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Original Article

# Effects of isoflurane, ketamine and dantrolene on apoptosis in the rat hippocampus

## *Sıçan hipokampüsünde izofluran, ketamin ve dantrolenin apoptozis üzerine etkileri*

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## Abstract

**Aim:** Since both isoflurane and ketamine were already known to cause neurodegenerative apoptotic effects and dantrolene was known to inhibit degeneration, we investigated whether dantrolene could play a cytoprotective role under isoflurane and/or ketamine anesthesia. Therefore, we aimed to determine caspase activation as a marker of apoptosis in hippocampus (CA1 and dentate gyrus regions) of rats exposed to either administration of isoflurane with or without ketamine and dantrolene or isoflurane+ketamine+dantrolene.

**Material and Methods:** Thirty Wistar male rats were randomly assigned to five groups. Only oxygen 100% was administered into the closed cage for 2 hours in the control group (group A) whereas in the four study groups (as B,C,D and E), either 1.4% isoflurane alone in 100% oxygen was administered (group B) or 1.4% isoflurane in 100% oxygen was administered 60 minutes after intraperitoneal (ip) injection of dantrolene 10 mg/kg (group C), subcutaneous (sc) ketamine 40 mg/kg (group D) or ip dantrolene + sc ketamine (group E). Rats were sacrified to perform histopathologic and immunohistochemical analysis (hematoxylin staining caspase activation).

**Results:** Isoflurane alone (group B) and isoflurane+ketamine (group D) exposure to rats resulted in a significantly increased caspase activation when compared to control (group A) and dantrolene inhibited isoflurane + ketamine induced apoptosis in the hippocampus.

**Conclusion:** Isoflurane with or without ketamine caused neuroapoptosis in rats and dantrolene attenuated the apoptotic effect of both isoflurane and isoflurane+ketamine by decreasing caspase activation. These results might have an important promising role in anesthetic choice for specific susceptible group after further clinical studies.

**Key words:** Isoflurane; ketamine; dantrolene; apoptosis

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## Öz

**Amaç:** Hem izofluran ve ketaminin nörodejeneratif apoptotik etkileri gösterilmiş hem de dantrolenin dejenerasyonu inhibe ettiği bilindiği için biz izofluran ve/veya ketamin anestezisi altında dantrolenin sitoprotekif bir rolü olup olamayacağını araştırdık. Bu nedenle sıçan hipokampüsünde (CA1 ve dentat girus bölgelerinde) apoptozisin belirteci olan kaspaz aktivasyonunu; izofluranı ketamin ve dantrolen varken veya yokken veya izofluran+ketamin+dantrolen varlığında belirlemeyi amaçladık

**Gereç ve Yöntemler:** Wistar 30 erkek sıçan rastgele beş gruba ayrıldı. Kontrol grubunda (grup A) 2 saat sadece %100 oksijen verilirken, çalışma grubunda (B, C, D ve E) ya sadece %100 oksijen içinde %1.4 izofluran (grup B) veya intraperitoneal (ip) dantrolen 10 mg/kg enjeksiyonundan (grup C), subkutan (sk) ketamin 40 mg/kg enjeksiyonundan (group D) ya da ip dantrolen + sk ketamine enjeksiyonundan (group E) 60 dk sonra %100 oksijen içinde %1.4 izofluran uygulanmıştır. Sonra sıçanlar histopatolojik ve immunohistokimyasal inceleme (hematoxylin boyama ile kaspas aktivasyonu) için sakrifiye edilmişlerdir.

**Bulgular:** Sadece izofluran (grup B) ve izofluran+ketamine (grup D) maruz kalan sıçanlarda kontrol grubuna (grup A) göre hipokampüsde kaspas aktivasyonunda önemli artış ile dantrolenin izofluran+ketaminle indüklenmiş apoptozisi inhibe ettiği gözlendi.

**Sonuç:** İzofluranın ketamine varken ya da yokken sıçanda nöroapoptozise neden olduğu ve dantrolenle; izofluran ve izofluran+ketaminin apoptotik etkisinin azalan kaspaz aktivasyonuyla olduğu gösterilmiştir. Bu sonuçlar ileri klinik çalışmalardan sonra duyarlı grupların anestezi seçimde rol oynabilir.

**Anahtar kelimeler:** Izofluran; ketamin; dantrolen; apoptozis

## Introduction

Neurodegeneration in developing animals following exposure to anesthetics has been established with intravenous or inhalational anesthetics [1]. Among commonly used general anesthetics in practice, isoflurane and ketamine were associated with neurodegenerative apoptotic effects [2-5]. Based on the biochemical evidences, caspase activation in execution of apoptosis include initiation of either intrinsic or extrinsic pathway. Both pathways converge at the activaton of effector caspase-3 which cleaves several cellular proteins, finally leading to apoptosis [6]. Additonally, isoflurane induced apoptosis was inhibited by dantrolene. Thus, a pivotal cytoprotective role for dantrolene, which is a ryanodine receptor antagonist drug specifically used to treat malign hyperthermia has been considered [7]. Therefore, we aimed to investigate whether dantrolene could play a cytoprotective role under isoflurane anesthesia with or without ketamine by determining caspase activation as a marker of apoptosis in hippocampus (CA1 and dentate gyrus regions) of rats.

## Material and Methods

After obtaining approval of Gazi University Animal Experiments Local Ethics Committee (Resaerch Project number G.Ü.E.T-10.088), all procedures was carried out in accordance with the 'Care and Use of Laboratory Animals' rules, 30 young adult male Wistar rats weighing approximately 200±25 gram were housed with access to food and water in special cages at 21±1°C within a room adjusted 12:12 hour (h) light–dark cycle with lights on at 6 am for 7 days before the onset of study. Afterwards rats were randomly assigned to five groups as control (group A) and four study groups to administer either isoflurane alone (group B), isoflurane+dantrolene (group C), isoflurane+ketamine (group D) or isoflurane+ketamine+dantrolene (group E) in order to investigate early apoptosis by detecting caspase activation (caspase 3, 8 and 9).

1. Group A (n=6)

Only 100% O2 was administered at 4 L/minute (min) for 2 hours in the control group.

2. Group B (n=6)

1.4% isoflurane in 100% O2 at 4 L/min was administered for 2 hours.

3. Group C (n=6)

Sixty minutes (min) after intraperitoneal (ip) injection of dantrolene 10 mg/kg, 1.4% isoflurane in 100% O2 at 4 L/min was administered for 2 hours.

4. Group D (n=6)

Sixty min after subcutaneous (sc) injection of ketamine 40 mg/kg, 1.4% isoflurane in 100% O2 at 4 L/min was administered for 2 hours.

5. Group E (n=6)

Sixty min after ip injection of dantrolene 10 mg/kg and sc injection of ketamine 40 mg/kg, 1.4% isoflurane in 100% O2 at 4 L/min was administered for 2 hours.

**Anesthesia procedure**

In all groups spontaneous breathing was maintained in the closed cage which was considered as an anesthesia chamber having gas inlet and outlet. Oxygen and end-tidal isoflurane concentration in the chamber, heart rate and oxygen saturation were monitorized and rectal temperature of rats were kept approximately at 37.5 °C. All the experiments were performed at the same time (7 am). Pressured oxygen tank was connected to standard isoflurane vaporizator (Ohmeda® Isotec 3 Abbott) to deliver 1.4% isoflurane in 100% O2 at a flow rate of 4 L/min which was also connected to anesthesia chamber via a line. Gas analyser detector was connected to gas outlet of the anesthesia chamber. Both control and study groups were kept 6 hours within the steel cage during spontaneous breathing.

**Sacrification procedure**

After ip administration of ketamine 60 mg/kg, intracardiac blood samples were collected by the principal investigator and rats were sacrificed by exsanguination. Afterwards brain tissue was removed rapidly. Tissue samples were placed at pH 7.4 1/15 µ phosphate buffered 2.5% glutaraldehyde fixation solution for further electron microscope evaluation. After fixation, hippocampus was identified by vertical section. Tissue samples of 1 cm3 fixed in 10% neutral formalin for 72 hours were embedded in paraffin for further evaluation under light microscope.

**Immunohistochemical procedure**

Four µm sections of brain tissues based on polylsine-covered microscope slides from control group and study groups were subjected to immunohistochemical staining. Caspase activation to elucidate apoptosis was investigated in the hippocampus (CA1 and dentate gyrus regions).

## Detection of caspase activation

Caspase-9 (Caspase-9 LAB6/Ab- 4) rabbit polyclonal antibody (Cat: RB- 1205-P, Lot:1205P306), caspase-8 (Caspase-8 FLICE/Ab-4) rabbit polyclonal antibody (Cat: RB-1200-P, Lot: 1200P708C) and caspase-3 (Caspase-3CPP32/Ab-4) rabbit polyclonal antibody (RB-1197-P, Lot: 1197P701) were applied. As a secondary kit, Ultravision Detection System Anti-Rabbit HRP (RTU) (Cat: TP- 125 HL, Lot: PBN70509, Lab Vision, Fremont, USA) and HRP/AEC (Cat: TA-007-HAC, Lot: 007HAC13565) were used.

Sections were kept overnight at 37$^0$C in oven and then temperature was raised to 57$^0$C for substracting the deparafinization and allowed for 1 hour. To complete the deparafinization process, slides were exposed to xylene for 15 min twice, respectively 100%, 96% and 80%. Afterwards alcohol with water was applied every 10 minutes followed by distilled water every 5 min twice to remove the alcohol.

For providing elucidation close to the receptor sites of formaldehyde, sections were performed to 1M citrate buffer in microwave (pH: 6. 0) (Cat: AP- 9003- 500, Lot: 9003LT13610, Lab Vision, Fremont, USA). After cooling room temperature within 20 minutes, endogenous peroxidase activity was blocked with hydrogen peroxide (Cat: TA-125-HP, Lot: 125HP14119, Lab Vision, Fremont, USA) for 15 minutes. Then, the cross sections were washed 3 times with PBS (Phosphate Buffer Saline) at pH: 7.4 after 3 minutes, epitopes were stabilized by application of serum blocking solution. Ultra V Block (Cat: TA-125-UB, Lot: AUB70803, Lab Vision, Fremont, USA) was applied for 5 minutes. Without washing, Caspase-8 (Cat: RB-1200-P, Lot: 1200P708C), Caspase-9 (Cat: RB-1205-P, Lot: 1205P306) and Caspase-3 (RB-1197-P, Lot: 1197P701) the primary antibodies were incubated for 60 minutes. The cross sections were washed 3 times with PBS at pH: 7.4 after 3 minutes. Then biotinylated secondary antibody (Cat: TR-125-BN, Lot: RBN70115, Lab Vision, Fremont, USA) was applied and streptavidin peroxidase (Cat: TS-125-HR, Lot: SHR70515, Lab Vision, Fremont, USA) was applied to the slides for 20 min.

After washing with PBS, AEC (3-amino-9-ethylcarbazole) (Cat:TA-007-HAC, Lot: 007HAC13565, Lab Vision, Fremont, USA) was used as chromogen to the sections. Finally, the slides were stained with Mayer's Hematoxylin (Cat: TA-125-MH, Lot: AMH70809, Lab Vision, Fremont, USA) and were covered with Ultramount (Cat: TA-125-UG, Lot: VM13518, Lab Vision, Fremont, USA). Slides were examined under light microscope (DM4000B Image Analyze System, Leica, Germany) and Leica DFC280 plus camera. The number of immune positive cells are measured manually by using Qwin software programme in consecutive areas for serial cutaways taken from. A semi-quantitative scoring system was used to assess the immunolabeling intensity as descibed.[8]

## Statistical analysis

Statistical analysis was performed with SPSS 15.0 computer program. Results were presented as Mean ± Standard Deviation (SD). After descriptive statistics, mean caspase 3, 8 and 9 values were compared with Kruskal-Wallis Variance Analysis. Then, Mann Whitney U test followed by Bonferroni correction was used to compare the differences among the groups. A p value less than 0.05 was considered as statistically significant.

## Results

Comparisons with respect to control (group A) and combination of all three drugs (group E) were presented in the tables 1 and 2.

**Table 1.** Caspase activity in the hippocampus (CA1 region) with respect to groups.

| | Caspase-3 | Caspase-8 | Caspase-9 |
|---|---|---|---|
| Group A | 15.7±1.9 | 15.2±1.6 | 16.0±1.8 |
| Group B | 21.5±2.0*# | 21.4±1.4*# | 22.0±1.6*# |
| Group C | 16.5±0.5 | 16.0±0.5 | 16.6±0.6 |
| Group D | 29.6±1.7*#§ | 28.3±1.0*#§ | 30.0±4.0*#§ |
| Group E | 19.9 ±1.6# | 19.0±1.5*# | 20.4±1.2*# |

Data (immunoreactive cells) are expressed as Mean ± SD
Group A (control)
Group B (isoflurane)
Group C (isoflurane+dantrolene)
Group D (isoflurane+ketamine)
Group E (isoflurane+ketamine+dantrolene)
*: p< 0.05 versus (vs) group A
#:p< 0.05 vs group C
§:p<0.05 vs group E

**Table 2.** Caspase activity in the hippocampus (dentate gyrus region) with respect to groups.

| | Caspase-3 | Caspase-8 | Caspase-9 |
|---|---|---|---|
| Group A | 18.5±2.2 | 16.5±1.3 | 18.4±1.8 |
| Group B | 24.7±0.9*#§ | 22.7±0.9*# | 29.7±3.8*#§ |
| Group C | 19.4±1.8 | 18.1±1.0 | 19.9±1.6 |
| Group D | 34.2±1.4*#§ | 30.2±2.0*#§ | 39.3±3.1*#§ |
| Group E | 21.0±1.4 | 20.4±1.8 | 22.9±1.7* |

Data (immunoreactive cells) are expressed as Mean ± SD
Group A (control)
Group B (isoflurane)
Group C (isoflurane+dantrolene)
Group D (isoflurane+ketamine)
Group E (isoflurane+ketamine+dantrolene)
*: p< 0.05 versus (vs) group A
#:p< 0.05 vs group C
§:p<0.05 vs group E

### Caspase activity (3, 8 and 9) in the hippocampus CA1 region

Isoflurane alone (group B) and isoflurane+ketamine (group D) resulted in increased caspase activation (caspase 3, 8 and 9) with respect to control (group A) (p=0.004). After exposure of isoflurane+dantrolene (Group C), caspase activity did not differ from the control group which can be explained by the inhibitory effect of dantrolene on isoflurane induced caspase activation. There was an increased caspase activity in group D with respect to control. There was a decreased caspase activity in group E because of inhibitory potential of dantrolene against neuroapoptosis.

There was a markedly increased caspase activity by isoflurane+ketamine in group D which was affected by addition of dantrolene to isoflurane+ketamine in group E. After exposure of isoflurane+ketamine+dantrolene (Group E), caspase 3 activity did not show any significant difference with respect to control but caspase 8 and 9 were significantly higher with respect to control group (p=0.004) (table 1).

### Caspase activity (3, 8 and 9) in the dentate gyrus region

After exposure of isoflurane (group B) and isoflurane+ketamine (group D), there was a significantly increased caspase activation (caspase 3, 8 and 9) when compared to group A.

Exposure of isoflurane (group B) and isoflurane+ketamine (group D) resulted in signicantly increased caspase activity when compared to control. Of note, group E (isoflurane+ ketamine + dantrolene) and group A (control) were similar. Caspase 3 and 9 in group B and caspase 3,8 and 9 activity in group D were inhibited by isoflurane+ketamine+dantrolene (group E) (p=0.004) (table 2).

### Histopathologic findings

The immunoreactive cells in the hippocampus cross-sections indicated with black arrow were displayed (figures 1 A, B, C, D, E and F).

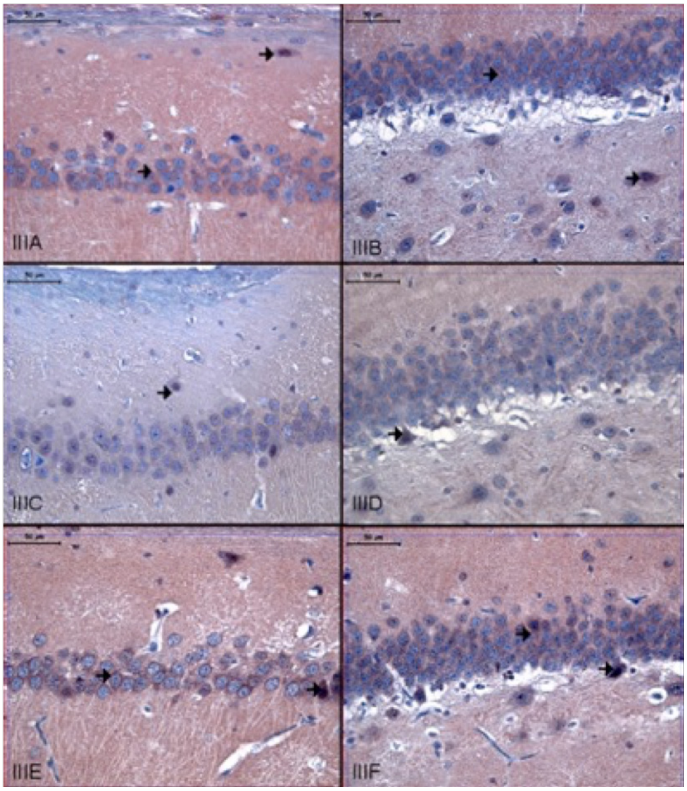


**Figure 1.** Caspase 3 immunoreaction in the hippocampus crossections indicated with an arrow (>). Immunoperoxidase & hematoxilen staining, magnification X 400.
**(A)** CA1 area in group B
**(B)** dentate gyrus regions in group B
**(C)** CA1 area in group C
**(D)** dentate gyrus regions in group C
**(E)** CA1 area in group D
**(F)** dentate gyrus regions in group D

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## Discussion

In the present study, we demonstrated that exposure of isoflurane with or without ketamine significantly increased the caspase activity (caspase 3, 8 and 9 positive neurons) in the hippocampus where it is the major target site for general anesthetics in the central nervous system. Our current data also provided a couple of insights. First, although isoflurane and ketamine were individually known to be associated with neurodegeneration, more prominent neuroapoptotic effect was observed after exposure to isoflurane plus ketamine in terms of increased caspase activation. The reason how we designed the study groups and why we selected these anesthetics are; ketamine is an intravenous anesthetic agent used for induction of anesthesia and isoflurane is an anesthetic commonly used for maintenance of anesthesia which means these two anesthetics are used together in a consecutive manner in a standard anesthesia practice. Secondly, we might indirectly suggest that addition of dantrolene to isoflurane+ketamine could have attenuated neuroapoptosis via a potential inhibitory neuroprotective by decreasing caspase activation in the current experimental setting.

Apoptotic effect of isoflurane was investigated by detecting immunohisthochemical activity for caspase activity in rats exposed to isoflurane at different concentrations with different durations. Earlier trials reported that either a 6 h exposure of isoflurane in combination with midazolam and nitrous oxide resulted in a widespread apoptotic neurodegeneration or a 4-h administration of isoflurane as a single anesthetic agent provoked brain cell death followed by learning impairment, memory retention tests and spatial learning later in adulthood [3,4].

After exposure of 1.4% isoflurane to prefrontal cortex of 5 to 6 month-old rats for 3 times with a 2-h duration every 15 min, significantly higher caspase 3 expression than the control group was observed [9]. When isoflurane 1%, 1.5% or 2% was administered to 2 to 3-month old rats for a period of 1 h) [10], prominent neurodegeneration was encountered 3 hours after 1% isoflurane exposure in the hippocampus CA1 region of the rats. Similarly, we found a significantly increased caspase activation in the hippocampus after 2 h administration of 1.4% isoflurane with or without ketamine. In a study by Xie et al, when rats were exposed to 1.4% isoflurane in 100% O2 for 2 hours in closed cages [9], maximum immunohisthochemical activity for caspase 3 at the cerebral cortex was found after 6 hours. Therefore, we particularly evaluated the caspase activity in the hippocampal CA1 and dentate gyrus areas 6 hours after 1.4% isoflurane administration in order to find the maximum effect.

Similar to isoflurane induced apoptotic effects, ketamine caused an accelerated neurodegeneration/neuronal apoptosis and neurocognitive deficit via caspase-3 activation in newborn rats [5] Single or multiple consecutive doses of ketamine (5 mg/kg, 10 mg/kg and 20 mg/kg) were administered to 7 day-old rats subcutaneously to evaluate caspase 3 activity at frontal cortex 6 hours after anesthesia. Six consecutive doses of 5 mg/kg and 10 mg/kg ketamine and single dose or 3 consecutive doses of 20 mg/kg ketamine did not result any neurotoxic effect whereas 6 consecutive doses of 20 mg/kg ketamine elevated caspase 3 activity [11]. Based on these findings, we designed our study to use a single dose of 40 mg/kg ketamine and observed elevated caspase activity in rats treated with 1.4% isoflurane for 2 hours.

Apoptotic effect of isoflurane and ketamine were investigated in cell culture studies as well. Both isoflurane and ketamine were associated with increased caspase 3 activity [7, 12]. Our results also demonstrated neuroapoptosis with both agents; a higher degree of caspase activity with isoflurane+ketamine than isoflurane alone.

There are mainly two major biochemical pathways for caspase activation in execution of apoptosis. The intrinsic (mitochondrial) pathway of apoptosis involves mitochondrial dysfunction, release of cytochrome c and subsequent activation of caspase-9 at the apoptosm. The extrinsic (death receptor) pathway is initiated by binding of death receptors to the death ligands and subsequent recruitment of an adaptor protein and caspase-8 into the death-induced signaling complex. These pathways converge to activate effector caspase-3 which cleaves several cellular proteins leading to apoptosis involving DNA fragmentation in the nucleus. [6] Currently, we identified neuroapoptosis in the hippocampus using immunostaining for elucidating the activated suicide enzyme caspase-3 which was significantly decreased by dantrolene particularly in isoflurane+ketamine induced apoptosis.

General anesthetics produce drug-specific and distinctive effects by modulating either excitatory and/or inhibitory synaptic transmission via different pathways in the central nervous system. Gamma (γ) amnino butyric acid (GABA) is the major inhibitory neurotransmitter and the density of GABAA receptor was highest in CA1 and dentat gyrus subregions of hippocampus. Glutamate is the major excitatory neurotransmitter in the hippocampus and there are two functional subtypes as N-Methyl D-Aspartate (NMDA) and non-NMDA glutamate receptor [13]. Volatile anesthetic

isoflurane, enhances GABAA receptor activity whereas intravenous anesthetic ketamine blocks NMDA glutamate receptors [14]. We already know that neurodegeneration following exposure to isoflurane and ketamine was established in developing animals [1]. As for ketamine induced apoptosis which was found to be concentration and time dependent via mitochondrial pathway was completely prevented by caspase inhibition [15]. It was also reported that apoptosis inducing effect of ketamine is unlikely to involve NMDA receptor [16]. Thus, exposure to GABAA receptor agonist and/or NMDA receptor antagonists leading to neuronal inhibition do not necessarily result in neuroapoptosis.

Regarding dantrolene`s inhibitory effect on isoflurane induced cytotoxicity [7], it blocks Ca+2 release from sarcoplasmic reticulum to cytosol [16]. Calcium access to the cytosol activates Ca+2 dependent enzymes and induces irreversible cell damage, impairs functions of mithocondrium and endoplasmic reticulum and finally results in cellular death. Dantrolene was shown to be highly neuroprotective in 7 day-old rats with hypoxic brain damage [17]. In a cell culture study, pretreatment with 30 μM dantrolene for 30 min significantly decreased the number of isoflurane induced apoptotic cells from 17% to 7% [7]. In the current study we found that pretreatment with ip administration of dantrolene 10 mg/kg attenuated isoflurane and isoflurane plus ketamine induced apoptosis by decreasing caspase 3 activity, which is the main effector, around 24% and 35%, respectively.

Protective and neurotoxic effects of commonly used anesthetics either in ischemia-reperfusion or apoptosis settings were investigated in animal models [18,19]. After rats anesthetized with 1.4% isoflurane, ketamine or 70% nitrous oxide and fentanyl were subjected to either incomplete or near-complete ischemia induced by bilateral carotid occlusion, the brain was maintained at normothermia during 22 h long ischemia. Five days later, no difference among anesthetic agents was observed during incomplete ischemia. However, , isoflurane was found to be protective when compared with fentanyl and ketamine during near-complete ischemia with no difference between fentanyl and ketamine [18]. In a comparative study, increased number of activated caspase-3 positive neurons were identified by immunostaining in the cerebral cortex and basal ganglia of 7-day old mice with xenon or isoflurane anesthesia but exposure of both xenon and isoflurane resulted less neuroapoptosis. These results suggested that xenon could have exhibited a potential neuroprotective effect during isoflurane anesthesia [19]. Additionally, when exposed to these commonly used anesthetics, susceptibility of neurons both within the developing and adult rodent brain to neuroapoptosis was addressed. [19]

Based on the information related to complete organogenesis associated with low risk of preterm labor, the dogma related to the best timing of non-obstetric surgery during 2nd trimester has been recently revisited. Collectively, fetal brain was reported to be more vulnerable to the adverse neurodevelopmental effects of inhalation anesthetics and ketamine during the 3rd trimester [20].

## Conclusion

Neurodegenerative effect of isoflurane with or without ketamine decreased after adding dantrolene in rats. Presumably, underlying mechanism is through intrinsic and/or extrinsic apoptotic pathways. Despite lack of available data supporting the selection of one anesthetic over the others and timing of surgery, these results might have important clinical implications when planning anesthesia for specific surgeries like non-obstetric surgery.

## Declaration of conflict of interest

This study was granted with TUBITAK (Project Number:110S514. There is no conflict of interest.

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