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ORIGINAL ARTICLE

Erythropoietin diminishes isoflurane-induced apoptosis in rat frontal cortex

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What is already known

- Anesthetic drug exposure during the brain growth spurt period may affect developing neurons leading to cellular and behavioral changes in the developing brain.

What this article adds

- This study showed that low-dose erythropoietin administered intraperitoneally can diminish isoflurane-induced neuroapoptosis.

Keywords
erythropoietin; rats; isoflurane; apoptosis; learning; memory

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Summary

Background: During the brain growth spurt, anesthetic drugs can cause cellular and behavioral changes in the developing brain. The aim of this study was to determine the neuroprotective effect of erythropoietin after isoflurane anesthesia in rat pups.

Methods: A total of 42, 7-day-old Wistar rats were divided into three groups. Control group (GC; $n = 14$): Rats breathed 100% oxygen for 6 h; Isoflurane group (GI; $n = 14$): Rats were exposed to 1.5% isoflurane in 100% oxygen for 6 h; Isoflurane + erythropoietin group (GIE; $n = 14$): 1000 IU·kg^{-1} (intraperitoneal; IP) Erythropoietin was administered after isoflurane anesthesia. Each group was divided into two groups for pathology and learning and memory tests. Silver, caspase-3, and fluoro-jade C staining were used for detecting apoptotic cells in frontal cortex, striatum, hippocampus, thalamus, and amygdala. Morris water maze was used to evaluate learning and memory.

Results: There was a significant increase in apoptotic cell count after isoflurane anesthesia in the frontal cortex when compared with control group (29.0 ± 9.27 vs 3.28 ± 0.75 [$P = 0.002$], 20.85 ± 10.94 vs 2.0 ± 0.81 [$P = 0.002$] and 24.57 ± 10.4 vs 5.14 ± 0.69 [$P = 0.024$] with silver, caspase-3, and fluoro-jade C staining, respectively). The apoptotic cell count in the frontal cortex was significantly higher in GIE than GC with caspase-3 staining (9.14 ± 3.13 vs 2.0 ± 0.81, $P = 0.002$). The apoptotic cell count in GIE was significantly reduced in the frontal cortex when compared with GI (4.0 ± 0.81 vs 29.0 ± 9.27 [$P = 0.002$], 9.14 ± 3.13 vs 20.85 ± 10.94 [$P = 0.04$] and 4.0 ± 1.63 vs 24.57 ± 10.4 [$P = 0.012$] with silver, caspase-3, and fluoro-jade C staining, respectively).

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Conclusions: A total of 1000 IU·kg^{-1} IP erythropoietin diminished isoflurane-induced neuroapoptosis. Further experimental studies have to be planned to reveal the optimal dose and timing of erythropoietin before adaptation to clinical practice.

Background

The rapid growth of developing brain is called the brain growth spurt. In rats, this period occurs in postnatal 2 weeks, while in humans it starts in gestation and continues for several years after birth (1). Anesthetic drugs used in this period may affect developing neurons leading to cellular and behavioral changes in the developing brain. General anesthetics can trigger neuroapoptosis in developing rodent brain and may cause learning and memory deficits (2,3).

Isoflurane is commonly used in daily practice. Like other volatile anesthetics, isoflurane mediates its effects through modulation of $GABA_A$ receptors. Studies of newborn rats and mice reported that neuronal cell death occurs several hours after isoflurane anesthesia (3). It has been shown that exposure of newborn rats to isoflurane causes neurocognitive deficits that persist for a third of the lifespan (4). Additionally, some human cohort studies have reported that behavioral or developmental abnormalities can occur in children who have been exposed to anesthetics in early childhood (3,5).

Erythropoietin (EPO) is secreted from the kidney and it stimulates the production of erythrocytes. EPO receptors are also expressed on many other cells including neurons (6), and there is a high density of receptors in embryonic, fetal, and adult brain of rats and humans (7,8). EPO exerts antiapoptotic, anti-inflammatory, antioxidative, angiogenic, and neurotropic properties (8,9). Therefore, in addition to protecting neurons directly by preventing apoptosis, EPO modulates inflammation (10) and also plays a restorative role by stimulating neurogenesis.

Our primary goal of the current work was to determine whether a low dose of EPO can reduce the neuroapoptotic effects of isoflurane anesthesia. A secondary goal of this study was to find out whether EPO can improve learning and memory skills of young rats that were exposed to isoflurane anesthesia when they were 7 days old.

Methods

Animals

Forty-two, 7-day-old Wistar rats weighing 9–11 ***g*** were involved in this study. Animals were housed under standard conditions (12 h light/12 h dark cycle at 22°C) in the Animal Research Laboratory at Dokuz Eylul University. The study protocol was approved by the Animal Research Committee of Dokuz Eylul University. All efforts to reduce the number of animals and their suffering were made.

Rats were randomized into three groups;

Control group (GC; $n = 14$): Rats breathed %100 oxygen for 6 h,
Isoflurane group (GI; $n = 14$): Rats were exposed to 1.5% isoflurane in 100% oxygen for 6 h,
Isoflurane + EPO group (GIE; $n = 14$): After 6 h of 1.5% isoflurane in 100% oxygen, 1000 IU·kg^{-1} (intraperitoneal; IP) EPO was administered.

Each group was divided into two groups for pathology (acute group) and learning and memory (chronic group) tests. In the acute group, rats were sacrificed by decapitation 24 h after the end of the anesthesia. All of the animals were anesthetized with isoflurane (for <5 min) before decapitation to prevent suffering. In the chronic group, rats were kept at standardized conditions for 5 weeks and then underwent Morris water maze (MWM) testing. For adaptation purposes, rats were moved to MWM testing location and kept there at standardized conditions for 1 week before the tests.

Anesthesia apparatus, induction, and maintenance

Separate glass jars (450 ml) were used for every rat. Jars had gas-in and gas-out systems. About 1.5% isoflurane (Forane; Abbott Lab., İstanbul, Turkey) in 6 l·min^{-1} O_2 was administered by isoflurane vaporizator (Isoflurane, Vapor 19.1; Abbott Lab., Lubeck, Germany). Inspired oxygen and volatile agent concentrations were monitored (Anesthesia Gas Monitoring 1304; Brüel & Kjær Sound & Vibration Measurement A/S Nærum, Copenhagen, Denmark) and kept constant, and respiratory effort and skin color were also evaluated during the study period. All jars were maintained in a constant temperature water-bath at 37°C.

Histomorphological evaluation

Brain tissues were processed through graded alcohols and xylene and were embedded in paraffin blocks. Brain sections were taken using a rotary microtome

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(Leica RM 2135; Leica Instruments, Nussloch, Germany) with microtome blades (Type N35; Feather Company, Osaka, Japan) and serial 5-μm thick slices in a coronal plane from paraffin blocks were obtained. The whole rat brain including frontal cortex (fig. 77 in the atlas of the developing rat brain), striatum (fig. 81), hippocampus (fig. 93), thalamus (fig. 97), and amygdala (fig. 99) (11) was examined. Attached pre- and postserial sections (for every brain region) were also morphologically assessed. All sections were stained with silver stain, active caspase-3, and *fluoro-jade C stain* for stereological and histomorphological evaluations.

Silver staining

Parafin sections (5 μm) were processed using the Gomori reticulum silver method. Sections were deparaffinized and hydrated to distilled water. They were oxidized in potassium permanganate, 0.5%, and then washed in tap water. Sections were differentiated in potassium m-bisulfite, 2%, and then they were washed in tap water. They were sensitized in ferric ammonium sulfate, 2%, washed in tap water, and transferred through two washes of distilled water. Tissues were impregnated with ammoniacal silver solution. Tissues were stained with Gomori's ammoniacal silver solution which contained potassium hydroxide, silver nitrate, and ammonium hydroxide. Tissues were fixed in formalin, 20% and rinsed in tap water. They were reduced in potassium m-bisulfite, 2%, and transferred to sodium thiosulfate, 2%. They were washed in tap water for 2 min. Dehydrated in 95% and 100% alcohol and cleared in xylene. They were mounted in entellan. Tissues were examined with an Olympus light microscope (12).

Caspase-3 immune staining method

Sections were incubated in a humid chamber for 18 h at +4°C with antibody active caspase-3 (AB3623, Polyclonal antibody; Millipore, Temecula, CA, USA); thereafter with biotinylated IgG, and then with streptavidin conjugated to horseradish peroxidase for 15 min (Invitrogen-Plus Broad Spectrum 85-9043). Sections were finally stained with 3,3 diaminobenzidine tetrahydrochloride (Roche, Mannheim, Germany) and counter-stained with mayer hematoxylin and analyzed by using a light microscope. For quantitative measurement of the number of cells that underwent apoptosis, the images were analyzed by using a computer-assisted image analyzer system consisting of a microscope (Olympus BX-51, Tokyo, Japan) equipped with a high-resolution video camera (Olympus DP-71, Tokyo, Japan). In the serial section, the counting frame was placed randomly five times on the image analyzer system monitor and the numbers of neurons were counted (UTHSCA Image Tool for windows version 3.0 software) and the average was taken. A total of 1000 cells are counted for each subject and numbers of apoptotic cells are presented as percentage (13).

Fluoro-Jade C staining

Parafin sections were deparaffinized and hydrated to distilled water and incubated in 0.06% KMnO4 solution for 10 min. Slides were then transferred for 10 min to a 0.0001% solution of fluoro-jade C (AG325; Millipore) dissolved in 0.1% acetic acid vehicle. The proper dilution was accomplished by first making a 0.01% stock solution of the dye in distilled water and then adding 1 ml of the stock solution to 99 ml of 0.1% acetic acid vehicle. The slides were rinsed through three changes of distilled water for 1 min per change. The air-dried slides were cleared in xylene and then coverslipped with DPX (Sigma-Aldrich, St. Louis, MO, USA) nonfluorescent mounting media. The fluoro-jade C staining was examined with an Olympus light microscope equipped with epifluorescence (14).

Morris water maze (MWM) test

Learning tests were performed inside a round pool (water tank) made up of black Plexiglas which has 120 cm diameter, 80 cm depth. A hidden platform of 10 cm diameter was placed inside the tank. Rats were required to find this platform using environmental clues consisting of colored geometrical figures or patterns. Platform was fixed in the middle of the quadrants, 3–4 mm under water. The water was made opaque. Temperature of the water was set to 23 ± 1°C. Learning trials are conducted for 4 days, four times a day with 10-min intervals, where rats were released from a different pole every day and platform finding durations and thigmotaxis, tendency to swim close to the walls of the tank, and time were recorded (learning trial). If the rat could not find the platform within 2 min, it was directed to platform and placed carefully on the platform for 30 s. Then, it was taken away from the platform and dried with paper towel. The 5th day constituted the test phase; during test phase (probe trial), the platform was removed and rats were allowed to swim for 30 s. The duration of rat's stay on platform quadrant (target quadrant) was evaluated and expressed as a percentage. During the experiments, all the phases were recorded and analyzed using "HVS image" system. This system includes a

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CCD camera and software which analyses the captured images.

Statistical method

Statistical analysis was performed using SPSS for Windows software program 15.0 version (SPSS Inc, Chicago, IL, USA). Values were reported as mean ± SD. Morris water maze data were analyzed using a repeated-measures general linear model of ANOVA. Probe trial data were analyzed using one-way ANOVA *post hoc* Scheffe tests. For histomorphological data, we used nonparametrical statistical methods. Kruskal–Wallis test was used for comparing three groups. Differences between the two groups were examined with the Mann–Whitney *U*-test. $P < 0.05$ was accepted as statistically significant.

Results

All the rats in the acute groups survived the experiment, so 21 rats were sacrificed for histomorphological evaluation. One rat from GI and two rats from GIE died at the end of the anesthesia, so totally 18 rats performed the learning and memory tasks.

Apoptotic cell count

There was a significant increase in apoptotic cell count after isoflurane anesthesia in the frontal cortex when compared with control group (difference [95% CI]: −18.85 [−27.89 to −9.81]; $P = 0.002$, 20.85 ± 10.94 vs 2.00 ± 0.81 with caspase-3, difference [95% CI]: −25.71 [−33.37 to −18.05]; $P = 0.002$, 29.00 ± 9.27 vs 3.28 ± 0.75 with silver and difference [95% CI]: −19.42 [−28.01 to −10.84]; $P = 0.024$, 24.57 ± 10.40 vs 5.14 ± 0.69 with fluoro-jade C staining; Figures 1–3).

The apoptotic cell count in the frontal cortex was significantly lower in GC than GIE with caspase-3 staining (difference [95% CI]: −7.14 [−9.80 to −4.47]; $P = 0.002$, 2.00 ± 0.81 vs 9.14 ± 3.13). There were no differences between other brain regions (Figure 1).

We also observed that in GIE, apoptotic cell count was significantly reduced in the frontal cortex when compared with GI (difference [95% CI]: 25.00 [17.33–32.66]; $P = 0.002$, 4.00 ± 0.81 vs 29.00 ± 9.27 with silver, difference [95% CI]: 11.71 [2.33–21.09]; $P = 0.04$, 9.14 ± 3.13 vs 24.57 ± 10.40 with caspase-3 and difference [95% CI]: 20.57 [11.89–29.24]; $P = 0.012$, 4.00 ± 1.63 vs 24.57 ± 10.40 with fluoro-jade C staining; Figures 1–3).

The apoptotic cell count did not differ between groups in the other four brain regions (striatum, hippocampus, amygdala, and thalamus) examined (Figures 1–3).

Learning and memory test

In MWM tests, there were no differences between groups in time to reach the platform (latency), the distance traveled to the platform (path length), or speed.

All of the groups learned the place of the platform in MWM tests {latency (difference [95% CI]: 17.70 [10.47–24.93]; $P < 0.001$, Day 1: 28.06 ± 2.19 vs Day 4: 10.36 ± 0.93) and path length (difference [95% CI]: 3.51 [2.44–4.58]; $P < 0.001$, Day 1: 5.54 ± 0.53 vs Day 4: 2.03 ± 0.24) decreased significantly during the study period}, and there were no differences between the groups in learning process (Figure 4).

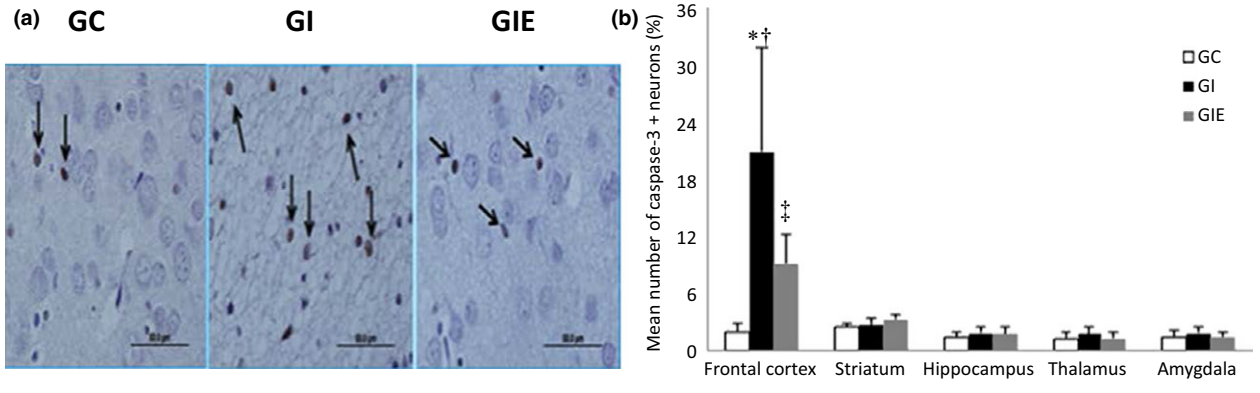


Figure 1 (a) Frontal cortex of three groups with caspase-3 staining (top, scale bar = 500 μm). Arrows are marking some of the cells stained with apoptosis marker caspase-3 (bottom, scale bar = 50 μm). (b) Isoflurane anesthesia in neonatal period increases the apoptotic cell number (% per section) in frontal cortex and erythropoietin diminishes its neurotoxicity. Columns represent mean number of positively stained cells with caspase-3 for each brain region (error bars show the SDs). *$P = 0.002$ compared with GC, †$P = 0.04$ compared with GIE, ‡$P = 0.002$ compared with GC. GC, Group Control; GI, Group Isoflurane; GIE, Group Isoflurane + Erythropoietin.

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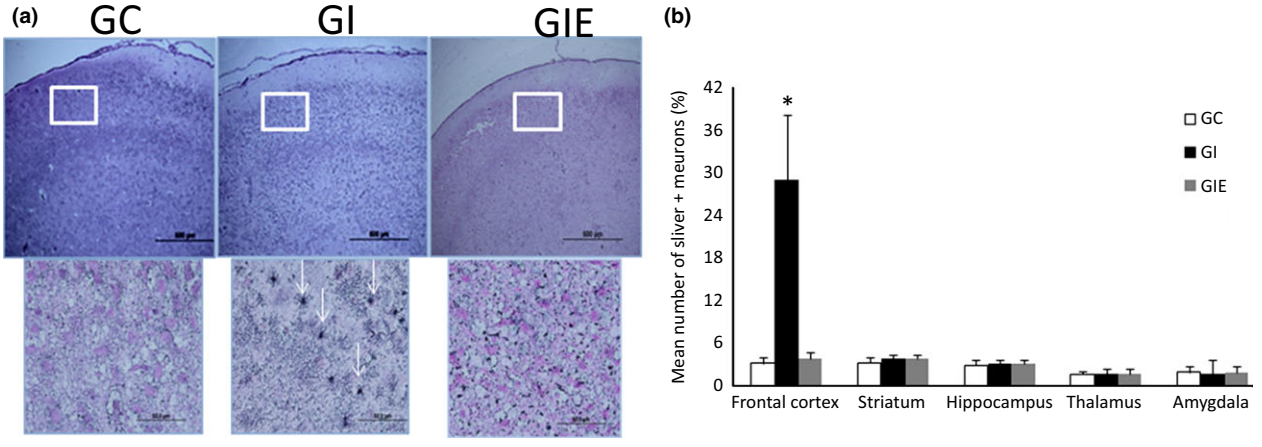


Figure 2 (a) Silver staining in frontal cortex (top, scale bar = 500 μm). Arrows show the positively stained apoptotic cells after isoflurane anesthesia (bottom, scale bar = 50 μm). (b) Isoflurane anesthesia in neonatal period increases the apoptotic cell number in frontal cortex and erythropoietin decreases this effect. Columns represent mean number of positively silver-stained cells for each brain region (error bars show the SDs). *P = 0.002 compared with GC and P = 0.002 compared with GIE. There were no differences between GC and GIE. GC, Group Control; GI, Group Isoflurane; GIE, Group Isoflurane + Erythropoietin.

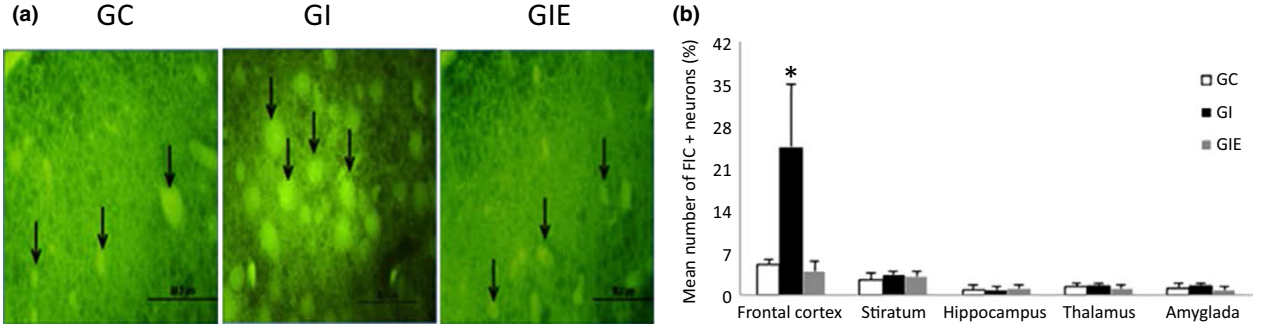


Figure 3 (a) Fluoro-jade C staining of frontal cortex of rats (top, scale bar = 200 μm). After isoflurane anesthesia, cell death increased and erythropoietin decreased apoptotic cell number in frontal cortex. Arrows show the Fluoro-jade-positive neurons (bottom, scale bar = 50 μm). (b) After isoflurane anesthesia in neonatal period, the apoptotic cell number in frontal cortex increased and erythropoietin decreased this effect. Columns represent mean number of positively stained cells with fluoro-jade C for each brain region (error bars show the SDs). *P = 0.024 compared with GC and P = 0.012 compared with GIE. There were no differences between GC and GIE. GC, Group Control; GI, Group Isoflurane; GIE, Group Isoflurane + Erythropoietin.

On probe trial, the time interval spent on target (original platform location; difference [95% CI]: −4.17 [−15.60 to 7.25]; P = 0.634, GC; 36.85 ± 8.94 vs GI; 41.03 ± 9.04, difference [95% CI]: 4.09 [−7.33 to 15.52]; P = 0.645, GC; 36.85 ± 8.94 vs GIE; 32.76 ± 7.0, difference [95% CI]: 8.26 [−2.77 to 19.30]; P = 0.167 GI; 41.03 ± 9.04 vs GIE; 32.76 ± 7.0 s) or opposite (across of the original platform) quadrant (difference [95% CI]: 1.35 [−9.02 to 11.73]; P = 0.942, GC; 11.28 ± 8.34 vs GI; 9.93 ± 4.58, difference [95% CI]: 0.04 [−10.34 to 10.42]; P > 0.999, GC; 11.28 ± 8.34 vs GIE; 11.24 ± 9.15, difference [95% CI]: −1.31 [−11.34 to 8.71]; P = 0.942, GI; 9.93 ± 4.58 vs GIE; 11.24 ± 9.15 s), and the rats' swimming speed (difference [95% CI]: 0.14 [−0.38 to 0.67]; P = 0.767, GC; 2.15 ± 0.64 vs GI; 2.01 ± 0.23, difference [95% CI]: 0.13 [−0.38 to 0.66]; P = 787 GC; 2.15 ± 0.64 vs GIE; 2.01 ± 0.11 and difference [95% CI]: −0.007 [−0.51 to 0.50]; P = 0.999 GI; 2.01 ± 0.23 vs GIE; 2.01 ± 0.11 $m \cdot s^{-1}$) did not differ among the groups. Animals in all groups stayed significantly longer in the target quadrant (Figure 5).

Discussion

In this study in which we aimed to identify the potential effects of systemic EPO after isoflurane anesthesia, our findings can be summarized as; first,

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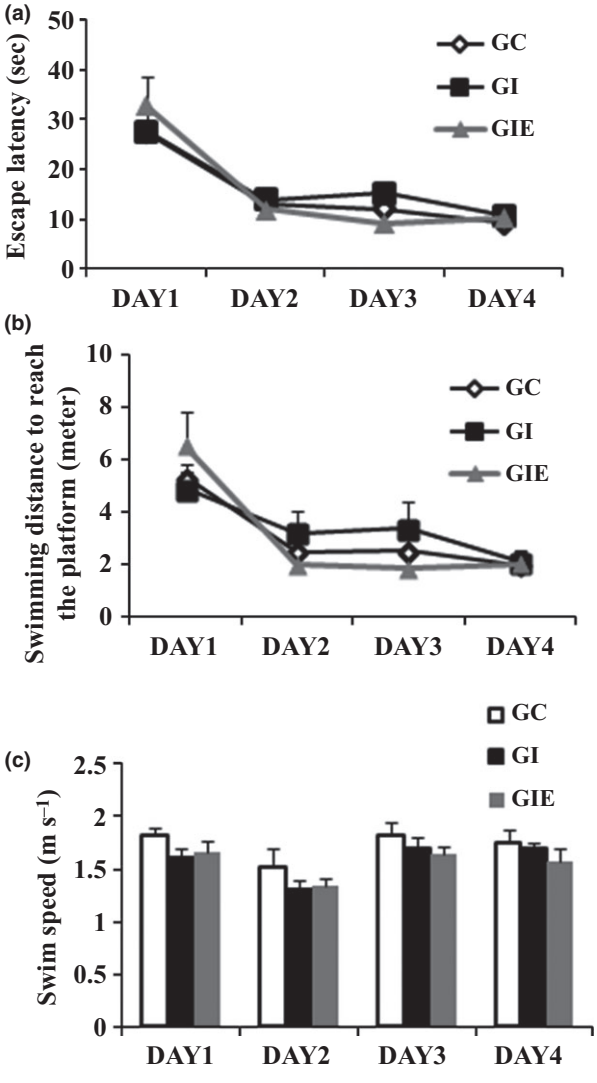


Figure 4 The Morris water maze performance of the groups. The daily change in (a) escape latency, (b) path, and (c) speed. There were no differences among the groups (error bars show the SDs). GC, Group Control; GI, Group Isoflurane; GIE, Group Isoflurane + Erythropoietin.

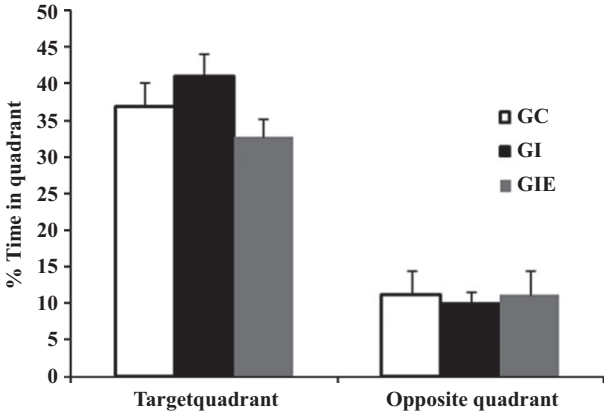


Figure 5 Time spent in the target and opposite quadrant in probe trial. There were no differences between groups (error bars show the SDs). GC, Group Control; GI, Group Isoflurane; GIE, Group Isoflurane + Erytropoietin.

isoflurane increased neural cell death in frontal cortex. Second, systemically administered EPO diminished the increase of neuroapoptosis. Third, isoflurane did not cause any learning and memory deficits in MWM tests.

Developing brain is vulnerable to the neurotoxic effects of the drugs that act on $GABA_A$ or NMDA receptors such as anesthetics, sedatives, or anticonvulsants (2,3). In many experimental studies, it has been shown that isoflurane exposure during this period is associated with neuroapoptosis (3,14,15) and learning and memory deficits (3,16,17). The mechanism of neuroapoptosis or cognitive impairment is not clear. It is accepted that isoflurane's neurotoxicity is dose- and time-related (2,3,14). As our goal was to investigate the neuroprotective effects of EPO, we used an isoflurane concentration (1.5%) which is relevant to clinical use and has been associated with significant neuroapoptosis (15,16,18). The minimum alveolar concentration (MAC) of isoflurane in humans has been found approximately 67% of the MAC in rats (16). About 1.5% isoflurane in rats is comparable to 1% concentration in humans which is within clinically used ranges.

Apoptotic cell increase in the frontal cortex after isoflurane anesthesia is comparable to the studies which used lower doses of isoflurane in neonatal monkey (19) and rat brains (14). Likewise, Jevtovic-Todorovic *et al.* (16) found a widespread apoptosis in neonatal rat brain including frontal cortex after triple anesthesia cocktail. However, there are different histopathological results reported in the literature. While several studies did not show any neurodegenerative changes, many studies found neurodegeneration in different parts of rodent brain after isoflurane anesthesia during neonatal period (2). The modifications in isoflurane concentration, anesthesia duration, the time of the histopathological examination, the staining, and the kind of the rodent (mice or rat) may be the reason of these different results. Caspase-3, silver, and fluoro-jade stains are well known cell death markers and being used as a marker of anesthesia-induced neuroapoptosis (18). So we used these staining to show neuroapoptosis after isoflurane anesthesia.

It has been reported that children who had anesthesia more than 2 h before 4 years had a risk for learning disability. Because of the need for surgery and anesthesia in early ages and the concerns about anesthesia-induced neurotoxicity, different interventions to prevent

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anesthetic neurodegeneration has been used (2,20). Erythropoietin is one of these interventions (1,6,7). EPO and EPO receptors are expressed in rodent and human brain (2,8). It has been shown that the lack of EPO receptors in the mice brain results with massive apoptosis indicating that EPO has a role in brain development (21). EPO diminishes ketamine- (7), isoflurane- (6), and propofol (1)-induced neurotoxicity. Tanaka *et al.* (22) showed that isoflurane and other general anesthetics inhibit hypoxia-induced EPO upregulation in a dose- and time-dependent manner.

Several studies have demonstrated that EPO has a dose–response behavior (1,8,9,23). In the cell culture, increasing EPO concentration after an optimal dose caused a decrease in the cell rescue rate (7). But high concentrations of EPO results with a down-regulation in EPO receptors. EPO doses above 10 000 or 20 000 IU·kg^{-1} have not been associated with greater neuroprotection (1). It has been observed that for tissues with tight endothelial barriers, like blood–brain barrier, effective doses are between 500 and 5000 IU·kg^{-1} (minimum effective dose ~500 IU·kg^{-1}) IP or intravenous (9,23). Besides these experimental studies that show high doses of EPO is not correlated with better results, some clinical studies demonstrated that high-dose EPO can cause serious cardiovascular and thromboembolic events, tumor progression, and increased mortality (23). Also in newborns, erythropoietic doses of EPO can cause neutropenia so high doses of this drug should be used carefully in infants. Thus, finding the optimal dose of EPO for neuroprotection is an important issue.

Tsuchimoto *et al.* (6) used 50 000 IU·kg^{-1} EPO before isoflurane anesthesia and they showed its neuroprotective effect. But as they also mentioned this dose is higher than clinically relevant doses (100 IU·kg^{-1} for treatment of anemia). Known side effects of EPO in adults are hyper-reactive platelets (24), red cell aplasia due to anti-EPO antibodies (25), and hypertension (26). These side effects have not been reported for neonates but side effects of such high doses have not been investigated. It has been reported that effective neuroprotective doses of EPO range between 1000 and 30 000 IU·kg^{-1} in rats (27). We decided to use the lowest dose because we showed this dose is neuroprotective in a previous study (28). Our results showed that 1000 IU·kg^{-1} EPO diminished isoflurane induced apoptosis in frontal cortex.

Timing of EPO administration is also important for neuroprotection. In the studies of stroke models, it has been shown that administration of EPO before or 6 h after ischemia results with a reduction of infarction volume and apoptosis (8). We used the dose, that has been shown to be effective after spinal cord ischemic injury in rabbits (1000 IU·kg^{-1}) (28), immediately after isoflurane anesthesia. Anesthesia-related neuronal cell death can start 6 h after anesthesia exposure (29). We planned to give EPO after isoflurane anesthesia like Pellegrini *et al.* (30). We think EPO could prevent apoptosis when it is administered during the start of anesthesia-related apoptosis.

During the study period, the physiological changes were not controlled and this might be a possible limitation. It is known that isoflurane can increase blood glucose levels (31) and hyperglycemia can cause neurotoxicity (32). But distinctively of these findings, Loepke *et al.* (15) showed that metabolic changes like lactic acidosis, hypercarbia, and hypoglycemia occurred after isoflurane anesthesia. The researchers also demonstrated that hypoglycemia is not responsible from neurodegeneration. In contrast, Tsuchimoto *et al.* (6) could not detect any metabolic change after isoflurane anesthesia. We did not detect any signs of hypoxia during the experiment. And we treated both isoflurane and isoflurane + EPO groups in the same way during anesthesia and showed that while isoflurane caused neuroapoptosis, EPO diminished isoflurane-related neurodegeneration. Another limitation of this study can be the usage of 100% oxygen. But in our laboratory we could only use 100% oxygen as carrier gas. As we want to investigate the neuroprotective effects of EPO for anesthesia related neurotoxicity, we decided to use 100% oxygen in all groups.

In conclusion, in the present study, we demonstrated that 6 h exposure to 1.5% isoflurane enhance apoptosis in the frontal cortex of neonatal rats but does not cause any learning and memory deficits when they become juveniles. Administration of IP 1000 IU·kg^{-1} EPO immediately after isoflurane anesthesia decreased the apoptotic cell number. These results indicate that relatively lower dose of EPO can prevent anesthesia-induced neurodegeneration effectively. This finding is important as the side effects related to high-dose EPO can be avoided. Further experimental dose–response studies have to be planned to reveal the optimal dose and timing of EPO before adaptation to clinical practice.

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Conflict of interest

The authors report no conflict of interest.

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