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# The Preconditioning Effect of Normobaric Hyperoxia on Postoperative Cognitive Dysfunction After Repeated Anesthesia in Rats

## Sıçanlarda Normobarik Hiperoksinin Tekrarlayan Anestezi Sonrası Gelişen Postoperatif Kognitif Disfonksiyon Üzerine Önkoşullayıcı Etkisi

Ahmet Rıdvan Dogan[1], Ferda Yilmaz Inal[2], Hasan Kocoglu[2], Tulay Zenginkinet[3]

[1]Sakarya Training and Research Hospital, Clinic of Anesthesiology and Reanimation, Sakarya, Türkiye
[2]Istanbul Medeniyet University, Faculty of Medicine, Department of Anesthesiology and Reanimation, Istanbul, Türkiye
[3]Istanbul Goztepe Prof. Dr. Suleyman Yalcin City Hospital, Clinic of Pathology, Istanbul, Türkiye

### ABSTRACT

**Objective:** This study aimed to evaluate the neuroprotective effect of normobaric hyperoxia preconditioning on postoperative cognitive dysfunction following repeated sevoflurane anesthesia in a rat model.

**Method:** Twenty-four male Sprague-Dawley rats were randomly assigned to four groups (n=6/group): control, normobaric hyperoxia, sevoflurane, and normobaric hyperoxia followed by sevoflurane. Rats in the hyperoxia groups received 95% oxygen for 3 hours on alternate days for 1 week. Rats in the sevoflurane groups were exposed to 2.9% sevoflurane for 2 hours daily over 5 consecutive days. Cognitive performance was assessed using the Morris Water Maze test. Hippocampal tissues were examined histopathologically for signs of neuronal damage and apoptosis.

**Results:** The sevoflurane group exhibited significantly impaired learning and memory performance compared to the control group (p<0.01). Rats preconditioned with hyperoxia before sevoflurane exposure showed significantly improved escape latency and platform crossing performance compared to the sevoflurane-only group (p<0.05). Histopathological analysis revealed marked hippocampal neuronal degeneration in the sevoflurane-only group, which was substantially reduced in the combined treatment group. No significant neuronal damage was observed in the control or hyperoxia-only groups.

**Conclusion:** Preconditioning with normobaric hyperoxia may mitigate hippocampal neuronal injury and cognitive dysfunction associated with repeated sevoflurane exposure. These preliminary findings highlight the potential neuroprotective effect of hyperoxic preconditioning and support the need for further research with larger cohorts and molecular-level validation.

**Keywords:** Postoperative cognitive dysfunction, sevoflurane, hyperoxia, preconditioning, hippocampus

### ÖZ

**Amaç:** Bu çalışmanın amacı, tekrarlayan sevofluran anestezisi sonrasında gelişen postoperatif bilişsel disfonksiyon üzerinde normobarik hiperoksi önkoşullamasının nöroprotektif etkisini rat modelinde değerlendirmektir.

**Yöntem:** Çalışmada, 24 erkek Sprague-Dawley rat rastgele dört gruba (n=6) ayrıldı: kontrol, normobarik hiperoksi, yalnızca sevofluran ve normobarik hiperoksi ardından sevofluran. Normobarik hiperoksi gruplarına bir hafta boyunca gün aşırı 3 saat süreyle %95 oksijen uygulandı. Sevofluran uygulanan gruplara ise beş ardışık gün boyunca günlük 2 saat süreyle %2,9 sevofluran verildi. Bilişsel performans Morris Su Labirenti testiyle değerlendirildi. Hipokampal dokular histopatolojik olarak nöronal hasar ve apoptoz bulguları açısından incelendi.

**Bulgular:** Sevofluran uygulanan grup, kontrol grubuna kıyasla öğrenme ve hafıza performansında belirgin bozulma gösterdi (p<0,01). Normobarik hiperoksi ardından sevofluran uygulanan grupta, yalnızca sevofluran uygulanan gruba göre kaçış latansı ve platform geçiş sayısında anlamlı düzelme gözlendi (p<0,05). Histopatolojik analizde, yalnızca sevofluran uygulanan grupta belirgin nöronal dejenerasyon tespit edilirken, bu bulgu kombine uygulama grubunda önemli ölçüde azalmıştı. Kontrol ve yalnızca oksijen uygulanan gruplarda anlamlı nöronal hasar saptanmadı.

**Sonuç:** Normobarik hiperoksi önkoşullaması, tekrarlayan sevofluran maruziyeti sonrası gelişen bilişsel bozukluk ve hipokampal nöronal hasarı azaltmada etkili bulunmuştur. Bu bulgular, normobarik hiperoksinin nöroprotektif potansiyelini desteklemekte olup, daha geniş örneklemler ve moleküler düzeyde doğrulamalar ile ileri çalışmalara ihtiyaç vardır.

**Anahtar sözcükler:** Postoperatif bilişsel disfonksiyon, sevofluran, hiperoksi, önkoşullama, hipokampüs

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***Corresponding author:** Ahmet Ridvan Dogan • a.ridvandogan@gmail.com

Ahmet Ridvan Dogan 0000-0002-5481-8882/ Ferda Yilmaz Inal 0000-0001-6323-1410
Hasan Kocoglu 0000-0002-0363-9307 / Tulay Zenginkinet 0000-0003-3056-2074

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## INTRODUCTION

Postoperative cognitive dysfunction (POCD) is a well-recognized complication of anesthesia and surgery, marked by deficits in memory, attention, and executive function (1). It is associated with increased morbidity, mortality, and prolonged recovery, particularly in vulnerable populations such as the elderly (2). Although numerous risk factors have been identified—ranging from age and comorbidities to the type and duration of anesthesia—the underlying mechanisms remain incompletely understood, and no definitive preventive strategies have been established (3,4).

Experimental and clinical studies suggest that repeated exposure to inhalational anesthetics may contribute to neurodegeneration by promoting neuroinflammatory cascades, oxidative stress, and apoptosis (5). The U.S. Food and Drug Administration has issued warnings regarding the potential cognitive risks associated with prolonged or repeated anesthetic exposure during critical periods of brain development (6,7). These concerns have underscored the need to explore neuroprotective strategies that can mitigate anesthetic-induced cognitive impairment.

Normobaric hyperoxia preconditioning (NBO-PC) has emerged as a candidate intervention due to its reported effects in modulating oxidative stress and inflammation in various models of ischemia and neurodegeneration (8–11). Preclinical data indicate that brief, intermittent exposures to high concentrations of oxygen can induce adaptive cellular responses that confer resilience against subsequent neural insults. However, the role of NBO-PC in the context of anesthesia-induced neurotoxicity, particularly under conditions of repeated exposure to volatile agents like sevoflurane, remains insufficiently characterized. Given the emerging evidence that repeated anesthetic exposure may exacerbate neuronal injury and cognitive decline, there is a pressing need to investigate interventions that could counteract these effects. Therefore, this study aimed to evaluate the potential neuroprotective impact of NBO-PC in a rat model subjected to repeated sevoflurane anesthesia. Cognitive performance and hippocampal histopathology were assessed to determine whether NBO-PC could mitigate anesthesia-related neurocognitive impairment.

## MATERIAL and METHODS

This study was conducted with the approval of the Yeditepe University Experimental Animal Ethics Committee (approval no. 2021-055). The study was carried out between November and December 2021 and encompassed a one-month experimental period. All procedures were performed at the Yeditepe University University Experimental Research Centre, and brain tissue analyses were conducted at the Istanbul Medeniyet University Faculty of Medicine, Department of Medical Pathology Research Laboratory. This animal research was conducted following the ARRIVE guidelines.

A total of 24 healthy, male, three-month-old Sprague-Dawley rats, weighing 300–320 g each, were included in this study. Male rats were chosen for the study because the effects of sevoflurane on cognitive functions and neuronal apoptosis in the hippocampus are more pronounced in males compared to females (12). The animals were housed under controlled environmental conditions: 20–24˚C room temperature, ≤ 4˚C daily temperature variation, 40–60% relative humidity, and a 12-hour light and dark cycle. Throughout the study, all of the rats had unrestricted access to food and water.

The rats were randomly divided into four groups (n = 6 per group): the control group (group C), which received no treatment; the normobaric oxygen group (group O), which underwent normobaric hyperoxia (NBO); the sevoflurane group (group S), which was exposed to repeated sevoflurane anesthesia; and the combined group (group OS), which received both NBO and sevoflurane.

Rats in groups O and OS were subjected to NBO (95% oxygen at 1 atm) for three hours on the 1$^{st}$, 3$^{rd}$, 5$^{th}$, 7$^{th}$, and 9$^{th}$ days using air-tight chambers (65 × 25 × 45 cm). In contrast, the rats in groups C and S were exposed to normobaric normoxia (21% oxygen at 1 atm) using the same protocol. Following the oxygen exposure sessions, rats in groups S and OS were anesthetized with 2.9% sevoflurane (Sevorane®, AbbVie Ltd., Maidenhead, UK) in 30% humidified oxygen for two hours in sealed induction chambers (13 × 13 × 20 cm). The concentrations of sevoflurane, oxygen, and carbon dioxide were continuously monitored using a gas analyzer (Vamos, Dräger, Lübeck, Germany). Meanwhile, rats in groups C and O were placed in identical chambers but did not receive sevoflurane.

The NBO-PC protocol used in this study (95% oxygen, 3 hours, on alternating days) was adapted from previous preclinical work. In particular, Bigdeli et al. demonstrated that repeated exposure to 95% oxygen for 4 hours daily over six consecutive days enhanced antioxidant enzyme activity in rat brain tissue. While our exposure duration was slightly shorter and spaced over alternate days, it was intended to maintain efficacy while minimizing cumulative oxygen-related stress (8).

Blood samples were collected from the rats in groups S and OS after the first anesthesia session to evaluate their respiratory and metabolic parameters, including pH, partial pressure of carbon dioxide ($PaCO_2$), partial pressure of oxygen ($PaO_2$), and oxygen saturation ($SpO_2$). These measurements were performed using a blood gas analyzer (i-STAT 1, Abbott Laboratories, Istanbul, Turkey).

Following the preconditioning and anesthesia sessions, the rats' cognitive functions were assessed through neurobehavioral testing using the Morris Water Maze (MWM) test from the 10th to the 14th days of the study. The MWM test was performed to evaluate spatial learning and memory, which are critical indicators of cognitive performance. On the 14th day, after completing the behavioral tests, the animals were euthanized under general anesthesia. Brain tissues were then collected, fixed in formaldehyde, and processed for histopathological examination to assess neuronal damage, apoptosis, and cerebral edema in the hippocampus (Figure 1).

The MWM test was employed to evaluate the spatial learning and memory performance of the rats. This test was conducted between the 10th and 14th days of the study in a circular pool measuring 150 cm in diameter and 50 cm in depth, with black walls and water maintained at a temperature of 23 ± 1°C to a depth of 30 cm. A hidden platform, 10 cm in diameter, was submerged 1 cm below the water surface in a fixed location within one quadrant of the pool. To ensure the platform was not visible, nontoxic black dye was added to the water.

The test consisted of two phases. In the learning phase, also known as the place navigation test, the rats underwent four trials per day from the 10th to the 13th days. In each trial, the rats were placed into the pool from one of four randomized starting points, facing the wall, and given 60 seconds to locate the hidden platform. If a rat successfully located the platform, it was allowed to rest on it for 15 seconds; if not, it was gently guided to the platform and given the same resting period. The time taken to find the platform, known as escape latency, was recorded for each trial. On the 14th day, the probe trial, or memory phase, was conducted. In this phase, the hidden platform was removed from the pool. Each rat was placed in the quadrant opposite to the previous platform location and allowed to swim freely for 60 seconds. The parameters recorded included the number of platform crossings, the time spent in the target quadrant, and the swimming path. All behavioral data were recorded and analyzed using EthoVision XT software (version 15.0.1418, Noldus, Wageningen, Netherlands). The test was carried out in a quiet and controlled environment to minimize external distractions.

The brain tissues were fixed in 10% formaldehyde for proper preservation and subsequently embedded in paraffin blocks. Sections measuring 3 µm in thickness were prepared from the hippocampal region and stained with hematoxylin and eosin for microscopic evaluation. The stained sections were examined under a light microscope (Olympus BX51, Olympus Lifescience, Tokyo, Japan) to assess neuronal morphology and damage. Acute neuronal damage was evaluated semi-quantitatively using a grading scale, where 0 indicated normal morphology, 1 indicated mild neuronal shrinkage and increased nuclear basophilia, and 2 indicated severe shrinkage and nuclear pycnosis. In addition to neuronal damage, cerebral edema in the hippocampal region was assessed using a four-

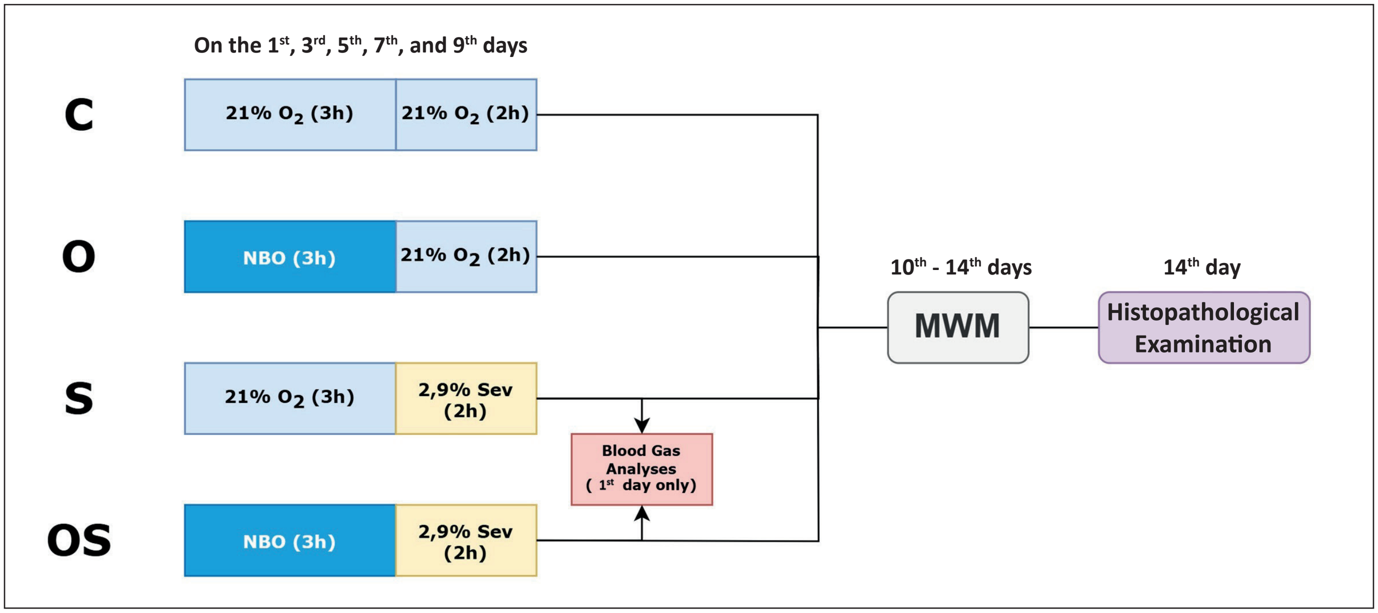


**Figure 1.** Experimental protocol timeline. Four groups of rats were exposed to different gas and anesthetic regimens on the 1st, 3rd, 5th, 7th, and 9th days. Group C (control) and Group S (sevoflurane only) received normobaric normoxia (21% $O_2$) for 3 hours; Group S additionally received 2.9% sevoflurane for 2 hours, with arterial blood gas analyses performed after the first exposure. Groups O (normobaric hyperoxia [NBO]) and OS (NBO + sevoflurane) received 95% oxygen for 3 hours. Group OS also underwent 2.9% sevoflurane anesthesia for 2 hours. Behavioral testing using the Morris Water Maze (MWM) was conducted between the 10th and 14th days, and histopathological analysis was performed on day 14.

point scale: 0 for no edema, 1 for mild, 2 for moderate, and 3 for severe edema. This detailed examination allowed for the identification of histopathological changes associated with neuronal apoptosis, inflammation, and tissue injury resulting from repeated anesthesia and preconditioning treatments.

### Statistical Analysis

The sample size was determined a priori using one-way analysis of variance (ANOVA) for repeated measures with within-between interactions based on a power of 0.80 and an effect size of 0.2. This calculation was performed using G*Power software (version 3.1.9.4, University of Düsseldorf, Germany) and informed by a similar, previous study (13). Statistical analysis was conducted using the Number Cruncher Statistical System 2019 software (NCSS, LLC, Kaysville, Utah). Descriptive statistics, including mean, standard deviation, median, frequency, percentage, minimum, and maximum values, were used to summarize the study data. The normality of the quantitative variables was evaluated using the Shapiro-Wilk test and graphical methods.

For comparisons of quantitative variables with a normal distribution among more than two groups, ANOVA was applied, and post-hoc pairwise comparisons were performed using the Bonferroni correction. For non-normally distributed quantitative variables, comparisons among groups were made using the Kruskal-Wallis test followed by Dunn's test with Bonferroni correction for pairwise comparisons. Within-group comparisons of non-normally distributed quantitative variables were conducted using the Friedman test, with pairwise comparisons carried out using the Bonferroni-corrected Wilcoxon signed-rank test. A p value of less than 0.05 was considered statistically significant.

## RESULTS

Normality analysis showed that arterial blood gas parameters followed a normal distribution (Shapiro–Wilk $p>0.05$), whereas all Morris Water Maze outcomes and histopathological scores demonstrated non-normal distribution ($p<0.05$). Therefore, statistical analyses were performed using appropriate parametric or non-parametric methods based on the distributional characteristics of each variable.

The blood gas parameters, including pH, $pO_2$, $pCO_2$, and $SpO_2$, showed no statistically significant differences among the groups (Table I).

The escape latency during the learning trials did not differ statistically between the groups (Table II). However, statistically significant differences were observed in the changes in escape latency over the course of the days within group C, group O, and group OS (p=0.026, p=0.042, p=0.035, respectively; $p<0.05$). In the pairwise comparisons within these groups, a significant decrease in the time required to find the platform on the fourth day compared to the first day was noted (p=0.031, p=0.027, p=0.014, respectively; $p<0.05$) (Figure 2). During the learning trials, the rates of time spent in the target quadrant on the first and second days did not show statistically significant differences between the groups ($p>0.05$) (Table III). However, significant differences were observed on the third and fourth days (p=0.032, p=0.034; $p<0.05$, respectively). Specifically, the time spent in the target quadrant on these days was significantly higher in group C compared to group S (p=0.002, p=0.004; $p<0.01$, respectively) (Figure 3).

During the probe trial, a statistically significant difference was observed between the groups in the number of platform crossings (p=0.021; $p<0.05$) (Table IV). In the pairwise comparisons, the number of platform crossings in group C and group O was significantly higher than in group S (p=0.031, p=0.031; $p<0.05$, respectively) (Figure 4). The grade of edema in the hippocampus did not show statistically significant differences between the groups ($p>0.05$). However, the red neuron degeneration grade in the hippocampus differed significantly among the groups (p=0.003; $p<0.01$) (Table V). In the pairwise comparisons, the red neuron degeneration grade was significantly higher in group S compared to group OS (p=0.002; $p<0.01$) (Figure 5).

## DISCUSSION

Repeated exposure to volatile anesthetics during early developmental stages has been associated with neurodegenerative responses in animal models, which may increase the risk of subsequent cognitive dysfunction (14). To the best of our knowledge, there is no established treatment for POCD caused by repeated anesthesia exposure. In this study, we investigated the effects of NBO-PC on cognitive dysfunction after repeated anesthesia exposure. Our findings suggest

**Table I.** Comparison of Arterial Blood Gas Parameters Between Sevoflurane-Only (S) and Sevoflurane + NBO (OS) Groups After the First Anesthesia Exposure

| | Group S | Group OS | p value |
|---|---|---|---|
| pH | 7.40 ± 0.04 | 7.40 ± 0.04 | 0.827 |
| $PaO_2$, mmHg | 154 ± 15.5 | 165 ± 11.8 | 0.181 |
| $PaCO_2$, mmHg | 35.7 ± 2.51 | 36.3 ± 1.85 | 0.640 |
| $SpO_2$, % | 99.3 ± 0.3 | 99.3 ± 0.2 | 0.830 |

Data are presented as mean ± standard deviation. Between-group comparisons were performed using the independent-samples t-test. **$PaO_2$:** Partial pressure of oxygen in arterial blood, **$PaCO_2$:** Partial pressure of carbon dioxide in arterial blood, **$SpO_2$**: Oxygen saturation, **S:** Sevoflurane-only group, **OS:** Sevoflurane with normobaric hyperoxia group.

**Table II.** Comparison of Escape Latency Across All Groups During the Learning Trials by Days

| | Group C | Group O | Group S | Group OS | p value |
|---|---|---|---|---|---|
| 1st Day (sec) | **39.90 ± 20.50δ (median 40.9, IQR 21.4–60.4)** | **41.65 ± 21.24** (median 50.7, IQR 30.4–71.1)** | 46.74 ± 19.61 (median 60.0, IQR 43.5–76.5) | **41.45 ± 18.96† (median 44.0, IQR 27.6–60.5)** | 0.553 |
| 2nd Day (sec) | 35.49 ± 22.54 (median 23.46, IQR 0.64–46.28) | 36.32 ± 24.96 (median 47.3, IQR 21.02–73.59) | 45.61 ± 23.56 (median 60.0, IQR 42.30–77.71) | 39.44 ± 22.86 (median 53.2, IQR 31.11–75.29) | 0.337 |
| 3rd Day (sec) | 31.58 ± 20.75 (median 30.62, IQR 13.40–47.85) | 34.42 ± 22.92 (median 33.5, IQR 9.28–57.72) | 40.08 ± 21.59 (median 49.42, IQR 30.44–68.40) | 36.93 ± 21.52 (median 34.52, IQR 13.69–55.35) | 0.544 |
| 4th Day (sec) | **24.99 ± 19.09δ (median 15.7, IQR 0.61–30.79)** | **25.32 ± 16.58** (median 24.08, IQR 12.51–35.66)** | 39.11 ± 23.40 (median 53.36, IQR 30.97–75.75) | **27.36 ± 22.68† (median 18.42, IQR 0–43.57)** | 0.189 |

Data are presented as mean ± SD and median (IQR). Between-group comparisons were performed using the Kruskal–Wallis test with Bonferroni-adjusted Dunn post-hoc analyses. Within-group repeated measurements were evaluated using the Friedman test with Bonferroni-corrected Wilcoxon signed-rank tests. †, **, δ indicate statistically significant within-group differences between 1st and 4th days: **δ:** p = 0.031 (Group C), ****:** p = 0.027 (Group O), **†:** p = 0.014 (Group OS). **C:** Control group, **O:** Normobaric hyperoxia group, **S:** Sevoflurane-only group, **OS:** Sevoflurane with normobaric hyperoxia group, **sec:** Seconds.

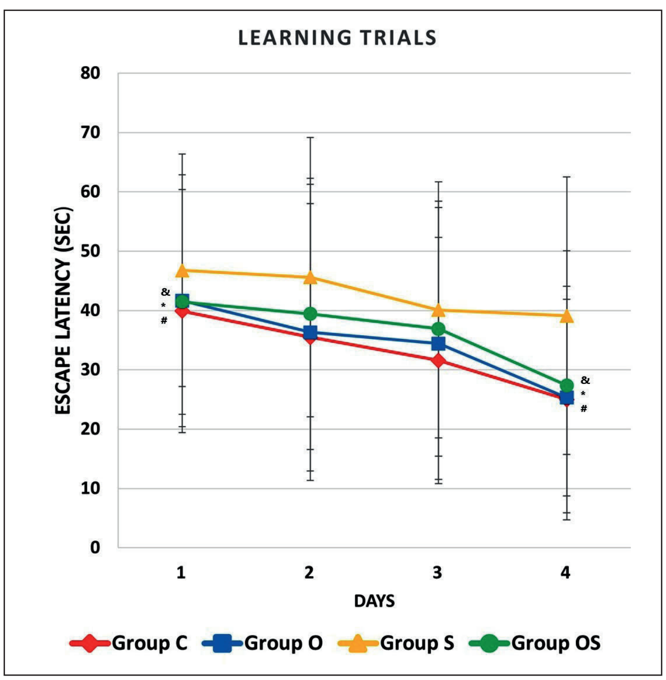


**Figure 2.** Escape latency (seconds) across four consecutive learning trials (Days 1–4) in the Morris Water Maze test for each group. Symbols (*, #, &) denote statistically significant within-group reductions from Day 1 to Day 4 based on the Friedman test followed by Bonferroni-corrected Wilcoxon signed-rank tests ($p<0.05$). Between-group differences at each time point were assessed using the Kruskal–Wallis test with Bonferroni-adjusted Dunn post-hoc comparisons. **Sec:** Seconds.

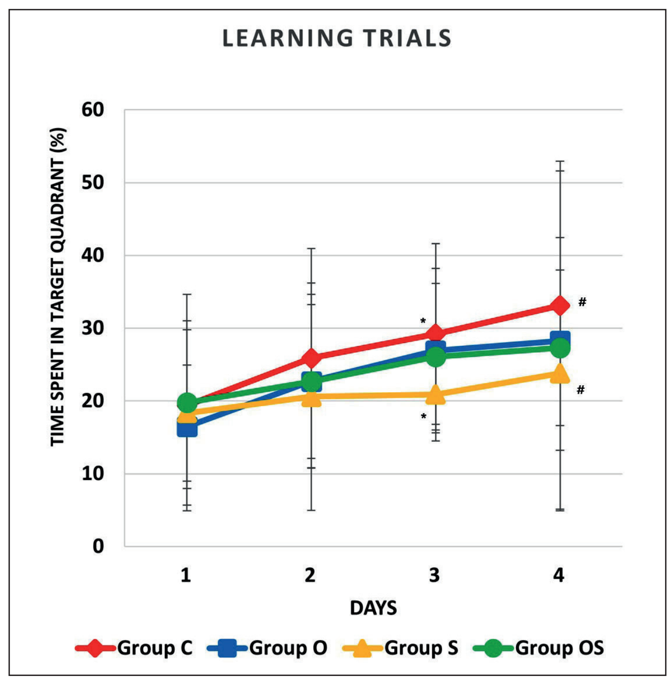


**Figure 3.** Percentage of time spent in the target quadrant during the Morris Water Maze learning trials (Days 1–4) for each group. Symbols (* and #) denote statistically significant differences between Group C and Group S on Day 3 (*p=0.032) and Day 4 (# p=0.034), respectively, based on the Kruskal–Wallis test followed by Bonferroni-adjusted Dunn post-hoc comparisons.

**Table III.** Time Spent in the Target Quadrant (%) by Each Group during the Learning Phase (Days 1 to 4) of the Morris Water Maze Test

| | Group C | Group O | Group S | Group OS | p-value |
|---|---|---|---|---|---|
| 1st Day (%) | 19.39 ± 10.40 (median 18.70, IQR 11.50–25.90) | 16.47 ± 8.48 (median 14.20, IQR 10.01–18.39) | 18.35 ± 12.66 (median 15.43, IQR 8.57–22.29) | 19.77 ± 14.87 (median 18.85, IQR 10.94–26.76) | 0.777 |
| 2nd Day (%) | 25.88 ± 15.05 (median 23.40, IQR 13.72–33.08) | 22.27 ± 11.96 (median 21.10, IQR 13.11–29.09) | 20.58 ± 15.64 (median 15.97, IQR 7.19–24.75) | 22.67 ± 10.58 (median 21.51, IQR 14.21–28.81) | 0.479 |
| 3rd Day (%) | **29.20 ± 12.43† (median 27.99, IQR 19.11–36.87)** | 26.91 ± 11.32 (median 24.53, IQR 18.21–30.85) | **20.87 ± 6.35† (median 20.91, IQR 15.78–26.04)** | 26.06 ± 10.08 (median 25.56, IQR 16.16–34.96) | **0.032** |
| 4th Day (%) | **33.08 ± 19.85** (median 31.43, IQR 19.27–43.59)** | 28.24 ± 23.35 (median 23.27, IQR 18.71–27.83) | **23.79 ± 18.64** (median 19.03, IQR 7.87–30.19)** | 27.31 ± 10.68 (median 25.95, IQR 16.91–34.99) | **0.034** |

Data are presented as mean ± SD and median (IQR). Between-group comparisons were performed using the Kruskal–Wallis test with Bonferroni-adjusted Dunn post-hoc analyses. Within-group repeated measurements were evaluated using the Friedman test with Bonferroni-corrected Wilcoxon signed-rank tests. †, ** indicate statistically significant pairwise differences between Group C and Group S: **†:** p = 0.002 (3rd day), ****:** p=0.004 (4th day). **C:** Control group, **O:** Normobaric hyperoxia group, **S:** Sevoflurane-only group, **OS:** Sevoflurane with normobaric hyperoxia group.

**Table IV.** Number of Platform Crossings during the Probe Trial for All Groups. A Higher Number of Crossings Indicates Better Memory Retention

| | Group C | Group O | Group S | Group OS | p-value |
|---|---|---|---|---|---|
| Platform Crossings | **2.17 ± 1.17† (median 2.00, IQR 0.88-3.13)** | **2.17 ± 1.17† (median 2.00, IQR 0.88-3.13)** | **0.5 ± 0.55†,** (median 0.50, IQR 0.00-1.00)** | 2.33 ± 1.21 **(median 2.50, IQR 1.38-3.63)** | **0.021** |

Data are presented as mean ± SD and median (IQR). Between-group comparisons were performed using the Kruskal–Wallis test with Bonferroni-adjusted Dunn post-hoc analyses. †, ** indicate statistically significant pairwise differences compared to Group S: **†:** p = 0.031 (Group C vs. Group S), ****:** p=0.031 (Group O vs. Group S). **C:** Control group, **O:** Normobaric hyperoxia group, **S:** Sevoflurane-only group, **OS:** Sevoflurane with normobaric hyperoxia group.

**Table V.** Semi-Quantitative Grading of Hippocampal Edema and Red Neuron Degeneration Across Groups. Edema Graded on a 0–3 Scale; Red Neuron Degeneration Graded on a 0–2 Scale

| | Group C | Group O | Group S | Group OS | p-value |
|---|---|---|---|---|---|
| Hippocampus Edema Grade | 0.67 ± 0.52 (median 1.00, IQR 0.50–1.50) | 0.33 ± 0.52 (median 0.00, IQR 0.00–0.50) | 1.00 ± 0.00 (median 1.00, IQR 1.00-1.00) | 0.67 ± 0.52 (median 1.00, IQR 0.50-1.50) | 0.124 |
| Hippocampus Red Neuron Degeneration Grade | 0.67 ± 0.52 (median 1.00, IQR 0.50–1.50) | 0.33 ± 0.52 (median 0.00, IQR 0.00–0.50) | **1.33 ± 0.52† (median 1.00, IQR 0.50-1.50)** | **0† (median 0.00, IQR 0.00-0.00)** | **0.003** |

Data are presented as mean ± SD and median (IQR). Between-group comparisons were performed using the Kruskal–Wallis test with Bonferroni-adjusted Dunn post-hoc analyses. † indicates statistically significant pairwise difference in red neuron degeneration grade between Group S and Group OS (p=0.002). **C:** Control group, **O:** Normobaric hyperoxia group, **S:** Sevoflurane-only group, **OS:** Sevoflurane with normobaric hyperoxia group.

that NBO-PC may mitigate neurodegeneration and possibly cognitive dysfunction associated with repeated anesthesia exposure, and it provides a novel approach to addressing this significant clinical challenge. This result is consistent with recent discussions in the literature on the mechanisms of POCD and suggests that NBO-PC could be considered among other experimentally tested neuroprotective strategies (15,16).

Sevoflurane is known to suppress spontaneous respiration, which leads to hypercarbia and hypoxia at higher concentrations (17,18). In our study, sevoflurane at a concentration of 2.9% did not suppress spontaneous respiration or cause hypercarbia, as the blood gas analysis remained within normal limits. Given that blood gas analyses remained within normal ranges, the observed neuronal injury may be attributable to

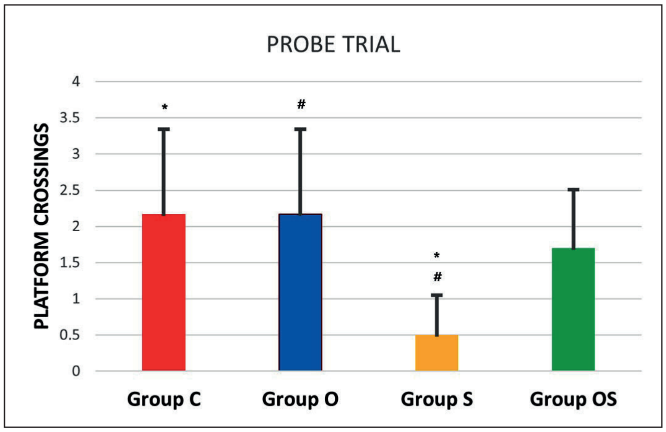


**Figure 4.** Number of platform crossings during the probe trial, indicating memory retention performance. Group S showed significantly fewer platform crossings compared with Group C (*p=0.031) and Group O (#p=0.031), based on the Kruskal–Wallis test followed by Bonferroni-adjusted Dunn post-hoc comparisons.

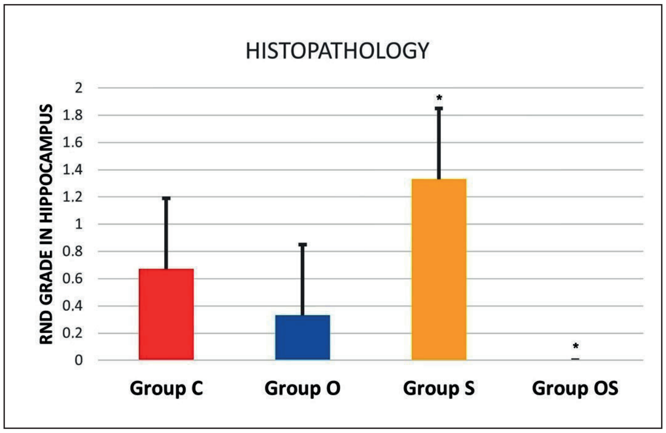


**Figure 5.** Red neuron degeneration (RND) grades in the hippocampus across experimental groups. Group S demonstrated significantly higher RND scores compared with Group OS (p=0.002), based on the Kruskal–Wallis test followed by Bonferroni-adjusted Dunn post-hoc analysis.

direct effects of sevoflurane rather than secondary to hypoxia or hypercarbia. Many studies investigating the effects of anesthetic drugs on cognitive function have produced conflicting results. While some studies have suggested that anesthetic agents do not impair cognition, others reported neurotoxic effects (19-21). Neuroapoptosis appears to be a key mechanism underlying anesthesia-induced neurotoxicity (22,23). Our study's protocol, involving anesthesia every other day for a total of five sessions, contrasts with Huang et al., who investigated repeated anesthesia exposure during the neonatal and adolescent periods, and Shen et al., who administered multiple exposures within a single day (24,25). This intermittent exposure approach provides a unique perspective on the cumulative effects of anesthesia. Our findings align with studies showing that repeated exposure to higher doses of sevoflurane impairs learning and memory (21,24,26). Specifically, rats exposed to repeated sevoflurane in our study demonstrated longer escape latencies and reduced platform crossings, indicating impaired learning and memory. This highlights the importance of addressing cumulative exposure to anesthetics in clinical settings. In recent years, several experimental and clinical studies have also reported that cumulative exposure leads to more pronounced cognitive impairments compared to single anesthetic exposure (27).

Experimental studies have explored various strategies to prevent anesthesia-induced neurocognitive dysfunction (28-30). Wang et al. demonstrated the anti-apoptotic properties of NBO-PC in mitigating sevoflurane-induced neurocognitive impairment (31). Consistent with their findings, our study showed that although NBO-PC did not produce statistically significant improvements in all behavioral metrics, it was associated with shorter escape latencies and increased platform crossings in some comparisons. These observations suggest a potential behavioral benefit of NBO-PC, albeit within the limitations of our small sample size and variable outcomes. The histopathological examination in our study also showed that NBO-PC appeared to reduce red neuron degeneration in the hippocampus. While this finding is consistent with Wang et al.'s results suggesting modulation of apoptosis-related markers, the absence of molecular or immunohistochemical confirmation limits the strength of this observation (31). Notably, the hippocampal edema scores did not significantly differ between groups, which suggests that NBO-PC's effect may be more closely linked to cellular integrity rather than gross tissue swelling. The histopathological analysis further supported these findings, as the red neuron degeneration in the hippocampus was significantly higher in the rats exposed to repeated sevoflurane. Wang et al. conducted a study in which anesthesia was administered as a single five-hour session, whereas our protocol involved repeated shorter exposures combined with NBO-PC (31). Additionally, their study did not include a group receiving NBO alone, which left the potential toxicity of NBO unexplored. By including an NBO-only group, our study demonstrated that NBO alone does not have harmful effects, thereby strengthening its safety profile. Gao et al., for instance, administered 40% oxygen to Alzheimer's model mice, whereas we employed 95% oxygen in rats, reflecting a methodological distinction that may have influenced the observed outcomes (32). Guo et al. observed similar results, showing increased apoptosis and hippocampal changes after repeated sevoflurane exposure (21). In line with this, Huang et al. reported that repeated anesthesia exposure, but not single exposures, led to neurotoxicity and hippocampus-dependent cognitive impairment (24). These findings emphasize the cumulative impact of repeated anesthetic exposure on the brain and underscore the need for protective interventions like NBO-PC. Taken together, these findings highlight the cumulative neurotoxic potential of repeated anesthetic exposure and strengthen the rationale for investigating strategies such as NBO-PC that target apoptosis and hippocampal integrity. This direction is also emphasized in the recent literature focusing on the molecular mechanisms of POCD (33).

The most significant limitation of this study was the relatively small sample size, as determined by power analysis. This limitation may have reduced the statistical power needed to detect significant differences, which highlights the need for future studies with larger animal cohorts. Another notable limitation is the absence of immunohistochemical or molecular analyses (e.g., cleaved caspase-3, brain derived neurotrophic factor, inflammatory cytokines), which precludes definitive conclusions about the mechanisms underlying the observed neuroprotection. Incorporating such techniques could have provided deeper insights into the underlying mechanisms of neurodegeneration and the protective effects of NBO-PC. Despite these limitations, this study emphasizes the potential of NBO-PC in mitigating cognitive dysfunction and neurodegeneration associated with repeated anesthesia exposure.

## CONCLUSION

This study demonstrates that repeated exposure to sevoflurane induces cognitive dysfunction and neurodegeneration in rats, which is consistent with the neurotoxic effects reported in previous studies. Normobaric hyperoxia preconditioning significantly ameliorated the cognitive deficits and histopathological changes caused by repeated sevoflurane exposure, reducing neuroapoptosis, edema, and red cell degeneration in the hippocampus. These findings raise the possibility that NBO-PC may serve as an experimental neuroprotective approach against anesthesia-induced cognitive impairment, but further validation is necessary. Future studies should investigate the underlying mechanisms of its effects and assess its clinical applicability, particularly in vulnerable populations, such as young children and the elderly.

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### AUTHOR CONTRIBUTIONS

**Conception or design of the work:** ARD, FYI
**Data collection:** ARD, TZ
**Data analysis and interpretation:** ARD, HK
**Drafting the article:** ARD
**Critical revision of the article:** ARD, FYI, HK
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The author (ARD, FYI, HK, TZ) reviewed the results and approved the final version of the manuscript.

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