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Neonatal isolation provokes hippocampal apoptosis and recognition memory impairment in rats

Hussein A. Ghanimi
College of Nursing, University of Al-Ameed, Karbala, PO Box 198, Iraq & Rayan Research Center for Neuroscience and Behavior, Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
Email: hughanimi@alameed.edu.iq

Hawraa M. Kadhim
College of Medicine, University of Al-Ameed, Karbala, PO Box 198, Iraq
Email: hawraakadhim4@gmail.com

Hawraa Hamid Hussein
College of Medicine, University of Al-Ameed, Karbala, PO Box 198, Iraq
Email: hawraa@alameed.edu.iq

Arash Abdolmaleki
Department of Engineering Sciences, Faculty of Advanced Technologies, University of Mohaghegh Ardabili, Namin, Iran
Email: abdolmalekiarash1364@uma.ac.ir

Ali Moghimi
Rayan Research Center for Neuroscience and Behavior, Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
Email: moghimi@um.ac.ir

***Abstract*---**Neonatal isolation (NI) has detrimental consequences on the hippocampal neurons of rat neonates. It has been reported to enhance neuronal cell death and impair memory behaviors. We conducted this study to assess the effects of NI on hippocampal apoptosis and recognition memory impairment in the hippocampus of rat neonates. One group of male Wistar rat neonates exposed to NI; rat neonates reared with 1-hour neonatal isolation (NI) for eight consecutive days (P2-P9). On the other hand, the control group reared normally. Novel object recognition test (NOR) test used to evaluate the effects of NI on recognition memory impairment. On day 22 (P22), a TUNEL assay was done. NOR demonstrated that rat neonates who

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***Corresponding author**: Ghanimi, H. A.; Email: hughanimi@alameed.edu.iq*
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experienced NI had long-term memory deficits ($P<0.01$). TUNEL assay results showed that NI increased the number of TUNEL positive neurons in CA1, CA3, and DG subfields of the hippocampus ($P<0.05$, $P<0.001$, and $P<0.001$). The present results indicated that NI exerted apoptotic effect and induced recognition memory impairment in the rat neonate's hippocampus

***Keywords*---**neonatal isolation, hippocampus, rat, memory, apoptosis.

Introduction

Neonatal stress (NS) can induce severe behavioral alterations and cognitive impairments during adolescent and adulthood periods (Girardi et al., 2014; Takatsuru and Koibuchi 2015). Also, it may disrupt the normal growth and development of rat brain neurons (Omotoso et al., 2020). It impairs the learning, memory, and cognitive abilities of rats (Aisa et al., 2009). Moreover, it compromises adaptive immunity and enhances susceptibility to diseases (Garcia-Flores et al., 2020). NS is categorized into many events including: Neonatal isolation (NI) (de Azeredo et al., 2017), Febrile seizure (FS) (Atabaki et al., 2020), neonatal shaking (Ueda et al., 2020), malnutrition (Cabral et al., 2011), sepsis (Sato et al., 2020), sleep deprivation (Han et al., 2020), hypoxia (Gehrand et al., 2020), or any traumatic experiences (RaiseAbdullahi et al., 2019; Uniyal et al., 2019). NI enhances altered social behavior during adulthood in rats (Tada et al., 2016). Also, it can disrupt learning and memory (Vivinetto et al., 2013). Also, it enhances apoptosis in rat hippocampal neurons (Baek et al., 2012). NI is applied by isolating rat neonates from their mothers for 1 hr. per day on P2-P9 (Cheng et al., 2019). Previously, rats exposed to NI exhibited anxiety, autistic-like behaviors, and degenerative changes in the hippocampal neurons (Arafat and Shabaan 2020; Bahi 2016). NI impaired recognition and spatial memories in rats (Banqueri et al., 2017; Cheng et al., 2019). On the other hand, NI affected the neuronal and glial cell densities in adolescent rats' brains (Majcher-Maślanka et al., 2019). Moreover, rats exposed to NI showed a neuroinflammatory response in glial cells (Banqueri et al., 2019). On top of that, NS can also interrupt the function of many types of neuronal cells, such as; cholinergic (Marković et al., 2014), serotonergic (Xue et al., 2013), and glutamatergic neurons (Toya et al., 2014). The study's objective was to assess the harmful effects of NI on the hippocampus of rat neonates.

Materials and Methods

Ethics statement

All the experiments and animal handling procedures carried out under the guidelines of the Ethics Committee for Human and Animal Care of Ferdowsi University of Mashhad; therefore, they approved (Approval number: IR.UM.REC.13

Experimental animals and grouping

Pregnant female Wistar rats provided by the Animal House of the Faculty of Medicine, Mashhad University of Medical Sciences (Mashhad, Iran). They maintained in the Animal House of the Faculty of Science, Ferdowsi University of Mashhad (one rat/cage). They were housed under standard environmental conditions (12 hr. of light/dark cycle, 22+2 °C, free access to food and water). They were checked daily, and the day they gave birth considered as P0. Male rat neonates divided into two experimental groups (n=20 in each). Rat neonates were reared with 1-hour neonatal isolation (NI) for eight consecutive days (P2-P9). The control (Ctrl) group was reared normally (See fig. 1).

Neonatal isolation

Mothers and their neonates were housed in clear plastic cages (38 cm×23 cm×20 cm). Male neonates used in this study (N= 40). Neonates (50% of them) were separated from their mothers and put in individual opaque round containers (7 cm in diameter and depth of 8 cm) without bedding for one hour per day on P2-P9 (09:00 a.m.-12:00 p.m.), (NI group). The rest of the neonates were maintained under standard conditions without any isolation (Ctrl group) (Cheng et al., 2019).

Novel object recognition test (NOR)

Apparatus of NOR is a Plexiglas box with dimensions of 32 × 52 × 30 cm. It is categorized into habituation, familiarization, and test trials. Rat neonates were explored the NOR box freely for 10 minutes/session/day for three days (P17-P19) to habituate. On the next day (P20), familiarization (FAM) trials were done. Accordingly, rat neonates put in the box with two similar objects (A1 and A2) for 5 minutes.)de Lima et al., 2005; Reger et al., 2009(Test trials are classified into STM and LTM test trials. STM trials applied 1.5 hours following familiarization sessions; rat neonates allowed to explore two objects (Familiar, A1 and novel, B) in the same positions during familiarization trials for 5 minutes. LTM trials carried out 24 hours (P22) following familiarization sessions. Rat neonates put in the box to explore two objects (Familiar, A1 and novel, C). Objects put in the same positions mentioned previously for 5 minutes.) de Lima et al., 2005; Schroder et al., 2003

Object exploration was video recorded by a camera placed 1 meter above the center of the box. Rats' exploratory behaviors characterized by sniffing and touching the objects. To inhibit perceiving olfactory signals by rat neonates, the NOR box and objects were cleaned continuously after each trial with 75% ethanol. NOR index documented as the time spent by neonates in exploring novel objects per the total time spent for exploring familiar and novel objects (Dong et al., 2018)(fig. 2).

TUNEL assay

At P22, rat neonates were decapitated, and then brains collected, washed with normal saline. Fixation of brains was applied using 10% neutral buffered formalin for 24 hours, and after that, the samples were embedded in paraffin. In the

present study, TUNEL carried out under accordance with the instructions of the manufacturer (In situ cell death detection kit, Roche, Germany) to detect apoptosis in the rat brains (Hippocampus subfields; CA1, CA3, and DG), coronal sections of 10 µm were made using microtome (Leitz 1512, Germany) (Hussein et al., 2019) . In brief, sections processed by xylene, ethanol, double distilled water, and PBS. After that, Sections were treated with 20 µg/ml proteinase K (20 minutes/room temperature), and then sections were washed with PBS again (Jung et al., 2011). Permeabilization applied by adding 0.1% Triton X-100 and 0.1% sodium citrate to sections. Then, they washed with PBS, and 50 µl of labeling solution (TdT and dUTP) added (In dark moisture chamber for 60 minutes at 37 °C). DNase I added to both negative and positive control slides. Finally, sections were washed (With PBS), air dried, covered (Abdolmaleki et al., 2020; Farhadi Moghadam and Fereidoni 2020). Slides antifaded and examined under a fluorescence microscope (Olympus BX51, Japan) to detect TUNEL positive neurons. Photomicrographs captured from the hippocampus (CA1, CA3, and DG subfields) of both hemispheres. Six high-power fields of sections with 150 µm intervals from each group were randomly selected and analyzed. Accordingly, six slides from each rat visualized, and in each slide, six different fields examined. Photomicrographs of hippocampal neurons were selected randomly, and the apoptotic neurons were counted in 1472.44×103 µm2 by Image J software.

Statistical analysis

The data are defined as means ± SEM. The GraphPad Prism 7.0 software (GraphPad Software Inc., USA, version 7) was used for statistical analysis. A two-tailed unpaired t-test was used to verify the differences between groups. $P<0.05$ was considered statistically significant.

Results

NOR

NOR was used to evaluate the recognition memory deficits following NI. The results of the STM recognition index showed that NI was not different compared to the control group (Fig. 3 A, P = 0.492, F, DFn and Dfd values = 1, 9, 9, respectively, 95% confidence interval = -0.1-0.05). On the other hand, NI significantly decreased the LTM recognition index compared to the control group (Fig. 3 B, $P<0.01$, F, DFn and Dfd values = 1, 9, 9, respectively, 95% confidence interval = -0.1-0.02). Therefore, NI could induce recognition memory impairment in the hippocampus of rat neonates later during adulthood.

TUNEL

The number of TUNEL positive neurons counted in the hippocampus (CA1, CA3, and DG subfields) of rat neonates. The results indicated an increase in the count of apoptotic neurons in NI compared to the control group in CA1 {Fig. 4A and 5 (A and B), $P<0.05$, F, DFn and Dfd values = 1, 9, 9, respectively, 95% confidence interval = 0.1-3}, CA3 {Fig. 4B and 5 (C and D), $P<0.001$, F, DFn and Dfd values = 1, 9, 9, respectively, 95% confidence interval = 2-4} and DG {Fig. 4C and 5 (E and F), $P<0.001$, F, DFn and Dfd values = 2, 9, 9, respectively, 95% confidence

interval = 3-6} subfields. The present findings demonstrated that NI might induce apoptosis in the hippocampus of rat neonates.

Discussion

NS can associate with different detrimental consequences, including; inflammatory responses, psychopathologies, and somatic disorders (Fogelman and Canli 2019). NI can adversely affect the memory of rats (Hemb et al., 2010). As mentioned previously, NI could alter social behavior and memory in rats (Tada et al., 2016; Vivinetto et al., 2013). In accordance, it might provoke apoptosis in the hippocampal neurons of rats (Baek et al., 2012). In view of this, we performed the current research to assess the harmful consequences of NI on hippocampal neurons of rat neonates. Therefore, we evaluated the memory impairments in the hippocampus following NI.(Holubová et al., 2018) Holubova et al., 2018; and Menezes et al., 2020 (Menezes et al., 2020) showed that NI promoted long-term memory impairments in rats. Accordingly, our results confirmed that NI enhanced long-term memory deficits. Previous studies have been mentioned that NI could induce memory deficits via; changing the hippocampal neurogenesis (Lajud and Torner 2015), the activity of dopaminergic and serotonergic systems (Li et al., 2013), insulin content (Maghami et al., 2018), and the size of hippocampus (Tata et al., 2015).

The present results showed that NI increased the apoptotic neurons in the hippocampus compared to control. Similarly, Chen et al., 2018 study showed that NI enhanced degenerative changes and apoptosis in the hippocampus of rats (Chen et al., 2018b). Also, Yang et al., 2017 indicated that the early-life NI induced hippocampal neuronal apoptosis in the DG of rats (Yang et al., 2017). The present study demonstrated that NI increased apoptotic neurons in the hippocampus (DG subfield) rather than others. NI could induce hippocampal neuronal apoptosis in rats by; disrupting hypothalamic-pituitary-adrenal axis (HPA-axis) (Nishi 2020), mitochondrial dysfunction (Chen et al., 2018a), down-regulation of protein kinase B (AKT) phosphorylation (Yang et al., 2017), and modulating the expression of apoptosis-related proteins (Drastichova et al., 2021). NS has associated with the developing of different neurological disorders, cognitive deficits, and hippocampus dysfunction (Pervanidou et al., 2020; Qulu et al., 2015). At present, there are evidences from human records that infants are exposed to various stressful experiences that may cause long-lasting consequences during adulthood. Therefore, animal studies are used to study more details about the mechanisms of these consequences.

Conclusion

The present results revealed NI's effect on the recognition memory impairment and apoptosis in the hippocampus of rat neonates. NI enhanced the recognition memory impairment and induced apoptosis in the hippocampus (CA1, CA3, and DG subfields).

Conflicts of interest

The author declares no conflicts of interest.

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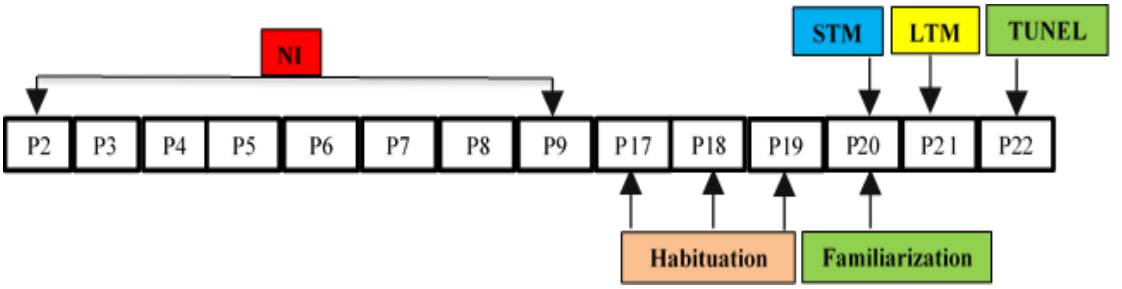

Figure 1: A schematic diagram of experimental timeline. P: Post neonatal day, NI: Neonatal isolation, STM: Short-term memory, LTM: Long-term memory and TUNEL: Terminal deoxynucleotidyl transferase- mediated dUTP nick end labeling

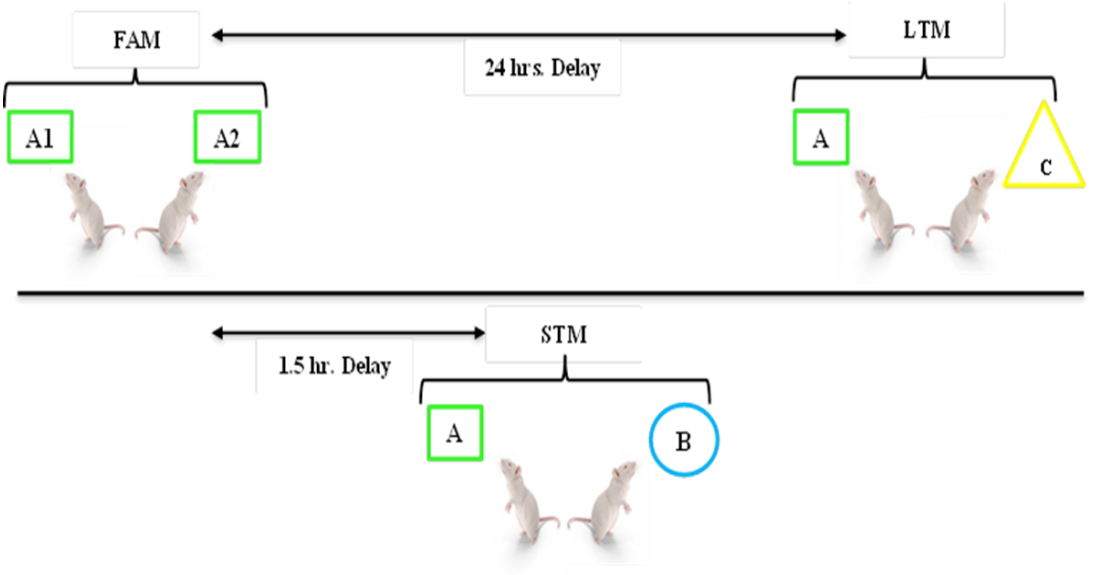

Figure 2: Novel object recognition test (NOR). FAM: Familiarization, STM: Short-term memory, LTM: Long-term memory, A: Familiar object, B and C: Novel objects.

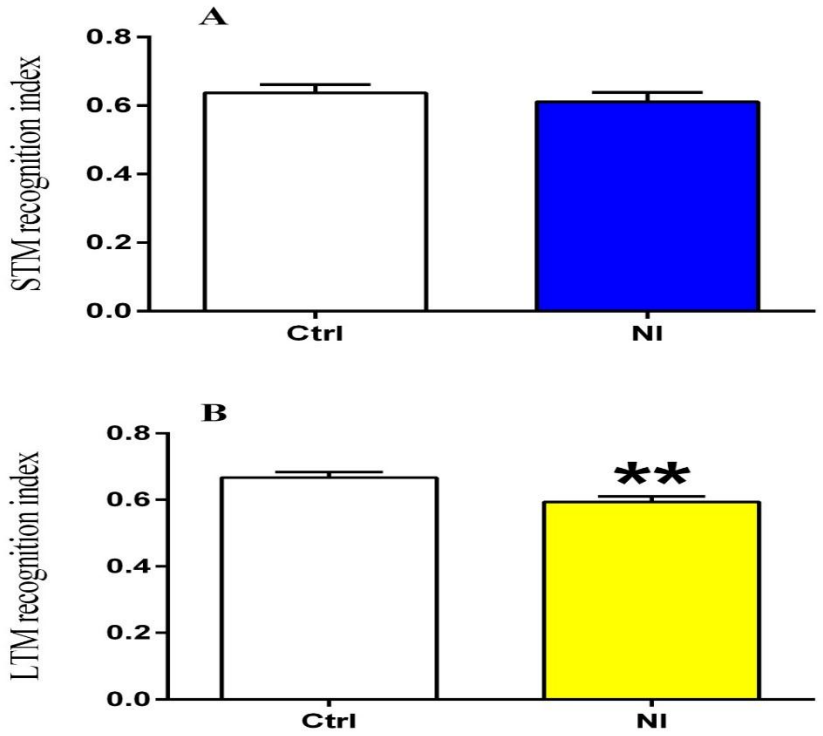

Figure 3: Recognition memory impairment in rat neonates exposed to NI. A. STM recognition memory wasn't affected in NI rat neonates compared to Ctrl. B. LTM recognition memory impairment in NI rat neonates compared to Ctrl. n=20. **= P< 0.01 different compared to control group. STM= Short-term memory, LTM= Long-term memory, Ctrl= Control, NI= Neonatal isolation.

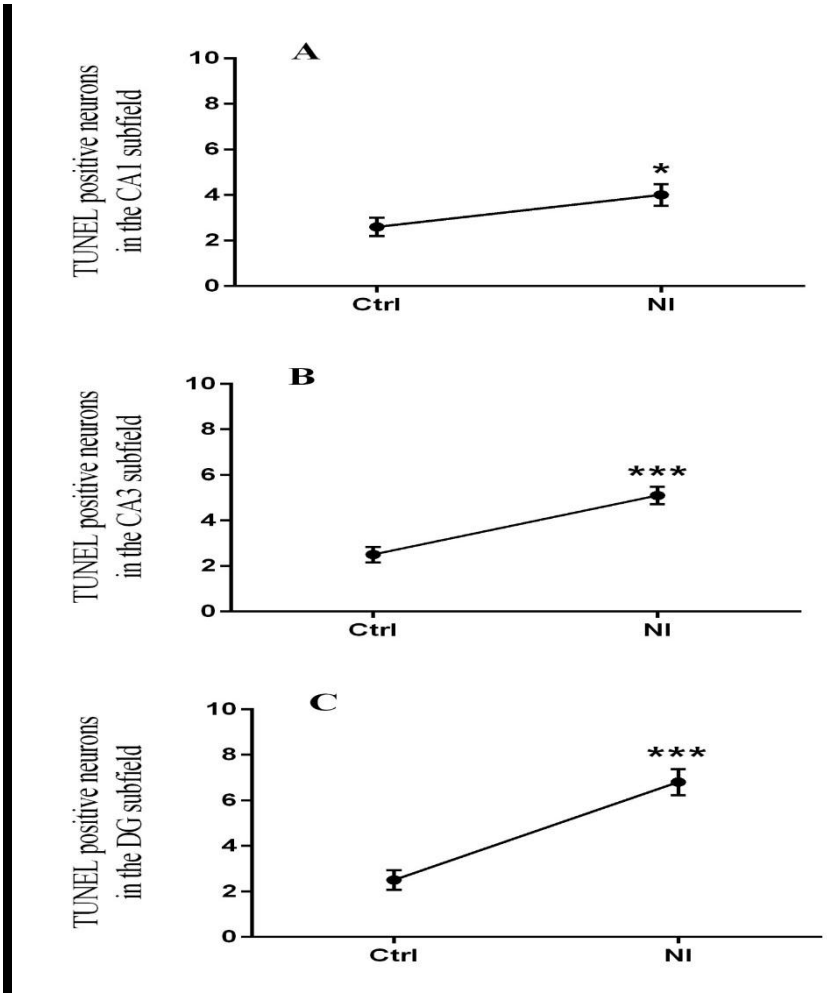

Figure 4: Effect of NI on TUNEL positive neurons in the hippocampus (CA1 (A), CA3 (B), and DG (C) subfields) of rat neonates. n= 20. *= P< 0.05 and ***= P<0.001 compared to control group. Ctrl: Control, NI= Neonatal isolation.

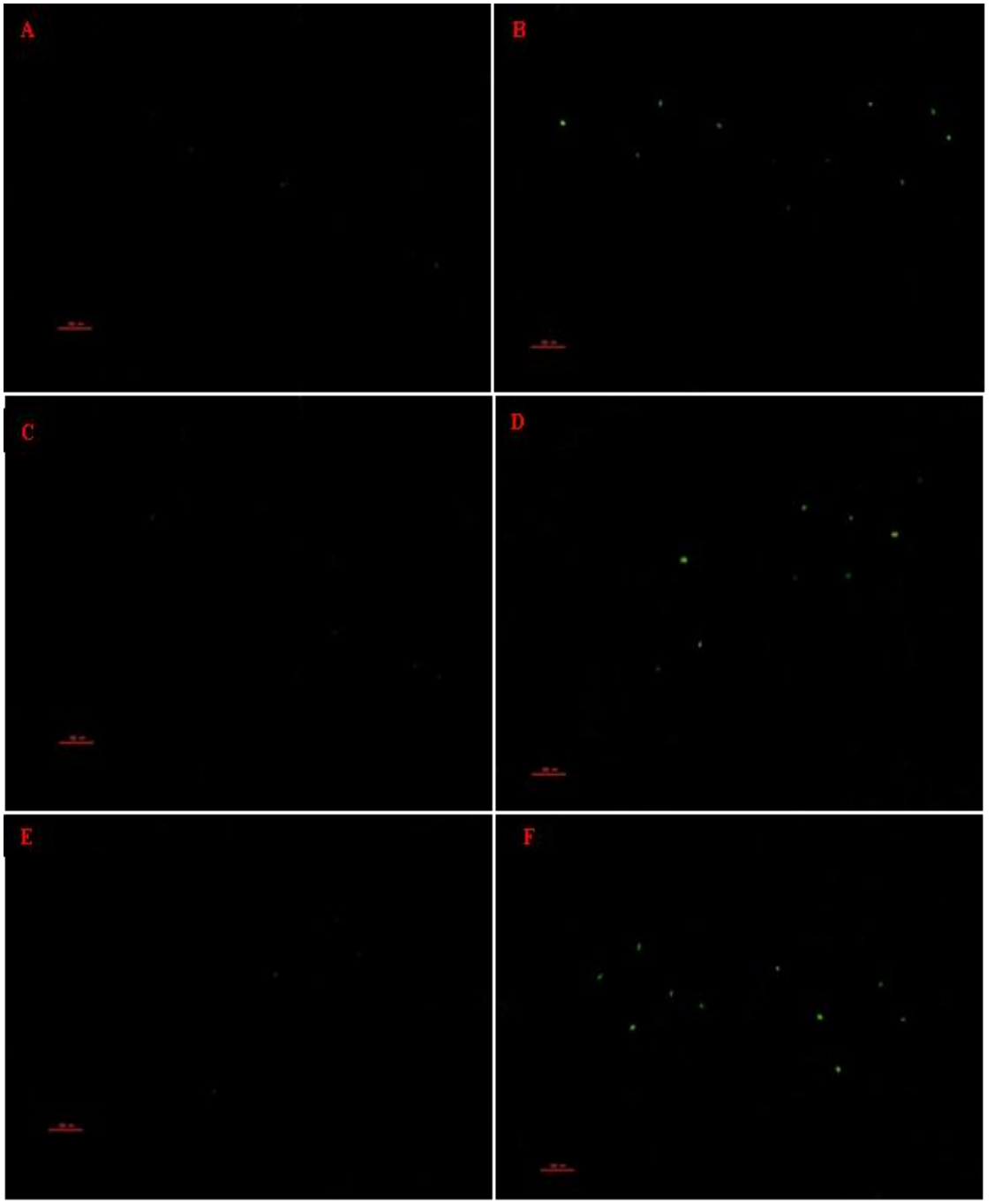

Figure 5: Photomicrographs of the rat neonates' hippocampus. TUNEL positive neurons increased in the hippocampus of neonatal isolated rats (B: CA1, D: CA3 and F: DG subfields) compared with non-neonatal isolated (Ctrl) rats (A: CA1, C: CA3 and E: DG subfields). Scale bars: 100 µm. Magnification: 400X.