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Effects of recurrent ketamine exposure on brain histopathology in juvenile rats

Ayşe Hande ARPACI1*, Süheyla Esra ÖZKOÇER2, Emel GÜNEN3, Çağdem ELMAS3, Berrin İŞIK4

1. Introduction
Ketamine (KET) was synthesized as an anesthetic agent by Calvin Stevens in 1962 and was initially used by Corsen & Domino in 1965. It has been widely employed in clinical practice since the 1970s [1]. It is the only agent with hypnotic (sleep-inducing), analgesic (pain-relieving), and amnesic (short-term memory loss) effects; thus, it is a “unique drug” [1]. It leads to dissociative anesthesia by noncompetitive blocking of N-methyl-D-aspartate acid and glutamate receptors [2]. It is widely used, especially in children, due to its rapid onset, short functional duration, hemodynamic safety, upper respiratory tract reflex protection, lack of respiratory depression, and strong analgesic properties. Currently, it is even used as an opioid-induced respiratory depression antagonist in treating chronic pain and treatment-resistant depression [2–6].

However, several studies demonstrated that KET could induce damage, especially in developing brain tissue, which could lead to behavioral changes later in life [7–11]. In the neonatal period, KET was reported to increase neuronal necrosis in the brain based on the dose and exposure, and it was reported to cause neuronal defects in the long term [12,13]. Experimental studies in rodents and nonhuman primates also report increased apoptosis in developing brain tissues and triggered necrosis [14–22]. Other studies demonstrated that KET induced apoptosis in the sensorimotor cortex and cerebellum in a dose- and exposure-dependent manner [23].

Caspases, an important gene family, are effective in maintaining homeostasis through the regulation of necrosis and inflammation in apoptosis mechanisms [24–27]. Caspases 3, 6, 7, 8, and 9 are associated with...
apoptosis, and caspases 1, 4, 5, and 12 are associated with inflammation. Cleaved caspase-3 is a reliable marker for cells that are dying or have already died due to apoptosis [28]. Clinical studies reported that the caspase-3 level affected the determination of the prognosis and local recurrence in cancer cases [29–31].

On the other hand, although it is known that exposure to an anesthetic KET dose increases inflammation, autophagy, apoptosis, and reactive oxygen species levels in neurodevelopmental stages, the neuroprotective properties of KET at subanesthetic doses have also been demonstrated [32].

It is unclear for how long and to what extent recurrent KET administration at different doses affects neuron damage and apoptosis; its effects in longer exposures in the juvenile period are also unclear. The present prospective randomized experimental study aimed to determine whether repeated (three times per day) KET administration at subanesthetic and anesthetic doses (5, 20, and 50 mg/kg) [33,34] for three consecutive days leads to undesired histopathological changes in the cerebral cortex, striatum, and hippocampus in juvenile rats ten days after the administration.

2. Materials and methods
This prospective, randomized experimental trial protocols were approved by the Ankara University Animal Experiments Local Ethics Committee (2020-36, 2020-7-6232). Rats were procured from Ankara University, School of Medicine, Experimental Animals and Research Laboratory. After the approval of the ethics committee, 32 Wistar Albino juvenile (30-day-old) male rats were randomized into four groups: 1 mg/kg serum saline (Group C), 5 mg/kg KET (Group 5), 20 mg/kg KET (Group 20), and 50 mg/kg KET (Group 50). Intraperitoneal (i.p.) KET [(Ketalar), ketamine HCl, 500 mg/10 mL injectable, Pfizer FE Pharmaceuticals, İstanbul, Turkey] or serum saline [0.9% sodium chloride (NaCl) solution] was administered to the rats with 23G, 0.5 mL injectors three times a day in three-h intervals for three days based on the randomly selected groups. The mean body weight change in rats throughout the experiment was 3.28 ± 0.11 g, and there was no statistical difference between the groups. Ten days after the last KET dose, the rats were sacrificed using anesthesia by sodium-thiopental (50 mg/kg Pental I.E. Ulagay Pharmaceuticals, Turkey). Brain tissues were removed. Rats' brain tissues were prepared and evaluated histopathologically in Gazi University, Faculty of Medicine, Department of Histology. This study started in 2020 and finished in 2021.

2.1. Histological studies
The cerebral cortex and striatum were fixed with 4% paraformaldehyde, and tissue processing was conducted with an automated tissue processor (TP1020, Leica Biosystems, Germany). Paraffin-embedded tissues were sectioned in 4-µm thickness. After deparaffinization, sections were rehydrated with decreasing alcohol series. Hematoxylin and eosin (H&E) staining was performed [35,36].

2.2. Cleaved caspase-3 immunohistochemistry
Hippocampi fixation and tissue processing were conducted for cerebral cortex and striatum samples. Sections were deparaffinized and dehydrated. Antigen retrieval was conducted with a citrate buffer at 60 °C overnight. After blocking endogenous peroxidase activity, primary antibody (ab2302, 1:50) incubation was done at room temperature for an h. Sections were incubated with secondary antibodies and 3-amin-9-ethylcarbazole as a chromogen. Hematoxylin was used for the ground stain.

Sections were evaluated with Leica DM4000 Light Microscopy Image Analysis Systems and Image J (NIH, USA) software by two blinded histologists. Neuron number per area (mm²) and cell size (micrometer) were evaluated in the cerebral cortex.

Statistical analysis was conducted with SPSS 21 software (IBM), a p-value <0.05 was considered statistically significant. The Kolmogorov–Smirnov test was used for the assessment of normality. Based on data distribution, either the Kruskal-Wallis or ANOVA test was used to analyze the difference between groups with Bonferroni post hoc analysis [35,36].

3. Results
3.1. Effect of ketamine on the morphology of the cerebral cortex
Histological evaluation of cerebral cortex sections revealed nondistinct layers except the molecular layer in all groups. The pyramidal cells with dark basophilic cytoplasm had a multipolar shape. The granule cells with large, euchromatin nucleus and prominent nucleolus had a small amount of pale cytoplasm. Degenerative signs, such as eosinophilic neurons, contracted pale-staining nuclei, and gliosis, were not inspected in the sections. The cell size (µm) and the number of cells in the area (mm²) of the groups are demonstrated in Table 1. Figure 1 (a, b, c, d, e, f, g, h) shows the micrographs of the cerebral cortex.

3.2. Effect of ketamine on the morphology of the striatum
Neuron bodies and neuropils were evaluated in striatum sections for all groups. The pyramidal and granule cells did not show degenerative changes. Mild gliosis was seen in Group 20, but gliosis was not prominent in Group 50. Figure 2 (a, b, c, d) shows micrographs of the striatum.

3.3. Immunohistochemistry results of ketamine effect on the CA1 region of the hippocampus
Cleaved caspase-3 was evaluated for apoptosis evaluation.
in the CA1 region of the hippocampus. Immune reactive cells were calculated per area (mm$^2$). The mean and SD values of groups are presented in Table 2. Caspase-3 positivity increased with the KET dose and was lower in Group 5 compared to Group C. However, the difference is statistically insignificant. Micrographs are presented in Figure 3 (a, b, c, d).

4. Discussion
The current experimental study findings revealed no differences between the groups in cell count or size in cortical morphometry. No degenerative changes were observed in pyramidal and granular cells in the striatum. Mild gliosis was observed in Group 20 and Group 50. Immunohistochemical analysis conducted to determine apoptosis in the CA1 region of the hippocampus revealed that caspase-3 positivity increased with the KET dose, although the difference was not statistically significant between the groups. While it was lower in Group 5 compared to Group C, it was similar in Group 20 and Group C and higher in Group 50 (p > 0.05).

Although several reports in the last three decades indicated that general anesthetic agents could lead to undesirable effects on the developing brain, others reported neuroprotective properties of KET [32].

Several studies conducted with various species, including nematodes and nonhuman primates, reported that early exposure to general anesthetic agents, especially KET, leads to neurodegeneration, which in turn leads to a decline in cognitive functions and socio-emotional development [14–23]. However, in most studies, KET was administered in the early stages of development, or the early postexposure findings were analyzed [32]. This raises a question about the nonacute effects of KET exposure.
in the juvenile period. This is because recurrent KET anesthesia in the clinic or chronic KET use due to other clinical indications could occur in early childhood and juvenile rather than fetal or neonatal periods.

Synaptogenesis in rats continues between 7–30 days postnatal [37,38]. One human year almost equals two rat weeks (365÷26.7 = 13.8 rat days) [39]. In this experiment to evaluate rapid brain development phase (juvenile phase), 30-day-old rats were preferred.

Cellular damage in neural tissue is determined by histopathological findings such as a decrease in cell count, change in cell size, eosinophilic neuron, pale-staining

### Table 2. Caspase-3 positive cells per area (mm$^2$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Caspase-3 positive cells per area (mm$^2$) Mean ± SD</th>
<th>p values</th>
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<tr>
<td>Group 5 mg/kg KET</td>
<td>130.65 ± 62.84</td>
<td>Group 20 mg/kg 1.000, Group 50 mg/kg 1.000, Group C 1.000</td>
</tr>
<tr>
<td>Group 20 mg/kg KET</td>
<td>143.89 ± 67.45</td>
<td>Group 50 mg/kg 1.000, Group C 1.000</td>
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<tr>
<td>Group 50 mg/kg KET</td>
<td>166.59 ± 30.87</td>
<td>Group C 1.000</td>
</tr>
<tr>
<td>Group C (1 mg/kg serum saline)</td>
<td>143.25 ± 73.12</td>
<td></td>
</tr>
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</table>

Mean±SD, the differences between groups were statistically insignificant p = 0.731
nucleus, gliosis, and increase in apoptosis. Damage to cerebral neurons is associated with regression in cognitive functions [40–42]. In vivo studies also reported that repeated KET administration leads to neuronal pyroptosis, apoptosis, and inflammation in the hippocampal region [43]. Experimental studies evaluating the neurotoxic effects of repeated KET administration on developing brain tissue have been performed on different animal groups and at different stages of neurodevelopment. Neurotoxic effects were evaluated by many histopathological, functional, or behavioral methods [14–23]. In the literature, it is reported that the KET dose, dose repetition, and KET exposure stage applied in animal studies are effective factors in neurological damage (Table 3). The time between KET exposure and histopathological analysis is also an important factor in determining the duration of cellular damage.

Zou X et al. analyzed the striatum, hippocampus, thalamus, and amygdala six h after KET administration in monkeys (20 mg/kg × 6 injections). They reported that KET led to an increase in caspase-3 positive apoptotic cell count; it was three-fold in the stratum, 2.5-fold in the hippocampus, 2.3-fold in the thalamus, and three-fold in the amygdala when compared to the control. The most obvious increase (10-fold) was identified in the frontal cortex [16]. Lyu et al. demonstrated that the administration of six 20 mg/kg KET doses at two-h intervals in postnatal seven-day-old Sprague-Dawley rats increased apoptosis in the hippocampus, and the apoptosis rate increased three h after administration and persisted even after six h [43].

In the current study, histopathological analysis of the rat brain tissues ten days after KET administration revealed no obvious cellular damage in the cortex. However, mild gliosis and an increase in caspase-3 in the CA1 region of the hippocampus were determined based on the KET dose, although the difference was not statistically significant in Group 20 and Group 50.

H&E staining was employed for morphological imaging and caspase-3 staining for immunohistochemical analysis to determine apoptosis in neural tissue [44,45].

The production of caspases that provide critical bonds for cellular regulation networks that control inflammation...
<table>
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<tr>
<th>Animal and stage</th>
<th>Method and doses of ketamine</th>
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<th>Evaluation method</th>
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<td>Subcutan 7 doses one every 90 min, of either saline, 10 mg/kg or 20 mg/kg ketamine, or a single dose of 20 mg/kg ketamine</td>
<td>Neurotoxicity Neuronal apoptosis</td>
<td>Silver staining histochemical results Fluoro-Jade B (FJ-B)</td>
<td>The repeated doses of 20 mg/kg ketamine increased the degenerating neurons in the dorsolateral thalamus [14]</td>
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<tr>
<td>Postnatal 7-day-old Sprague-Dawley rats</td>
<td>Intraperitoneal injections of a single dose of ketamine 25, 50, and 75 mg/kg, repeated doses of ketamine 25 mg/kg at 90-min intervals over 9 h</td>
<td>Degeneration of neurons</td>
<td>10 brain regions using the stereological dissector method Cupric-silver stain</td>
<td>Repeated doses of ketamine (25 mg.kg⁻¹) at 90-min intervals over 9 h increased degenerating neurones in seven out of 10 brain regions [15]</td>
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<tr>
<td>Postnatal day 5 or 6 newborn rhesus monkeys</td>
<td>Intravenously for 3, 9, or 24 h to maintain a steady anesthetic plane, followed by a 6-h withdrawal period</td>
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<td>Silver and Fluoro-Jade C stains and caspase-3 immunostain</td>
<td>No significant neurotoxic effects occurred if the anesthesia duration was 3 h but anesthetic durations of 9 h or greater are associated with significant brain cell death in the frontal cortex [16]</td>
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<td>Rhesus monkeys (N = 3 for each treatment and control group) at three stages of development 122 days of gestation and 5 and 35 postnatal days</td>
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<td>A shorter duration of ketamine anesthesia (3 h) did not result in neuronal cell death in the 5-day-old monkey. Earlier developmental stages appear more sensitive to ketamine-induced neuronal cell death than later in development [17]</td>
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<td>Cyclin D1, cyclin-dependent kinase 4, and E2F1, Bcl2-interacting mediator of cell death, and activated caspase-3</td>
<td>The expression of cell cycle proteins, mediator of cell death, and activated caspase-3 was determined</td>
<td>Ketamine induces aberrant cell cycle reentry, leading to apoptotic cell death in the developing rat brain [18]</td>
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<td>mTOR signaling pathway and apoptosis of rat hippocampal neurons (TUNEL)</td>
<td>The expressions of mTOR signaling pathway and apoptosis-related proteins in hippocampal neurons were examined by qRT-PCR and Western blot</td>
<td>Ketamine could dose-dependently promote the apoptosis of rat hippocampal neurons with upregulation of p-mTOR and its downstream regulators [19]</td>
</tr>
</tbody>
</table>
Postnatal day 7 Sprague-Dawley rat pups

Intraperitoneal ketamine treatment 25, 50, and 75 mg/kg, for three consecutive days

Hippocampal neurodegeneration and behavioral deficits in adulthood

Protein kinase C-Gamma (PKCγ) extracellular signal regulated kinase (ERK)1/2 and Bcl-2 expression

Ketamine at a dose of 75mg/kg in the developing brain results in hippocampal neurodegeneration and persistent learning and memory impairment, which is associated with the PKCγ-ERK signaling pathway [20]

Pregnant rats (P19)

Intravenous injection of ketamine (200 mg/kg) for 3h

Fetal hippocampus tissue

Fetal brain tissue Cultured PC12 cells in vitro to determine the relationship between ROS, autophagy, and apoptosis

Total Antioxidant Capacity, Reactive Oxygen Species, and Malondialdehyde. Changes in the levels of Cleaved-Caspase-3, Beclin-1, B-cell lymphoma-2, Bcl-2 Associated X Protein, Autophagy-related gene 4, Atg5, p62, and marker of autophagy Light Chain 3

Anesthesia with ketamine in pregnant rats may increase the rate of autophagy and apoptosis in the fetal hippocampus and the mechanism may be through inhibition of antioxidant activity and ROS accumulation [21]

Postnatal day 10 male mice

Subcutan a single dose of 7.5 g/kg ketamine One h after ketamine exposure, mice were whole body irradiated with 50–200 mGy gamma radiation. Behavioural observations were performed at 2, 4, and 5 months of age. At 6 months of age, cerebral cortex and hippocampus tissue were analysed for neuroprotein levels

Impaired learning and memory

Morris Water Maze

Coexposure to IR and ketamine can aggravate developmental neurotoxic effects at doses where the single agent exposure does not impact on the measured variables [22]

7 days old ICR mice

Subcutan 1.25, 2.5, 5, 10, 20, and 40 mg/kg ketamine in 0.9% NaCl or with 0.9% NaCl alone as control Righting reflex testing was performed and mouse brains were examined at 24, 48, and 72 h and 7 days after injection

DNA fragmentation and apoptosis

The number of degenerating neurons was measured using silver staining

The administration of ketamine in a clinically relevant single dose to 7-days old mice induced apoptosis in the sensorimotor cortex and cerebellum. This effect was dose-dependent and long lasting [23]
and necrosis as inactive zymogens and their acquisition of catalytic activities are under strict control. Activation of inflammatory caspases leads to an immune response through the release of active proinflammatory cytokines. Dysregulation of caspases underlies several diseases, including cancer and inflammatory disorders [46].

Soriano et al. reported that i.p. administration of KET (5, 10, and 20 mg/kg/dose) to postnatal seven-day-old rats five times (at 90-min intervals over six h) led to apoptotic necrosis in the cortical and thalamic regions of the baby rat brains [18]. Also, Zou et al. demonstrated that KET administration to seven-day-old rats led to an increase in neuronal apoptosis based on the dose and exposure period [47]. Similar findings were also reported in the experiment conducted with monkeys [16]. However, potential neurotoxic effects in these studies were analyzed six h after the final drug administration.

In our study, no degeneration was determined in cortex cells, while mild gliosis was identified in the striatum in Group 20 and Group 50. There was no statistically significant difference between immunoreactive cells in the CA1 region (p > 0.05). They were lower in Group 5 when compared to Group C, similar to Group C in Group 20, and higher in Group 50 when compared to Group C (Table 2).

Neurons in the CA1 region of the hippocampus are essential for spatial learning and memory functions. CA1-region neurons receive and process data from the entorhinal cortex or the CA3 region. The CA3 region is connected to the CA1 region via Schaffer collateral fibers. A solid CA3 and CA1-CA3 connection is required for reference memory. The cell count in CA1 and CA3 gradually increases after adolescence with spatial learning and memory functions [48]. Our experiment analyzed rat brain tissue, the cerebral cortex, and the striatum. The cell count in the cerebral cortex was measured. The pyramidal cells with dark basophilic cytoplasm had multipolar shapes. The granule cells with large, euchromatin nucleus and prominent nucleolus exhibited a small pale cytoplasm count. Degenerative signs, such as eosinophilic neurons, contracted pale-staining nuclei, and gliosis, were not determined in the sections. Neuron bodies and neutrophils were observed in striatum sections. The pyramidal and granule cells did not exhibit degenerative changes. Mild gliosis was observed in Group 20; however, it was not prominent in Group 50.

The main limitations of this experimental study were not measuring the blood KET level in the rats and not using methods other than histopathological methods to show cell damage to evaluate neurotoxicity.

5. Conclusion
Ten days after repeated KET administration in juvenile rats, 20 and 50 mg/kg KET doses had negative effects on the cerebral cortex, striatum, and hippocampus, although these changes were not obvious. Although there was no statistically significant difference between apoptosis in the hippocampus with KET administration compared to Group C, apoptosis decreased after the 5 mg/kg administration, was similar with the 20 mg/kg dose, and increased with the 50 mg/kg dose.

According to current data, it is not possible to avoid general anesthesia in clinical practice. In our study, repeated KET exposure did not significantly affect cerebral morphology and apoptosis in juvenile rat hippocampal cells, but KET doses are worth considering. The experimental design of this study means these results could not be totally adapted to human beings.

Conflict of interests
The authors declare that they have no conflicts of interest.

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Ethical approval
The study was approved by Ankara University Animal Experiments Local Ethics Committee, Turkey.

Consent for publication
The manuscript is approved by all authors for publication.

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