring the results with rodents to humans. This possibility can occupy an important place in clinical practice because IV propofol followed by inhaled sevoflurane is commonly used anesthetic technique for induction of pediatric anesthesia. If our current findings can be extrapolated to clinical practice, this study may provide clinicians with neurotoxicity data to make more informed decisions on anesthetia regimens for pediatric patients. Additional research is obviously needed to determine the potential neurotoxicity of various anesthetic drug combinations and possible neuroprotective strategies. In the interim, anesthesiologists

should be aware of all current neurotoxicology results to select the least harmful anesthetic agents for newborn and infant anesthesia.

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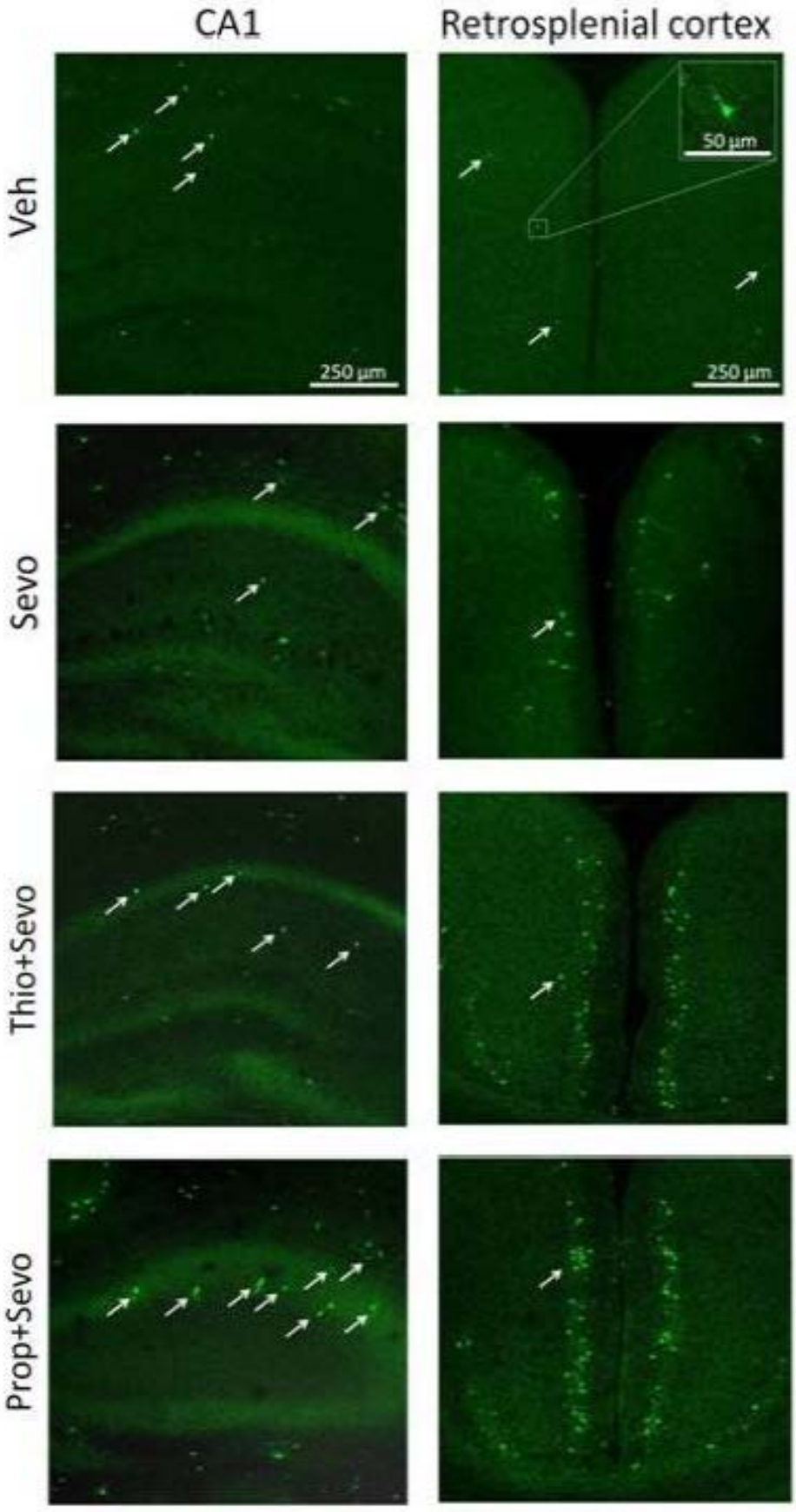
CA1
Retrosplenial cortex
Veh
Sevo
Thio+Sevo
Prop+Sevo
250 μm
50 μm
250 μm

**Figure Legends**

**Fig. 1** Expression pattern of the apoptosis marker activated caspase-3. Images were obtained from coronal brain sections from the hippocampal CA1 region (left column) and retrosplenial cortex (right column) under a laser confocal microscope immediately after Veh, Sevo, Thio+Sevo, or Prop+Sevo treatment. Cells positive for activated caspase-3 were stained bright green using a secondary antibody conjugated to Alexa Fluor 488. Arrows mark cells positive for activated caspase-3. The different treatments are symbolized as follows: (Veh) saline + air, (Sevo) saline + 3% sevoflurane, (Thio+Sevo) thiopental (5 mg/kg) + 3% sevoflurane and (Prop+Sevo) propofol (10 mg/kg) + 3% sevoflurane.

**Fig. 2** The box plots showing quantification of capase-3-positive cells for each treatment condition in the hippocampal CA1 reginon and retrosplenial cortex. Horizontal lines represent respective group medians; boxes, $25^{th}$-$75^{th}$ percentile; and whiskers, minimum-maximum value.The treatment abbreviations are summarized in Fig. 1. n = 8 per group. *P = 0.04 versus Sevo; **P = 0.001 versus Veh or Prop; + P = 0.04 versus Veh; ++ P = 0.006 versus Thio; # P = 0.001 versus Veh; ## P = 0.04 versus Thio.

## Tables

**Table 1** Percutaneous oxygen saturation in mice

| | $SpO_2$ (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| Time (h) | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Veh | 97.4±0.5 | 96.9±0.5 | 96.9±0.4 | 97.4±0.5 | 97.1±0.6 | 96.9±0.4 | 97.1±0.3 |
| Thio | 95.9±0.4 | 95.6±0.5 | 96.5±0.9 | 97.3±0.6 | 97.3±0.6 | 97.0±0.7 | 97.0±0.6 |
| Prop | 96.6±0.6 | 96.8±0.5 | 96.3±0.8 | 96.5±0.5 | 95.8±0.3 | 96.5±0.4 | 97.0±0.6 |
| Sevo | 97.1±0.4 | 95.9±0.9 | 96.0±0.9 | 96.0±0.4 | 95.8±0.7 | 96.5±1.2 | 96.8±0.6 |
| Thio+Sevo | 96.4±0.6 | 96.0±0.8 | 96.3±0.9 | 96.5±0.5 | 95.5±1.0 | 95.8±1.0 | 96.9±0.6 |
| Prop+Sevo | 96.8±0.5 | 95.6±1.0 | 96.1±0.8 | 96.4±0.7 | 96.4±0.6 | 96.3±0.6 | 95.8±1.0 |

Values are presented as mean ± SEM. Analysis of percutaneous oxygen saturation ($SpO_2$) revealed no significant differences in every time point among groups (two-way repeated measures ANOVA, groups: $P > 0.05$, time: $P > 0.05$, interaction between groups and time: $P > 0.05$, n = 8 per group).

**Table 2** Cerebral blood flow in mice

| | CBF (arbitrary perfusion unit) | | | | | | |
|---|---|---|---|---|---|---|---|
| Time (h) | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Veh | 27.5±2.1 | 26.6±1.2 | 22.5±1.7 | 24.4±1.7 | 23.8±1.6 | 25.9±1.9 | 22.8±1.2 |
| Thio | 22.1±1.1 | 28.6±2.6 | 26.9±2.5 | 27.0±2.4 | 26.5±1.8 | 24.1±2.3 | 24.5±3.4 |
| Prop | 20.8±3.1 | 24.6±1.4 | 25.5±2.3 | 26.0±2.6 | 25.9±2.0 | 22.9±1.4 | 22.0±1.1 |
| Sevo | 25.5±2.4 | 24.8±1.8 | 25.3±3.5 | 26.8±1.9 | 26.0±2.9 | 28.9±3.3 | 25.8±3.3 |
| Thio+Sevo | 22.3±2.2 | 26.0±1.2 | 28.6±3.1 | 25.6±1.0 | 26.1±2.2 | 26.3±1.3 | 26.6±2.1 |
| Prop+Sevo | 23.3±1.2 | 23.1±1.5 | 26.1±1.7 | 26.5±1.8 | 25.6±1.0 | 25.9±0.9 | 27.6±1.6 |

Values are presented as mean ±SEM. Analysis of cerebral blood flow (CBF) revealed no significant differences in every time point among groups (two-way repeated measures ANOVA, groups: $P > 0.05$, time: $P > 0.05$, interaction between groups and time: $P > 0.05$, n = 8 per group).