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Effect of Prenatal Exposure to Ketamine on Newborn Rat Frontal Cortex: Immunohistochemical Study with TUNEL Test

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Abstract

Background Ketamine as an analgesic drug is widely used to provide sedation for minor procedures. It was reported that the use of this drug causes deletion of large numbers of neurons from the developing brain.

Objectives To investigate the effect of prenatal ketamine exposure on the newborn rat frontal cortex using immunohistochemical TUNEL test.

Methods Seventy two pregnant rats were divided into three groups: I, II, and III (24 rat for each group), and exposed to ketamine at the 7th day, 11th day, and 18th day, of gestational age. Each group was subdivided into control subgroup A injected intraperitoneally with normal saline, and the subgroups B, C and D injected with intraperitoneal ketamine with 5 mg/kg, 10 mg/kg, and 20 mg/kg, respectively. Paraffin block sections of newborn rat frontal cortices were investigated by TUNEL test.

Results Counting of fluorescent cells showed progressive increase in mean values with increased dose of ketamine injection and advancing age of prenatal exposure to ketamine. Significant variability was demonstrated between the control subgroup A and the other subgroups, and between the subgroups of each group, whereas, non-significant variability was observed between the counted mean differences values obtained for subgroup B in all groups (I, II, and III) compared to that of the control subgroup A.

Conclusion Prenatal exposure to ketamine in a dose of 5mg/kg was a relatively non-toxic in all studied groups compared to the control subgroup. Apoptosis in frontal cortical tissue was involved in the mechanism of neuronal death caused by ketamine exposure during pregnancy.

Keywords Frontal, cortex, prenatal, ketamine, neurotoxicity, immnuohistochemistry.

Introduction

There are wide varieties of symptoms 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 [(1)].

Ketamine is used in the surgical emergencies requiring anesthesia; it has been suggested that ketamine can be used safely for anesthesia in infants and children [(2)].

Considering the effect on developmental tissue, ketamine is proved to affect 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 [(3)].

The current study is formulated to investigate the neurotoxicity of prenatal exposure to ketamine in rat using the immune-histochemical in situ direct DNA fragmentation assay (TUNEL-based detection kit).

Methods

In this study, female Wistar rats (*Rattus Norvegius Albinus*) aged 4-6 weeks and

weighted between 150-250gm were brought from Baghdad University, Medical College Laboratory Animal House. The study was performed during the period from November 2013 to May 2014. The 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 [4].

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) as seen in table 1. Animals of these groups were exposed to ketamine at different gestational periods (at the 7th day, 11th day, and 18th 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 (5 mg/kg, 10 mg/kg, and 20 mg/kg, respectively). Female rats which were found to have no signs of pregnancy were excluded.

Pregnant rats of the experimental subgroups received intraperitoneal ketamine hydrochloride 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 7th, 11th and 18th 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 [5].

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

The TUNEL-based detection kit reagent was provided by abcam (code ab66108).The fluorescein-labeled DNA was observed with a fluorescent microscope (Polyvar).

Statistical evaluation of the number of apoptotic cells that were stained in a bright yellow color was done by counting these cells in equidistant 6 linear fields along the cortical layers at 400X magnification in all the subgroups of each group.

Results

The immunohistochemical TUNEL test of the frontal cerebral cortex revealed three different fluorescent colors (Fig. 1-3):

1. Yellow color representing the apoptotic cells (positive color).
2. Orange color for the non-apoptotic cells (negative color).
3. Green color, also a negative color.

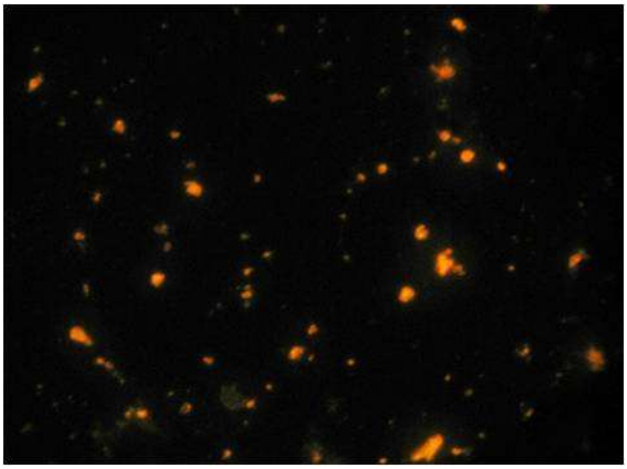

Fig. 1. Coronal section in frontal cerebral cortex from subgroup A (control) of group I showing the fluorescence activity of TUNEL test. 400X.

Counting of the apoptotic cells in group I showed mean value in subgroup B was (33 ± 3.7), in subgroup C was (49.3 ± 5.9), and that in subgroup D (83.6 ± 5.8) (Table 1).

Statistical analysis of the mean differences in group I showed significant variability between the control subgroup A and the subgroups B, C

and D ($p < 0.001$). ANOVA analysis of the mean differences of counting in group I showed that subgroup B varied significantly compared to subgroups C and D ($p = 0.00$ LSD). The mean difference of the counted values in subgroup C also varied significantly from subgroup D ($p < 0.001$).

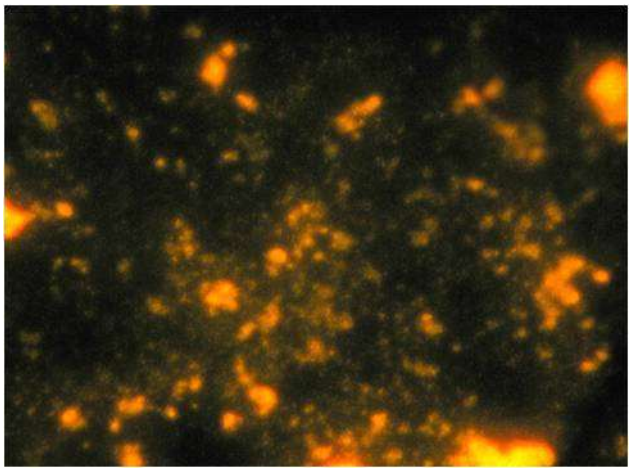

Fig. 2. Coronal section in frontal cerebral cortex from subgroup B (injected with 5mg/kg of ketamine) of group II showing the fluorescence activity of TUNEL test. 400X.

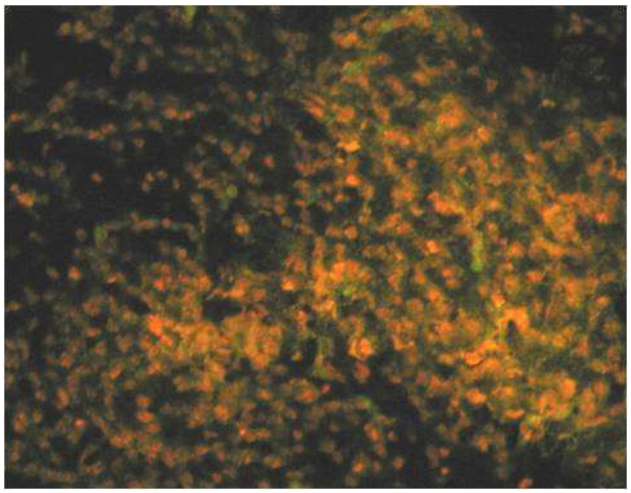

Fig. 3. Coronal section in frontal cerebral cortex from subgroup B (injected with 5mg/kg of ketamine)of group III showing the fluorescence activity of TUNEL test. 100X.

The mean values of subgroup D from group II showed the highest counting compared to subgroups B and C. The mean number of the positive fluorescent cells of subgroup D was (104.5 ± 8.2), while that of subgroup B (53.8 ± 5.3) and subgroup C (80.3 ± 8.06) (Table 2).

Table 1. Mean number of apoptotic cells revealed by TUNEL test in the subgroups of group I.

Subgroup	mean±SD
A	21.5 ± 4.08
B	33.0 ± 3.7*
C	49.3 ± 5.9*
D	83.6 ± 5.8*

*p value ≤ 0.05 is considered statically significant. Dunnett t-test treats one group as a control, and compares all other groups against it.

Table 2. Mean number of apoptotic cells revealed by TUNEL test in the subgroups of group II.

Subgroup	mean±SD
A	39.1 ± 3.7
B	53.8 ± 5.3*
C	80.3 ± 8.06*
D	104.5 ± 8.2*

*p value ≤ 0.05 is considered statically significant. Dunnett t-test treats one group as a control, and compares all other groups against it.

There was statistically significant variability between the counting of the number of positively reacting cells in subgroup A (the control subgroup) compared to the treated subgroups C, and D and B ($p < 0.001$). The multiple comparison test of the mean differences in counting the number of positive cells showed that subgroup B varied significantly in comparison to subgroups C and D ($p < 0.001$). The mean differences of the counted values in subgroup C also varied significantly from subgroup D ($p < 0.001$).

The counted number of the bright yellow apoptotic cells was much higher in the cerebral cortices of the neonates from pregnant rats treated with 20mg/kg (subgroup D) in all groups (I, II, and III). The mean counting number of these positive fluorescent cells in subgroup B was (83.6 ± 6.6) and for subgroup C

(128.3 ± 14.9), while that for subgroup D was (175.8 ± 12.3) (Table 3).

Table 3. Mean number of apoptotic cells revealed by TUNEL test in the subgroups of group III.

Subgroup	mean±SD
A	54.8 ± 4.5
B	83.6 ± 6.6*
C	128.3 ± 14.9*
D	175.8 ± 12.3*

*p value ≤ 0.05 is considered statically significant. Dunnett t-test treats one group as a control, and compares all other groups against it.

There was significant variability between the counting number of positively reacting cells in subgroup A (the control subgroup) compared to the treated subgroups B, C, and D ($p < 0.001$).

The multiple comparisons analysis of the mean differences in counting the number of positive cells showed that subgroup B varied significantly in comparison to subgroups C and D ($p < 0.001$). The mean difference in the counted values of subgroup C was also significantly variable from subgroup D ($p < 0.001$).

Discussion

The effect of the prenatal ketamine exposure on the frontal cerebral cortex development was the concern of this study as many authors described neurobehavioral impairments seen after exposure of the developing rodent brain to anesthetic drugs [(6)].

The question whether anesthetic drugs can trigger neuroapoptosis in the developing non-human primate (NHP) brain was first addressed by Slikker and colleagues in the rhesus macaque brain [(7)]. These reports were supportive to the results of this study.

The number of the reactive cells was the least in subgroup (B) compared to subgroup (C) and (D). The presence of positively reacting apoptotic cells in the sections of the cerebral cortices of the control subgroup (A) is in congruence to the developmental history of the cerebral cortex reported in the literatures. It was documented that the developing cortical neuroepithelium generates more neurons than what is retained in the adult. Neuronal attrition occurs developmentally, by apoptotic cell suicide. The cell death is partly a consequence of competition for limited target-derived growth factors [(8)].

The number of neurons in developing rat brain is derived from early proliferative phase and a later phase of selective death of differentiated neurons. The neuronal precursor cells recognized in rat embryo before the 12th day of gestation and proliferate before the date of neuronal differentiation. It was reported that there is no change in cell number between the end of neurogenesis on the 15th day of gestation and birth [(9)].

The distribution of apoptotic cells in rat cerebral cortex at different developmental stages was described from embryonic day 16 to adulthood [(10)]. This description supports the hypothesis of this study that prenatal exposure to ketamine produces apoptotic changes in the rat frontal cortex.

The statistical analysis showed an attribute for considering the dose of 5 mg/kg as a relatively non-toxic dose in all the groups compared to the subcontrol group. This survival of the newborn rat cortices from the neurotoxic effect was also reported by one study suggesting that there are some cells in the cerebral cortex, which have the ability to resist the action of some neurotoxins. The cellular survival was suggested to be based on the equilibrium between the toxic action and the ability of living cells to protect themselves against cellular reactive oxygen species action [(11)]. Thus, our findings were parallel to the findings of other studies regarding the susceptibility of fetal brain to ketamine's apoptogenic action [(12)].

The progressive abundance of TUNEL positive cells in frontal cortical area of our subgroups in each group and indicates that apoptosis was

involved in the mechanism of neuronal death caused by ketamine exposure during pregnancy. This explanation is in agreement with the suggestion of previous studies [13].

The results of this study showed exaggeration of the apoptotic changes in the frontal cortical tissue by the effect of ketamine. This phenomenon was seen in previous reports that detailed the explanation of the tissue injury in the CNS following virus infection concluding that apoptotic changes are seen in specific regions with neuronal ability to undergo apoptosis [14].

The number of the positively reacting apoptotic cells counted with the TUNLE test showed a progressively increased mean values starting from control subgroup (A) followed by the subsequent groups B, C and D. This result is in agreement with the previous studies reporting progressive cortical cell apoptotic behavior in rat embryo reaching a prenatal peak at the 15th day of gestation [8].

In support to the results of prenatal exposure to ketamine on newborn rat frontal cortex demonstrated in this study, there are many drugs that have been described to trigger widespread apoptotic death of neurons in the developing animal brain [15-19]. It was concluded that susceptibility to the apoptosis reaches its peak during the developmental period of rapid synaptogenesis (also called the period of brain growth spurt). This period was reported in rodents to be primarily during the first 2 weeks after birth, but in humans, it extends from about mid-gestation to several years after birth [12]. All the recent human epidemiological studies pertaining to developmental anesthesia neurotoxicity have focused on full-term infants and children [20,21], the focus of future human research should be expanded to include third trimester fetuses and prematurely born infants.

The results of this study proved that neuronal apoptosis in rat by the effect of ketamine showed progressive peak during prenatal neurogenesis, and it is not only a phenomenon occurring if the rat was exposed to ketamine during the period of brain growth spurt.

The neuroapoptosis by the effect of drugs had been studied at both light and electron microscopic levels [22,23] and by immunohistochemical procedures including caspase-3 (AC3) immunohistochemistry [24]. TUNEL test has been used in this study for marking dying cortical cells to have a new focus on ketamine induced developmental neuroapoptosis. Many animal studies which investigated single or brief exposure to clinically relevant doses of commonly used anesthetics (ketamine, midazolam, propofol, isoflurane, sevoflurane, chloral hydrate) [25,26] showed supportive evidences to the results of this study.

This study concluded that prenatal exposure to ketamine in a dose of 5 mg/kg was a relatively non-toxic in all studied groups compared to the control subgroup. Apoptosis in frontal cortical tissue was involved in the mechanism of neuronal death caused by ketamine exposure during pregnancy.

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Authors contributions:

Dr. Gaeb (a PhD candidate) performed the laboratory research work. Dr. Mobarak (supervisor) performed the interpretation of the results. Dr. Jaffar (supervisor) performed the production of the results.

Conflict of interest

The authors disclose no any financial and personal relationships with other people or organizations that inappropriately influence (bias) out work.

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