

## The effects of midazolam and etomidate on the antioxidant system in the rat liver

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**Abstract:** This study was conducted to investigate the histopathological and biochemical effects of midazolam and etomidate, which are intravenous anesthetics used in surgical operations, on rat liver. There were 3 study groups each consisting of 9 animals: the control, midazolam, and etomidate groups. Midazolam and etomidate were administered 10 times with 1-day intervals. At the end of the study, tissues were taken for histological examination and biochemical analysis. We determined superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels in the liver. There was a slight increase in MDA level in the midazolam-exposed group when compared with the controls, and this was not statistically significant. However, SOD and CAT activities were significantly increased in the midazolam group. We found slight damage in histological sections in parallel to biological findings. MDA levels were significantly increased in the etomidate group and SOD and CAT activities were also prominent in this group. More histological changes like hepatocyte degeneration, vascular congestion, and mononuclear cell infiltration were observed in the etomidate group and this was statistically significant. We can say that these intravenous anesthetic agents may cause dose-dependent damage in tissue. Therefore, we think that one must be cautious of the administration duration and dose levels of anesthetics during surgical operations.

**Key words:** Catalase, etomidate, liver, malondialdehyde, midazolam, superoxide dismutase

### 1. Introduction

Inhalation anesthetics exert their effects in a short time period. Some risk factors like air contamination in the surgery room and toxicity are present while using these agents. For this reason, intravenous anesthetics are widely preferred in current practice (Reves et al., 1987). An ideal anesthetic agent should fulfill some criteria such as rapid effect during surgical operations, absence of toxicity in tissues and organs, large confidence interval, and short awakening time right after the cessation of anesthesia application (Aitkenhead and Smith, 1990; Rang, 1995; Trevor and Miller, 1998). Although huge improvement has been established in intravenous agent research, there are still some issues with toxicity. Toxic effect can be caused by the agent or its solution in water (Sear, 1987). Midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo(1,5-a)(1,4) benzodiazepine) is a benzodiazepine-derived intravenous anesthetic agent with short half-life in the body (Khanderia and Pandit, 1987).

Midazolam is a widely used short-acting benzodiazepine with hypnotic, sedative, anticonvulsant,

muscle-relaxant, amnesic, and anxiolytic properties (Jabor et al., 2005; Emoto and Iwasaki, 2007). Midazolam is preferred over diazepam because of its shorter induction and recovery times and its marked amnesic properties (Lichtenstein et al., 2008; Riphaus et al., 2009; Correia et al., 2011). About half (40%–50%) the amount of midazolam is rapidly metabolized in the liver via hydroxylation and subsequent glucuronidation reactions (Vinik et al., 1981). At lower doses, midazolam has muscle relaxation, anxiolytic, amnesic, and sedative effects, whereas at higher doses it can lead to stupor and unconsciousness (Jong and Bonin, 1981; Riphaus et al., 2009). Etomidate is a carboxylated imidazole-derived general anesthetic agent (Reves et al., 1987). Etomidate is used especially in cases such as heart or lung transplantation, reactive airway disease, and intracranial pressure increment (Krumholz et al., 2001). Etomidate is eliminated primarily in liver via ester hydrolysis or N-dealkylation mechanisms (Reves et al., 1999). Etomidate shows its depressant effects on reticular formation, similar to those of the barbiturate derivatives, and it also has inhibitor effects on spinal

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neurons (Işık, 2004). However, etomidate at 0.4 µg/mL has no significant effect on isolated rat liver mitochondria, but at 4 µg/mL it can lead to disruption of mitochondrial energy metabolism of rat livers in vitro (Liu et al., 2009). In previous studies, it has been reported that some anesthetic agents administered via inhalation or intravenous routes could lead to decrease in hepatic blood flow or hepatotoxicity (Hanquet, 1972; Fassoulaki et al., 1986; Atiba et al., 1988).

Free radicals affect important cellular components such as lipids, proteins, DNA, and carbohydrates. They have affinity to macromolecules such as phospholipids, glycolipids, unsaturated lipid acids of glycerides, and membrane proteins (Codogan, 1973). There are many studies reporting that some intravenous anesthetic agents may lead to an increase in reactive oxygen species and consequent tissue damage (Fassoulaki et al., 1986; Murphy et al., 1993; Okutomi et al., 1995; Abidova, 2002). In this study, we aimed to investigate the effects of midazolam and etomidate on liver tissue, lipid peroxidation, antioxidant defense system, and histopathological changes.

## 2. Materials and methods

### 2.1. Experimental conditions

Twenty-seven male Wistar albino rats weighing between 180 and 240 g were obtained from the Süleyman Demirel University Laboratory of Animal Science (Isparta, Turkey). The rats were housed in Makrolon cages under standard laboratory conditions (light period 0700 to 1900 hours;  $21 \pm 1$  °C). Animals were maintained and used in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals as prepared by Süleyman Demirel University.

The rats were divided into three groups as follows: control group ( $n = 9$ ), midazolam group ( $n = 9$ ), and etomidate group ( $n = 9$ ). Midazolam (F. Hoffmann-La Roche Ltd., Switzerland) was intraperitoneally administered at a dose of 3 mg/kg for 20 days with 1-day intervals. Etomidate (B. Braun Melsungen AG, Germany) was intraperitoneally injected at a dose of 20 mg/kg for 20 days with 1-day intervals (Lee et al., 2000; Molina-Hernandez and Tellez-Alcantara, 2001). Two of the animals died during this period.

At the end of the experimental period, all rats were sacrificed and liver tissues were collected for histopathological and biochemical analysis.

### 2.2. Histopathological examination

Liver tissue samples were fixed with 10% neutral formalin and then routine light microscopic techniques were used on the tissue samples. Paraffin-embedded blocks were cut at 3–5 µm and stained with hematoxylin and eosin (H&E). The slides were then examined using a light

microscope (Olympus BX 50, Japan) and photographed. All histological evaluations were made twice under blind conditions (without knowledge of the treatment). The histopathological findings were graded according to the degree of changes (Abdel-Wahhab et al., 1999).

### 2.3. Biochemical examination

The frozen tissue samples of liver were weighed and homogenized (Ultra Turrax T25, Germany) (1:10, w/v) in 50 mM L<sup>-1</sup> phosphate buffer (pH 7.4) kept on an ice bath. The homogenate was then centrifuged at  $5000 \times g$  for 30 min to remove debris. The homogenate and supernatant were frozen at -20 °C in aliquots until used for biochemical assays. The protein content of the tissue was determined using the Lowry method (Lowry et al., 1951).

#### 2.3.1. Determination of MDA

Malondialdehyde (MDA) levels, an indicator of free radical generation, which increases at the end of the lipid peroxidation, were estimated by the double heating method of Drapper and Hadley (Drapper and Hadley, 1990). The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid with MDA. The results were given as nanomoles per gram wet tissue.

#### 2.3.2. Determination of SOD activity

Total (Cu-Zn and Mn) superoxide dismutase (SOD) activity was determined according to the method of Sun et al. (Sun et al., 1988). The method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Activity was expressed as units per gram protein.

#### 2.3.3. Determination of CAT activity

Catalase (CAT) activity was measured according to the method of Aebi (Aebi, 1984). The principle of the assay is based on the determination of the rate constant  $k$  (unit of measure: s<sup>-1</sup>,  $k$ ) of hydrogen peroxide decomposition. By measuring the absorbance change per minute, the rate constant of the enzyme was determined. Activities were expressed as  $k$  (rate constant) per gram protein.

### 2.4. Statistical evaluation

Statistical analysis was performed with SPSS 9.0 for biochemical parameters. One-sample Kolmogorov-Smirnov and ANOVA tests were used for group comparisons. P values of less than 0.05 were considered as significant.

SPSS 9.0 for Windows was used for statistical analysis of histopathological findings. The extent of histopathological changes of all experimental groups for liver tissues was semiquantitatively graded as follows: (-) indicates no changes, and (+), (++) and (+++) indicate

mild, moderate, and severe changes, respectively. These values were considered to be nonparametric; therefore, data were statistically analyzed by the Mann–Whitney U test and  $P < 0.05$  was considered significant.

### 3. Results

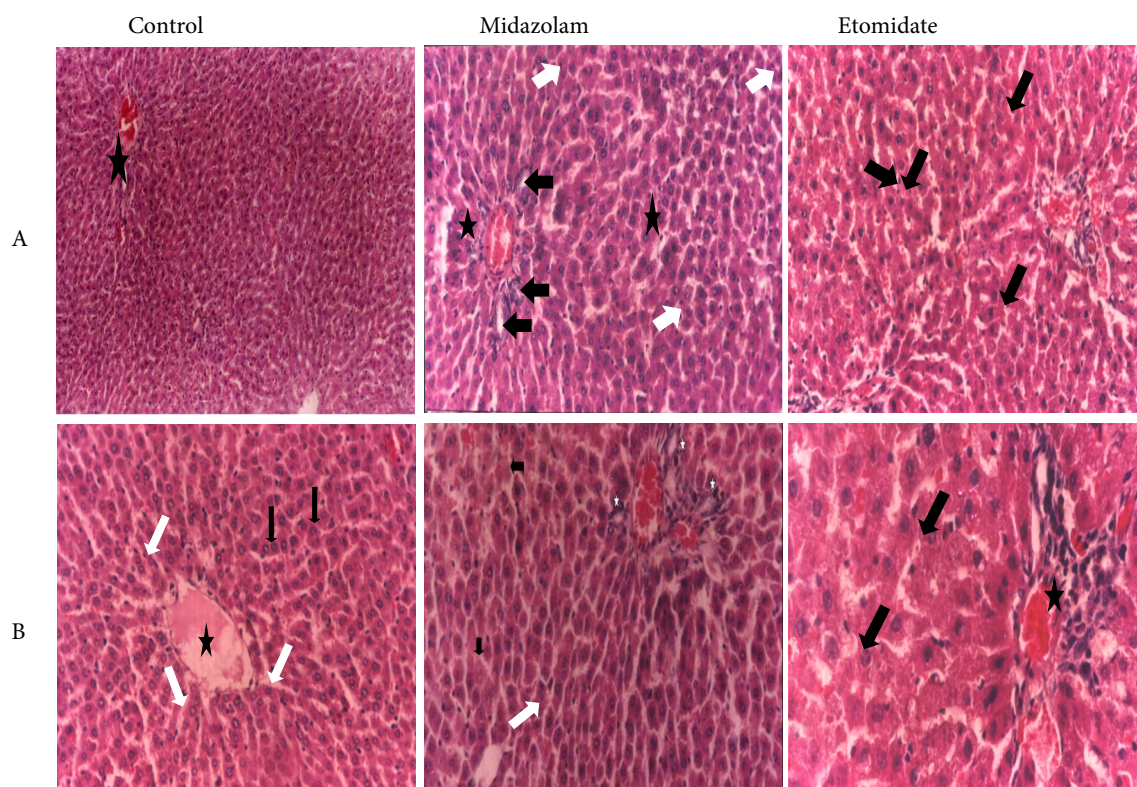
#### 3.1. Histopathological findings

Liver tissue samples of the control group were normal. Hepatocytes, portal triads, and sinusoids were normally observed, as shown in Figure 1. There were significant structural changes in the liver tissues of the midazolam group as compared with the controls, as shown in Table 1. Histopathological examination of liver tissues from the rats treated with midazolam revealed that there were some structural changes such as granular degeneration and pyknotic nuclei in hepatocytes, necrotic cells, vascular congestion, hemorrhage, bile duct proliferation, and mononuclear cell infiltration, shown in Table 1 and Figure 1. There were also some significant changes in liver tissues

of the etomidate group when compared with the controls, as shown in Table 1 ( $P < 0.05$ ). Histopathological changes such as granular degeneration in hepatocytes, vascular congestion, pyknotic nuclei, bile duct proliferation, and mononuclear cell infiltrations in perivascular and portal areas were observed in the etomidate group, as shown in Figure 1. Structural changes and semiquantitative evaluation parameters are given in Table 1.

#### 3.2. Biochemical findings

There was a slight increase in mean MDA levels in the midazolam group when compared with the controls, but this was not statistically significant (Figure 2). However, there was a significant increase in hepatic SOD and CAT levels in the midazolam group, as shown in Table 2 and Figure 2. There was a statistically significant difference between the etomidate group and controls in terms of MDA, SOD, and CAT levels, as shown in Table 2 ( $P < 0.05$ ). Mean values of SOD, MDA, and catalase are given in Table 2.

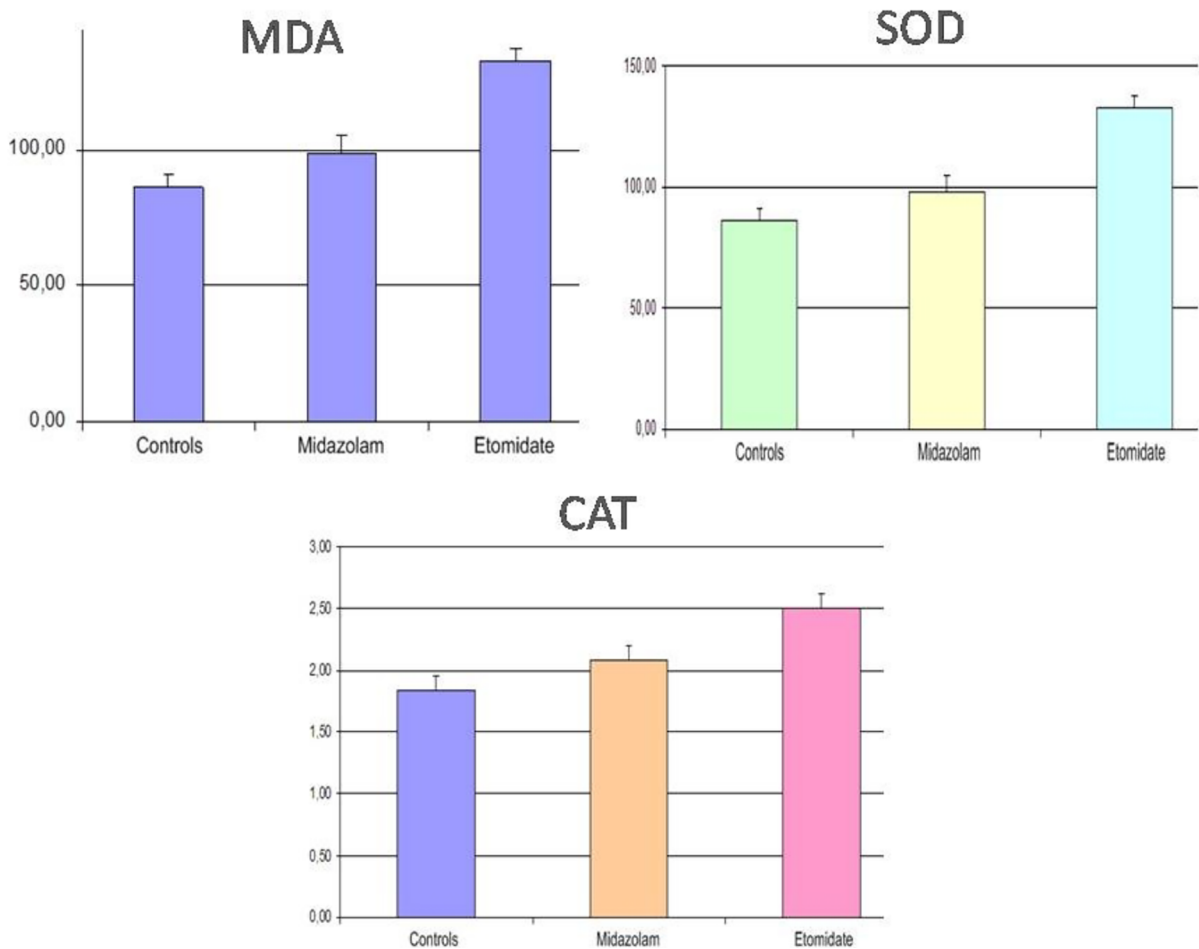


**Figure 1. Control group:** Hepatocytes (black arrow), sinusoids (white arrow), central vein (asterisks), and portal areas (asterisks) are normal in appearance (H&E) (A: 120 $\times$ ; B: 240 $\times$ ). **Midazolam group:** A) Bile duct proliferation (black arrow), hepatocyte necrosis (asterisks), and hepatocyte with pyknotic nucleus (white arrow) are present. B) There is granular degeneration of hepatocytes (black arrow), mononuclear cell infiltration in portal area (white asterisks), and hepatocyte with pyknotic nucleus (white arrow) (H&E; A: 240 $\times$ , B: 240 $\times$ ). **Etomidate group:** A) Hepatocytes with pyknotic nuclei (black arrows) are present. B) Granular degeneration of hepatocytes (black arrows) and mononuclear cell infiltration in portal area (asterisks) are evident. (H&E; A: 240 $\times$ , B: 480 $\times$ ).

**Table 1.** Grading of histopathological changes in liver tissues of the groups.

Experimental groups	Group I (control) n = 9					Group II (midazolam) n = 9					Group III (etomidate) n = 7					P-values
Parameters / score <sup>a</sup>	-	+	++	+++	O	-	+	++	+++	O	-	+	++	+++	O	
Hepatocyte degeneration	6	3	0	0	-	0	3	6	0	++	0	0	2	5	+++	P = 0.0001
Vascular congestion	8	1	0	0	-	2	7	0	0	+	0	2	5	0	++	P = 0.0001
Perivascular mononuclear cell infiltration	9	0	0	0	-	1	7	1	0	+	0	2	4	1	++	P < 0.0001
Hepatocyte necrosis	9	0	0	0	-	2	7	0	0	+	5	2	0	0	-	P < 0.0001
Hepatocyte with pyknotic nucleus	9	0	0	0	-	2	7	0	0	+	1	6	0	0	+	P < 0.0020
Mononuclear cell infiltration in parenchyma	9	0	0	0	-	1	7	1	0	+	6	1	0	0	-	P < 0.0001
Mononuclear cell infiltration in portal areas	9	0	0	0	-	1	7	1	0	+	0	1	6	0	++	P < 0.0001
Bile duct proliferation	9	0	0	0	-	2	7	0	0	+	6	1	0	0	-	P < 0.0001

n = Number of cases. \*Score (-): no meaningful histopathological changes. Score (+): mild degree. Score (++) : moderate degree. Score (+++) : severe degree.

**Figure 2.** Biochemicals parameter values of experimental groups in liver tissues.

**Table 2.** Changes in the parameters of SOD, CAT, and MDA.

Experimental groups	SOD (U/g protein)	CAT (k/g protein)	MDA (nmol/g wet tissue)
Control (n = 9)	86.01 ± 4.61	1.84 ± 0.11	10.84 ± 1.05
Midazolam (n = 9)	98.30 ± 6.62	2.08 ± 0.12	11.49 ± 0.94
Etomidate (n = 7)	132.69 ± 4.55	2.50 ± 0.11	16.86 ± 1.26
P values			
I-II	0.0001	0.002	n.s.
I-III	0.0001	0.0001	0.0001

Values are expressed as mean ± SD.

n.s.: not significant

#### 4. Discussion

Experimental studies have focused on the effects of anesthetic agents on various organs and systems and the liver has been the most emphasized organ in this regard because the localization of liver is suitable for metabolite storage, substance transformation, and/or other detoxification processes. Harmful effects of some drugs are prevented via oxidation, methylation, or conjugation mechanisms in the liver (Işık, 2004; Koçkar et al., 2010). Numerous studies have indicated that some inhalation or intravenous anesthetic agents may cause decrease in blood flow and hepatotoxicity (Hanquet, 1972; Fassoulaki et al., 1986; Atiba et al., 1988; Fee et al., 1997). It has also been reported that lower doses of midazolam have no effect on enzymatic induction, whereas administration of midazolam at repeated or high doses can lead to elevation in enzymatic activity levels, hepatic functional disorders, and tissue damage in the liver (Amrein and Hetzel, 1990; Nilsson, 1991). During the cellular metabolism process, some cytotoxic free radicals occur. Such compounds include superoxide anions, hydroxyl radical, hydrogen peroxide, and some other radicals. Interaction between the reactive oxygen species (ROS) and cellular macromolecules can result in oxidative damage and cellular dysfunction. ROS especially affect the polyunsaturated fatty acid unit of membrane phospholipids in cells and lead to the formation of hydroperoxides via the peroxidation pathway. Hydroperoxides in turn lead to an increase in ROS amount. This cascade may cause severe cellular damage and cell death (Özcan et al., 1992; Bachowski et al., 1997; Ceylan et al., 2011). In numerous studies, it has been reported that some intravenous anesthetic agents cause an increase in ROS production and tissue damage as an indicator of deleterious effects (Fassoulaki et al., 1986; Murphy et al., 1993; Okutomi et al., 1995; Abidova, 2002). Hoen et al. (2001) reported that intraperitoneal

administration of midazolam to rats at a dose of 50 mg/kg resulted in 4-fold increase in cytochrome P-450 (CYP-3A) isoenzyme expression. MDA is a substance produced as a result of the interaction between ROS and cell membranes and it is an important indicator for lipid peroxidation (Nazıroğlu et al., 2004). MDA can lead to damage in the plasma membrane, deterioration of cellular homeostasis, and loss in ion transport systems and intercellular gap junction communication (Özcan et al., 1992). The antioxidant enzyme system is the most important defense mechanism against membrane damage induced by lipid peroxidation in mammalian cells. CAT is another enzyme that catalyzes the dissociation of hydrogen peroxide and other lipid peroxides, thereby protecting the cell membranes and hemoglobins against oxidation (Bleeker et al., 1994; Nazıroğlu, 2012). CAT is present in virtually all cells in the body and it is especially effective in the case of high hydrogen peroxide concentrations (Bushnell et al., 1994; Nazıroğlu, 2012). CAT enzyme activity may show huge diversity in various organs. The liver and kidneys are the organs with the highest CAT activity. SOD enzyme protects oxygen-catabolizing cells against the harmful effects of superoxide free radical and inhibits lipid peroxidation (Niwa et al., 1990; Fatmi et al., 2013). SOD activity is high in tissues that use much oxygen and it increases in parallel to the partial oxygen pressure of the tissue (Lunec and Blake, 1990). SOD also plays a role in intracellular termination of the phagocytized bacteria (Kobayaski et al., 1977).

Our study is the first that evaluates the changes in hepatic MDA, SOD, and CAT levels following midazolam administration. There was a statistically significant difference between the midazolam group and the controls in SOD and CAT enzyme activity levels, suggesting the increase in the formation of superoxide radicals and hydrogen peroxide production. However, there was no



difference between the controls and the study groups in MDA levels. This may indicate that the antioxidant system is effective enough to prevent a possible lipid peroxidation reaction. There were some structural changes in liver tissue sections of the midazolam group when compared with the controls. These structural changes include moderate granular degeneration in hepatocytes and slight mononuclear cell infiltrations in the parenchyma.

Drug biotransformation is an important function of the liver. The biotransformation function is related to anesthesia in two ways: the sensitivity of microsomal enzymes to induction and the acceleration of drug biotransformation. It has been indicated that inhalation anesthetics and barbiturate-type drugs lead to enzymatic induction (Gillman et al., 1991). In a previous study, it was reported that etomidate led to a reversible inhibition in microsomal enzyme activity in rabbit liver. This inhibition may suggest the possibility that etomidate led to a deleterious effect in hepatic tissue via binding to the cytochrome P450 system with high affinity (Horai et al., 1985). In another study, it was indicated that etomidate causes inhibition in drug elimination in the human liver; however, this process could be carried out as hepatic

clearance rate of etomidate is low (Atiba et al., 1972). Our study is the first that aims to investigate the changes in hepatic MDA, SOD, and CAT enzymatic activity levels due to etomidate administration. There was a significant increase in hepatic SOD and CAT activity levels in rats given etomidate when compared with the controls. However, there was also a prominent increment in MDA levels in this group. Increase in antioxidant enzyme activity along with the MDA levels may suggest that the antioxidant defense system can interfere with free radical accumulation but cannot stop lipid peroxidation. We have observed that repeated administration of etomidate at high doses has led to some histopathological and biochemical changes, whereas midazolam administration did not cause lipid peroxidation but did give rise to some histopathological changes. As a result, we conclude that one must be cautious about the dose and duration in the administration of these anesthetic agents during surgical interventions.

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