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# Immunohistochemical MDA Changes of the Newborn Rat Frontal Cortex Affected by Prenatal Ketamine Exposure

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## Abstract

**Background** Ketamine has an excellent analgesic property and is widely used currently to provide "sedation" for minor procedures. Ketamine has been proved to potentiate deletion of large numbers of neurons from the developing brain.

**Objectives** To investigate the neurotoxic effect of prenatal ketamine exposure on newborn rat frontal cortex using malondialdehyde antibodies as immunohistochemical marker.

**Methods** Seventy two pregnant rats were divided into three groups I, II and III (24 rat for each group) and exposed to ketamine at different gestational periods (7th day, 11th day, and 18th day, respectively). Each group was subdivided into four subgroups including the control subgroup A injected intraperitoneally with normal saline, and the experimental subgroups B, C and D rats injected intraperitoneally with different doses of ketamine (5mg/kg, 10mg/kg, and 20mg/kg respectively). Paraffin sections of the frontal cortex of the newborn rats were immunohistochemically stained with Expose Mouse and Rabbit Specific HRP/DAB Detection Kit. The Aprio Image Scope v.9 software was used to evaluate the anti-MDA antibodies immunohistochemical reaction.

**Results** Non-significant variability between the subgroups B and C of group I, while significant variability was found between these two subgroups in groups II and III. The values obtained from subgroup B in all the groups I, II, and III had no significant variability compared to the control subgroup (A). The values obtained from subgroups (C) and (D) showed statistically significant changes compared to the control subgroup (A) in all the groups (I, II, and III). The results showed significant variability by comparing the results of subgroup (D) with both subgroup (B) and subgroup (C) in all the groups.

**Conclusion** The anti-MDA immunohistochemical reactivity shown in this study suggested that lipid peroxidation is an event occurring during ketamine induced neurotoxicity, this event leads to apoptosis.

**Keywords** Cortex, prenatal, ketamine, neurotoxicity, immnuohistochemistry.

**List of abbreviation:** MDA = malondialdehyde.

## Introduction

Neurotoxicity is defined as the abnormalities of the nervous system following exposure to a chemical, biological, or physical agent. The susceptibility to neurotoxicity is more during development as the blood brain barrier is not completely developed and neurogenesis and synaptogenesis are taking place at high rates [(1)].

The frontal lobes in human include several functionally different regions that are grouped into three regions; motor, premotor, and prefrontal. The frontal lobes connect to all cortical regions through association fibers. It receives mainly strong input from limbic cortex, amygdala and septal nuclei, and areas

involved in emotional responses [2]. There are wide varieties of symptoms that are associated with frontal lobe lesions; these include disorders of motor functions, failure of divergent thinking, impaired response inhibition and inflexible behavior, reduced memory, and impaired social and sexual behavior imaging [3].

Ketamine has been used in the surgical emergencies requiring anesthesia, it has been suggested that ketamine can be used safely for anesthesia in infants and children [4]. Ketamine is a nonbarbiturate, dissociative anesthesia used for short diagnostic and surgical procedures and to supplement low-potency anesthetics such as nitrous oxide. The adverse reactions associated with ketamine including visual hallucinations, nightmares or illusion, and post-anesthesia delirium [5].

Considering the effect of ketamine on developmental tissue, ketamine was placed in the class of other competitive and noncompetitive *N*-methyl-d-aspartate antagonists. Ketamine affects neuronal functioning in the developing brain of the rat, and significant decreases were found in neural cell adhesion molecules and postsynaptic densities after single exposure to ketamine during neuronal development [6].

The teratogenic potency of ketamine hydrochloride in mice has been proved experimentally [7]. It was suggested that ketamine has the potential to delete large numbers of neurons from the developing brain by a mechanism involving interference with the action of the neurotransmitters glutamate and gamma-amino butyric acid at *N*-methyl-d-aspartate and gamma-amino butyric acid receptors during the synaptogenesis period. This transient interference during the synaptogenesis period (the last trimester of pregnancy and the first several years after birth in humans) causes millions of developing neurons to commit suicide (die by apoptosis) [8].

The current study was formulated to investigate the prenatal ketamine neurotoxicity in rat. This aim was proposed as the neuronal number, location, differentiation, and type are affected by the events occurring in embryonic life before neuronal differentiation. It was reported that there are 2 distinct periods of cell proliferation in the nervous system, the first occurs embryonically and correlates with neurogenesis and the second occurs postnatally and correlates with gliogenesis [9].

Immunohistochemical antibodies to malon-dialdehyde (MDA) have been used in this study to investigate the prenatal ketamine neurotoxicity. MDA is a natural product formed in all mammalian cells as a product of lipid peroxidation. It is a highly reactive byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism. MDA can combine with many functional groups on proteins, lipoproteins, and DNA. MDA is toxic and has been implicated in many biological events [10].

## Methods

In this study, female Wistar rats (*Rattus Norvegius*) aged 4-6 weeks and weighted between 150-250g were brought from the laboratory animal house, College of Medicine, Baghdad University. The study was performed during the period from November 2013 to may 2014. These female rats were mated, and pregnancy was confirmed by the observation of vaginal plug.

All animals were treated according to National Institute of Health Guidelines for the Care and Use of Laboratory Animals [11].

The total number of pregnant rats used in this study was (72), these animals were divided into three groups I, II, III (24 rat for each group). Animals of these groups were exposed to ketamine at different gestational periods (at the $7^{th}$ day, $11^{th}$ day, and $18^{th}$ day, respectively).

The pregnant rats of each group were subdivided into four subgroups (six animals for each subgroup) including the control subgroup A, received intraperitoneal injections of normal saline, and the experimental subgroups B, C

and D, received intraperitoneal injections of ketamine in different doses (5mg/kg, 10mg/kg, and 20mg/kg, respectively). Female rats which were found to have no signs of pregnancy were excluded.

Pregnant rats of the experimental subgroups received intraperitoneal ketamine hydro-chloride injections (Kanox, ketamine 50 mg/ml preservative; chlorobutanol 5%, batch number 122228E, Duopharma), the control subgroup A received intraperitoneal normal saline injections.

The injections were done at 6 consecutive doses every 1.5 hours (for a total of 9 hours of therapy) at each of the $7^{th}$, $11^{th}$ and $18^{th}$ days of pregnancy.

Each female rat delivered (8-16) neonates, from which 6 neonates were selected randomly for this study.

Newborn animals were sacrificed by decapitation during the first hour on the first day of delivery, their brains were removed from the cranium, and coronal paraffin sections of 5µm thickness of the frontal cortex were prepared after fixation in 10% formalin [12].

Digital camera (Sony cyber shot) was used for documenting tissue staining and histology.

Anti-MDA antibodies

These antibodies were provided from Abcam (code no. ab6463). They are rabbit polyclonal antibodies containing small molecules of synthetic malondialdehyde conjugated to bovine serum albumin.

The immune-histochemistry detection kit is called Expose Mouse and Rabbit Specific HRP/DAB Detection IHC Kit from Abcam (code no. ab80436).

Six sections were randomly selected from the sections of the frontal cortex of neonates delivered by the rats of each subgroup (A, B, C, and D) in each of the groups (I, II, and III).

Aperio Image Scope version 9 software was used for the evaluation of MDA antibodies immunohistochemical reaction. This image analysis software involves counting the number of strong positive pixels to evaluate the immunohistochemical stain.

The list of positive pixel count algorithm includes parameters obtained from the application of this software to quantify the amount of a specific stain present in a scanned slide image.

These parameters when first selected have been pre-configured for brown color quantification. Pixels which are stained, but do not fall into the positive-color specification, are considered negative stained pixels.

## Results

Analysis of variance (ANOVA) statistical evaluation of the mean values of MDA immuno-histochemical reactivity in the frontal cortex of neonates delivered by rats of subgroup A (the control subgroup) showed non-significant differences among all the groups (I, II, and III).

***Anti-MDA reaction in the frontal cortex of neonates delivered by rats of group (I) (Fig. 1-3):***

The evaluation of the counted mean values obtained by the application of the Aperio Image Scope software in the cortex of neonates delivered by rats of subgroups (B, C, and D) revealed statistically significant variability compared to those of the control subgroup A.

This counting of the mean value of the number of strong positive pixels was highest in subgroup D (11562.6 ± 764.6). The mean values in subgroup B and C were 4400.1 ± 234.1 and 4925.6 ± 149.4 respectively. The *P* values were ≤ 0.001 for the subgroups B, C, and D.

The multiple comparison statistical tests done for the mean differences between the experimental subgroups B and C of group I showed none significant variability ($P \geq 0.072$), while high significant variability was shown between the subgroups B and C compared with subgroup D of group I ($P \leq 0.001$).

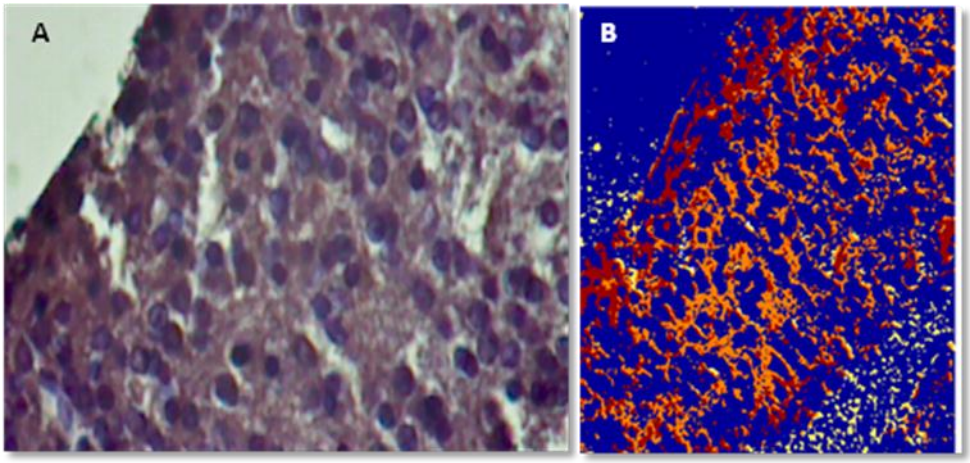


**Fig. 1. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup B of group I. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.**

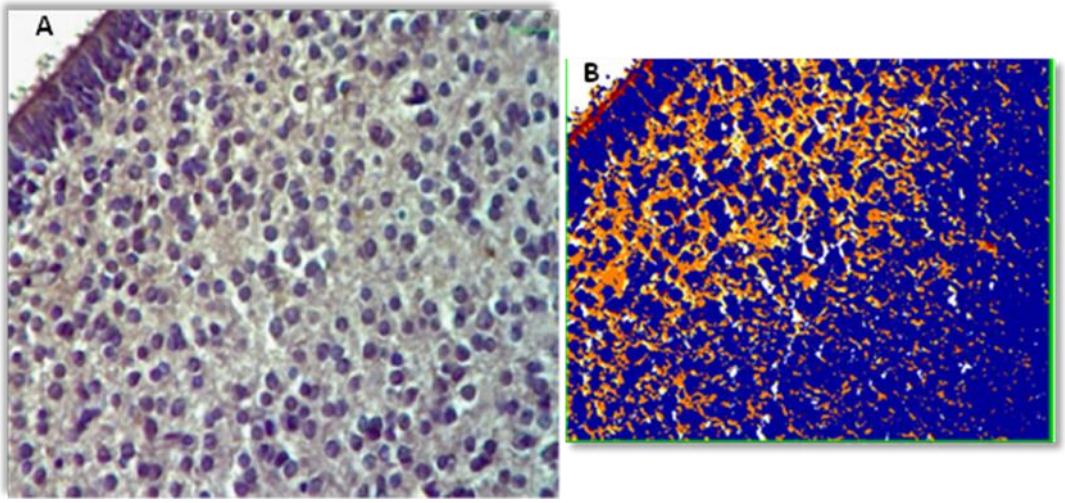


**Fig. 2. (A) Anti MDA reactivity in frontal cerebral cortex of neonate rat from subgroup C of group I. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.**

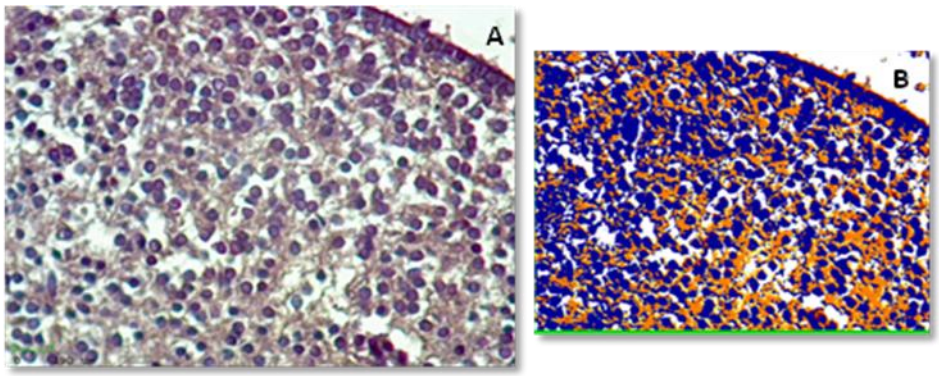


**Fig. 3. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup D of group I. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm (400X).**

***Anti-MDA reaction in the frontal cortex of neonates delivered by rats of group (II) (Fig. 4-6):***

The counting for the mean values of the number of strong positive pixels obtained from the cortex of the neonates delivered by female rat of the experimental subgroups (B, C, and D) and those of the control subgroup A of group II showed equivalent analysis compared to that of group I.

The highest mean value was in subgroup D (16791.3 ± 1325.9), the mean values of subgroup B and C were 5571.3 ± 348.3 and 9965.1 ± 436.6, respectively. The *P* value was ≤0.001 for subgroups B, C, and D.

The multiple comparison statistical tests done for the mean differences between the frontal cortices of neonates in experimental subgroups B, C, and D of group II showed high significant variability ($p \leq 0.001$).

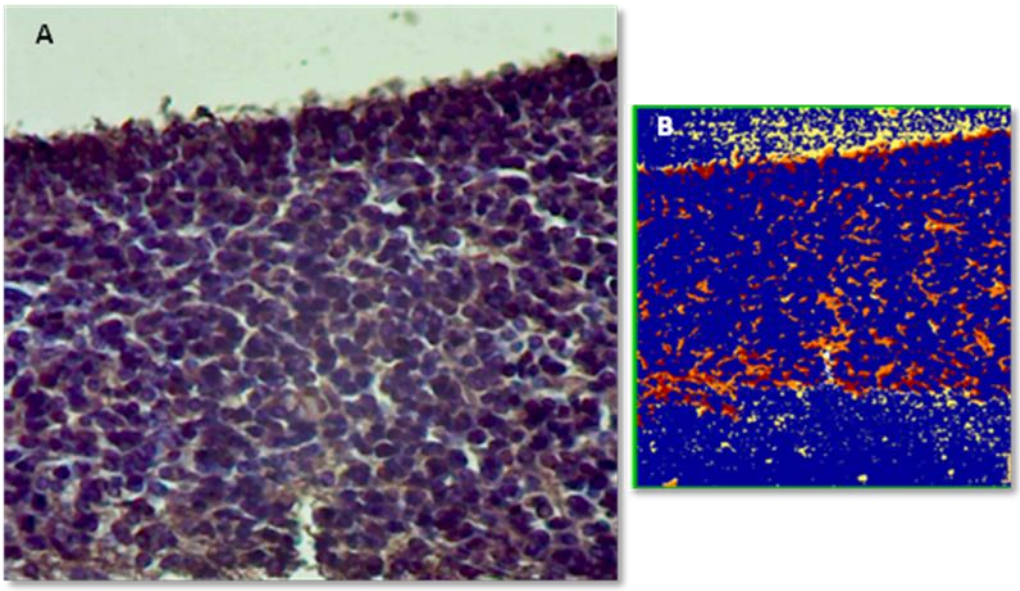


**Fig. 4. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup B of group II. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.**

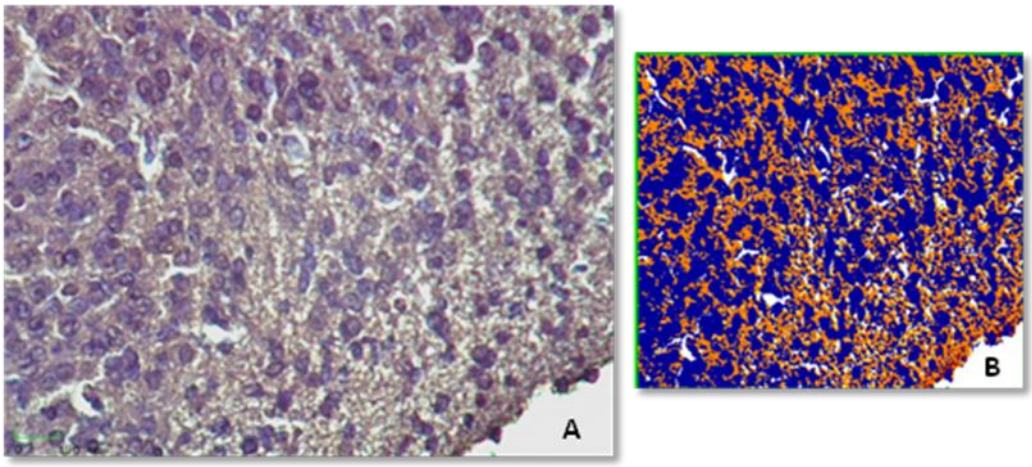


**Fig. 5. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup C of group II. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.**

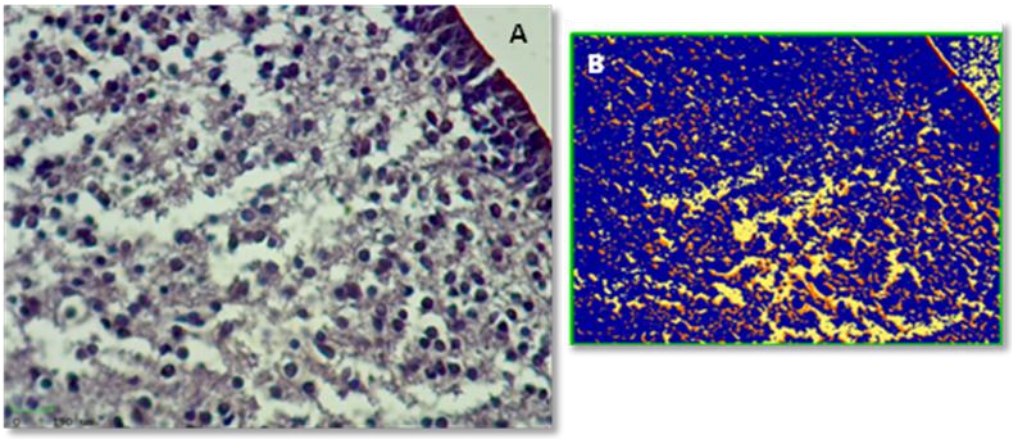


**Fig. 6. (A) Anti- MDA reactivity in frontal cortex of neonate rat from subgroup D of group II. Anti-MDA positive stain is seen in all layer of frontal cortex (400X) (B) The snap shoot of Aperio Positive Pixel Count Algorithm.**

***Anti-MDA reaction in the frontal cortex of neonates delivered by rats of group (III) (Fig. 7-9):***

The neonate frontal cortex of group III showed marked increase in the mean value of subgroup D (42147.1 ±1058.0) compared to that of the subgroups B and C (5544 ±447 and 13676.5 ±419.6, respectively), these results were parallel to the results obtained from the analysis of group I and II. A significant variation between the experimental subgroups (B, C, and D) was recorded at $p \leq 0.05$ compared to the control subgroup A.

Equivalent statistical results were obtained for the mean differences between the treated subgroups B, C, and D of group III. Highly significant variability ($P \leq 0.001$) was found between these subgroups B, C, and D.

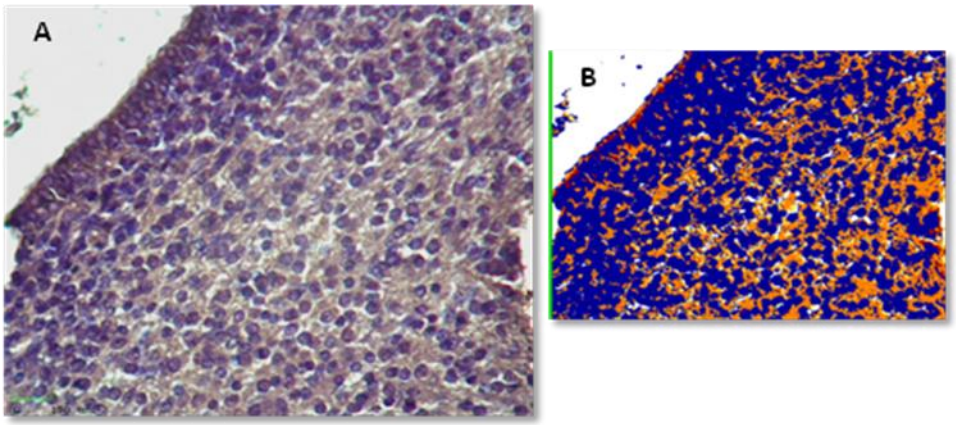


**Fig. 7. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup B of group III. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.**

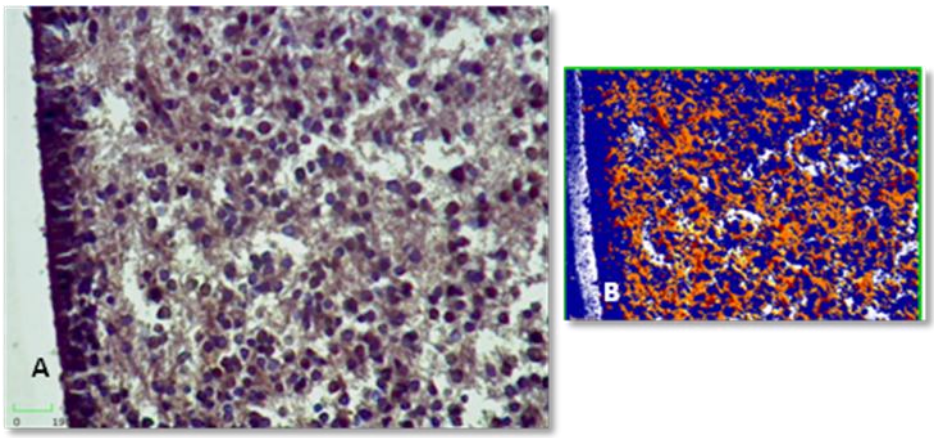


**Fig. 8. (A): Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup C of group III. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.**

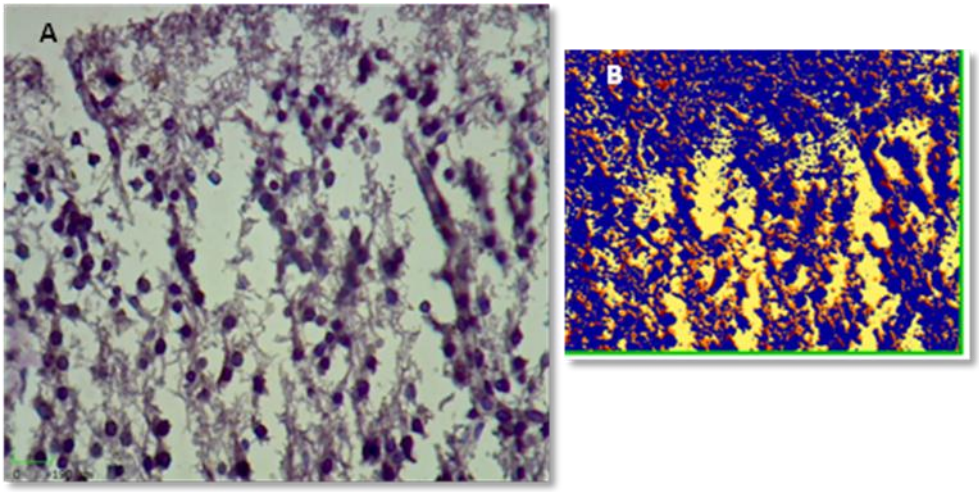


**Fig. 9. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup D of group III. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.**

***Appraisal of anti- MDA reaction of each subgroup in all the groups:***

ANOVA statistical analysis of the counted mean values obtained for subgroup B in all groups (I, II, and III) compared to that of the subgroup A (the control subgroup) showed non-significant variability ($P \geq 0.253$). While significant variability was found from the values of comparing the subgroup C with control subgroup A ($P \leq 0.024$), and a highly significant variability was found for subgroup D in all groups ($P \leq 0.001$).

***Appraisal of anti- MDA reaction of all the subgroups in each of the groups:***

ANOVA statistical analysis of the counted mean values for all the subgroups in group I showed non-significant variability compared to those of group II ($P \geq 0.179$), while the statistical analysis of these values in group I compared to group III showed highly significant variability ($P \leq 0.001$). Also, the analysis of the values obtained from group II compared to group III showed significant variability ($P \leq 0.01$).

## Discussion

***Evaluation of the anti-MDA immune-histochemical changes caused by 5mg/kg of ketamine exposure (subgroup - B):***

The anti-MDA immunohistochemical reactivity shown in the results of this study suggested that ketamine-induced lipid peroxidation is an event occurring during the process of ketamine neurotoxicity. Lipid peroxidation after cellular injury leads to apoptosis and autophagy; cellular membranes, because of their high lipid content, are especially susceptible to damage because lipid peroxidation reactions can alter the structure and function of critical membrane lipids leading to cell injury and cell death [13].

Therefore, the statistical analysis of the results in this study, showing the non-significant variability between subgroups B and C of group I, suggested that the cortical cellular membrane damage by lipid peroxidation reactions is of the same severity in the newborns of these subgroups. This explanation indicates that the doses of 5mg/kg and 10mg/kg of ketamine produce equivalent neurotoxic effect when used during the 7$^{th}$ day of gestation.

Statistical evaluation showed the least values from the subgroups (B) compared to the other subgroups in each of the groups; therefore, it could be assumed that the least neurotoxic effect of ketamine is seen when using the drug in dose of 5mg/kg.

The analysis of the results in subgroup B of all the groups I, II, and III have no significant variability compared to the control subgroup (A). This is a supportive evidence for considering 5mg/kg as a dose of least neurotoxicity.

***Evaluation of the anti-MDA immune-histochemical changes caused by 10mg/kg and 20mg/Kg of ketamine exposure (subgroups B and D):***

The newborn frontal cortex of these subgroups (C) and (D) showed statistically significant changes compared to the control subgroup (A) in each of the groups (I, II, and III).

The anti-MDA staining analysis showed significant variability by comparing the results of subgroup (D) with both subgroup (B) and (C) in each of the groups; this is considered as a supportive evidence that the injection of 20mg/kg is the highest toxic dose. The results of this study showed that the mean of the number of strong positive pixels counted to evaluate the histochemical reactivity in the cortex of the subgroup (C) have an intermediate values between that for subgroup (B) and (D) in each of the groups. The mean for the subgroup (D) was the highest in each of the groups.

Also the results of the counted values obtained from the subgroups (C) and (D) in all the groups showed significant variability from those of the control subgroup (A).

***The neurotoxic effect of ketamine injection on spatiotemporal development of the cerebral cortex:***

The important question whether anesthetic drugs can trigger neuroapoptosis in the developing non-human primate brain was first addressed by Slikker and colleagues [14] who reported that intravenous infusion of ketamine triggered neuroapoptosis in the (5) day old rhesus macaque brain. These reports are supportive to the results of this study, suggesting that lipid peroxidation detected by the anti-MDA immunohistochemical technique indicates neuroapoptosis in the frontal cortex by the neurotoxic effect of ketamine.

In agreement with the results of this study, it was reported that the pattern of augmented neuroapoptosis in the ketamine-exposed fetal brains was widespread [15]. Therefore, the results of this study supported the fact that ketamine exposure during development is responsible for inducing neuroapoptosis in the developing brain.

All of the recent human epidemiological studies pertaining to developmental anesthesia neurotoxicity [16] have focused on full-term infants and children; the focus of future human research should be expanded to include third

trimester fetuses and prematurely born infants.

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## Author contribution

Dr. Gaeb performs the laboratory research work; Dr. Mobarak and Jaffar interpret the results.

## Conflict of interest

The authors disclose no any financial and personal relationships with other people or organizations that inappropriately influence our work.

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## References

1. Ceccatelli S, Bose R, Edoff K, et al. Long-lasting neurotoxic effects of exposure to methylmercury during development. J Intern Med. 2013; 273:490-497.
2. Joaquin MF. Frontal lobe and cognitive development. J Neurocytol. 2002; 31:373-385.
3. Tullberg M, Fletcher E, DeCarli C. White matter lesions impair frontal lobe function regardless of their location. Neurology. 2004; 63:246-253.
4. Lönnqvist PA, Walker SM. Ketamine as an adjunct to caudal block in neonates and infants. Br J Anaesth. 2012; 109:138-140.
5. Jennings PA, Cameron P, Bernard S. Ketamine as an analgesic in the pre-hospital setting: a systematic review. Acta Anaesthesiologica Scand. 2011; 55:638-643.
6. Cheng W, Natalya S, Charlotte H, et al. Blockade of N-Methyl-D-Aspartate receptors by ketamine produces loss of postnatal day 3 monkey frontal cortical neurons in culture. Toxicol Sci. 2006; 91:192-201.
7. Abdel-Rahman MS, Ismail EE. Teratogenic effect of ketamine and cocaine in CF-1 mice. Teratology. 2000; 61:291-6.
8. Bita M, Barbara A, Anita V, et al. Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex. J Neurosci. 1997; 17:2921-2927.
9. Frederiksen K, McKay RDG. Proliferation and differentiation of rat neuroepithelial precursor cells in vivo. J Neurosci. 1998; 8:1144-1151.
10. Del RD, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis. 2005; 15:316-28.
11. Janet CG, Wayne R, Joseph T, et al. guide for care and use of laboratory animals. 8th Eds. National academies press. Washington; 2011.
12. Kiernan J. Histological and Histochemical Methods: Theory and Practice. 4th edition. Scion publishing. London; 2008.
13. Juliann GK, Risaku F, Nikolai V. Genetics and Molecular Biology. Chapter 13; Lipid Peroxidation. Uniformed Services University of the Health Sciences USA; 2012.
14. Slikker W, Zou X, Hotchkiss CE, et al. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. Toxicol Sci. 2007; 98:145-158.
15. Ansgar M, Brambrink, Alex S, et al. Ketamine induced neuroapoptosis in the fetal and neonate Rhesus Macaque brain. Anesthesiology. 2012; 116:372-384.
16. DiMaggio C, Sun LS, Kakavouli A, et al. A retrospective cohort study of the association of anesthesia and hernia repair surgery with behavioral and developmental disorders in young children. J Neurosurg Anesthesiol. 2009; 4:286-291.

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