Avicenna J Med Biochem. 2016 September; 4(2):e33530. doi: 10.17795/ajmb-33530.

Published online 2016 August 28. **Research Article**

The Effects of Different Types of Chronic Stress on Morphometric Changes and Apoptosis of Betz Cells in the Internal Pyramidal Layer of the Cerebral Cortex of Rats

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Received 2015 October 01; **Revised** 2016 May 24; **Accepted** 2016 May 24.

Abstract

Background: Technology-induced increases in stress can have adverse effects on the nervous system.
Objectives: The aim of the present study was to investigate the effects of chronic multiple stressors on morphometric changes in Betz cells in the rat cerebral cortex.
Methods: Eighteen Wistar rats were divided randomly into two equal groups. One group was then exposed to different types of stress (forced swimming, restraint, water deprivation, isolation, and food deprivation) for 10 days, and the other group (control) remained in cages and was not exposed to any stress. The animals were then weighed and anesthetized, and their brains were removed and weighed. After fixation, the brain samples were prepared for light microscopy study. The number, size, and incidence of apoptosis of Betz cells in the internal pyramidal layer were determined using the ImageTool software program, and the data were analyzed using SPSS software and a t-test, with a value of $P < 0.05$ considered statistically significant.
Results: The mean number and size of Betz cells in the stressed group decreased significantly compared to those in the control group ($P < 0.05$). Qualitative observations revealed chromatolysis of Nissl bodies, nucleus condensation, and a decrease in neural processes. The mean number of apoptotic cells in the stressed group were also significantly increased compared to that of the control group ($P < 0.001$).
Conclusions: The results showed that chronic multiple sequential stress can have negative effects on the cerebral cortex of rats by reducing the size and number of Betz cells and increasing the incidence of apoptosis. More studies are needed to confirm these results.

Keywords: Apoptosis, Brain, Pyramidal Cells, Rats, Stress

1. Background

Emotional disturbance or emotional stress refers to responses to harmful effects of external stimuli (1). In the long term, the variety and type of stress can lead to stress (2). Studies have reported that chronic social stress caused changes in the secretion of neurotransmitters and in neural structures and led to a variety of disorders and diseases (3). Studies of the impact of various types of chronic stress, such as food deprivation, water deprivation, immobilization, forced swimming, and social isolation, on animals found that stress-induced physiological changes depended on the amount, frequency, and duration of exposure of the animals to the stressor (4, 5). Stress was shown to have both physiological and psychological effects and cause mental and behavioral changes. Experimental evidence suggested that stress gave rise to structural changes in the neural network, such as the hippocampus, prefrontal cortex, and the amygdala (6). Apoptosis is a programmed process of cell death, which can be induced by physiological and pathological stimulation. Research also demonstrated that stress increased apoptotic markers in most tissues, such as the neural system. Stress-related disorders were also shown to trigger neurochemical changes and subsequently the apoptotic process (7). According to one study, anything that prevented the growth of normal cells, such as exposure to toxic agents or freezing, had the potential to trigger cellular apoptosis (8). Various physiological and psychological stressors were reported to affect the hypothalamic-pituitary-suprarenal cortex, hypothalamic-pituitary–gonadal system, adrenomedullary system, and sympathetic system and to cause wonderful effects in some

members the body, with dramatic changes in the sympathetic nervous system (9). The internal prefrontal cortex is involved in cognitive functions, including the regulation of the stress response (10). This region is also involved in stress-related depression. Research clearly showed that stress caused dysfunction of the internal prefrontal cortex in animal models and that chronic stress induced morphological changes in Betz cells of the internal prefrontal cortex (11). The cortex is composed of six layers. Betz cells, which are found in the internal pyramidal layer, and stellate cells account for the majority of cortical neurons (12). A previous study showed that chronic stress was associated with molecular, morphological, and ultrastructural changes in the rat hippocampus that could potentially give rise to cognitive problems (13).

2. Objectives

Given the central role of Betz cells in motor activities and the dearth of studies in this field, this study aimed to evaluate the effects of different types of stress on morphometric parameters of Betz cells in the internal pyramidal layer of the cerebral cortex of rats.

3. Methods

Eighteen Wistar rats (200 - 300 g) were randomly and equally divided into a stressed and nonstressed group (control). The control group remained in cages and was exposed to no disruption for 10 days. The stressed group was exposed to different types of stress for 10 days (Table 1) (14). To minimize predictive capability, the animals were subjected to different types of stress at various times of day. To simulate restraint-induced stress, the animal was placed in a 21×6 cm plastic tube-shaped chamber, so that it could not move. Forced swimming-induced stress was achieved by placing the animal in a glass tank (44 × 33 × 30 cm, depth of 22 cm) filled with water at a temperature of 23 ± 2°C. The animals in each group were provided with adequate food and water.

After 10 days, both groups of mice were anesthetized by an injection of ketamine (60 mg/kg) and xylazine (6 mg/kg). The tissues were prepared (fixation, dehydration, clearing, and impregnation) using a tissue processor. Thin 5 μm slices were prepared by a rotary microtome. To avoid double counting of cells, five slices, numbers 5, 8, 11, 14, and 17, were selected from each sample. The sections were stained with hematoxylin and eosin (H & E), and microscopic slides were prepared and imaged using a Nikon camera. The images were transferred to a computer, and the number and size of Betz cells in the inner layer of the cerebral cortex of the rats were identified using the ImageTool program. To determine the size of the pyramidal cells, the first large and small diameter of the cells was determined. The average cell size was then obtained using the following formula: $x = \sqrt{a^2 + b^2}$. A t-test was used for the data analysis, and P values less than 0.05 were considered statistically significant.

Table 1. Sequential Multiple Stressors Used in This Study

Days	Items Used	Times
1	Forced swimming	10 min
2	Limitation	3 h
3	Forced swimming in water with a temperature of 3°C	24 h
4	Forced swimming in water with a temperature of 4°C	1.5 h
5	Isolation	24 h
6	Food deprivation	24 h
7	Water deprivation	24 h
8	Forced swimming in water with a temperature of 4°C	2 h
9	Food deprivation	24 h
10	Forced swimming	10 min

3.1. TUNEL Staining

The paraffin sections were dewaxed in xylene and rehydrated through a graded ethanol/water series, followed by washing in PBS. The tissues were incubated with 20 μg/mL of proteinase K in 10 mM Tris and 5 mM EDTA for approximately 15 minutes at 37°C and then washed in PBS. So they were affected by Triton X at room temperature, and they were then washed in PBS. The sections were incubated with H_2O_2 for about 15 minutes at 37°C and then washed again in PBS. The tissue sections were incubated with TUNEL labeling solution (1 unit of enzyme and 9 units of labelling solution) for about 60 minutes at 37°C. Excess TUNEL solution was removed by washing in PBS. Finally, the microscopic slides were imaged using a fluorescent Nikon microscope. The number of apoptotic cells was determined using the ImageTool computer software program.

3.2. Statistical Analysis

The results are expressed as mean ± standard deviation (SD). Differences between groups were assessed by a t-test (SPSS software). A P value of less than 0.05 was considered statistically significant. A t-test was used for the data analysis.

4. Results

As shown in Table 2, the number and size of Betz cells in the stressed group were significantly decreased compared to those in the control group ($P < 0.05$). The qualitative analysis revealed chromatolysis of Nissl bodies, nuclear condensation, and loss of neuropils (Figure 1). Moreover, the mean number of apoptotic cells in the stressed group was significantly increased compared to that of the control group, as indicated in Figure 2 ($P < 0.001$).

Table 2. Mean Size, Number, and Apoptotic Incidence of Betz Cells in the Rat Cerebral Cortex[a]

Variables	Control	Stress
Mean diameter of pyramidal cells, μm	279.94 ± 57.257	238.00 ± 53.55[b]
Number of pyramidal cells, μm	12.92 ± 4.68	6.48 ± 3.00[b]
Apoptotic cells, %	6.3 ± 2.9	15.1 ± 5.6[c]

[a] Values are expressed as means ± SD.
[b] $P < 0.0$.
[c] $P < 0.001$ compared to the control.

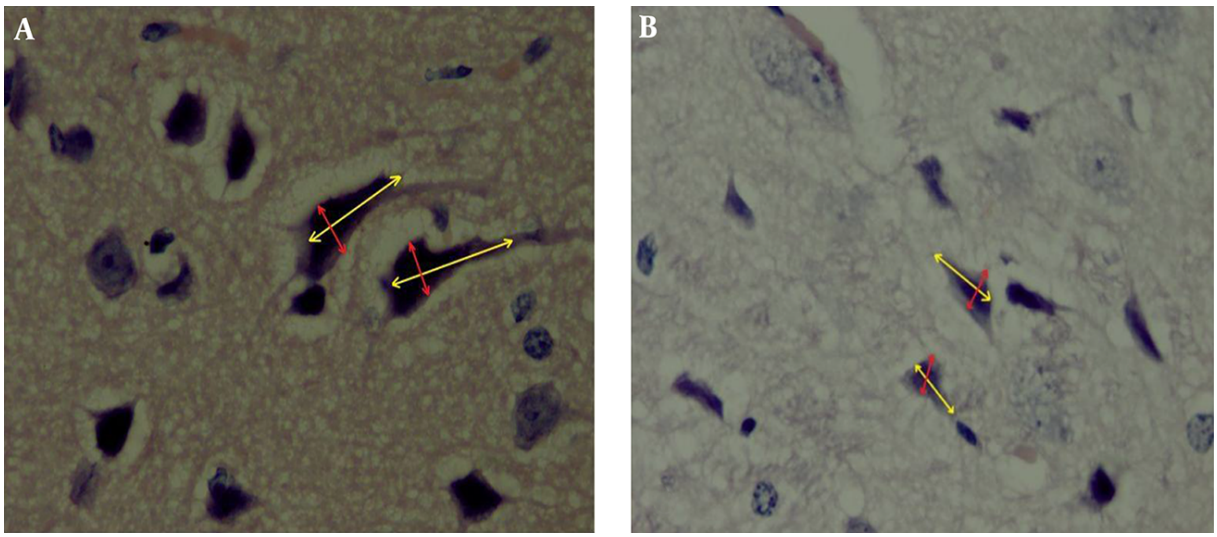
5. Discussion

The findings of this study revealed significant differences between large and small diameter Betz cells in the internal pyramidal layer of the cerebral cortex and significant differences in the numbers of Betz cells between the stressed and control groups. These results indicate that chronic sequential stress reduces the number of Betz cells in the cerebral cortex by causing cell damage and degeneration, leading to reduced cell sizes, cell death, and apoptosis. A previous study reported that astrocytes and Betz cells underwent apoptosis in the CA1 region in response to activation of the endoplasmic reticulum stress response and ischemia by caspase 12 (15). In another study, social stress reduced the expression of genes associated with neurogenesis and thereby reduced cell proliferation in the brain (16). A short period of stress (6 days) caused atrophy of Betz cells in the hippocampus (17). In that study, the authors prepared sections of CA3 and CA1 areas of the hippocampus and observed that exposure to stress for longer periods resulted in neurons with shorter trunks and fewer branches as compared to those of a control group. The same study reported long-trunk neurons in the CA3 and CA1 regions, but this finding was not significant. They concluded that a short period of chronic stress caused atrophy of neurons in the hippocampus. Both the hippocampus and gonadal, thyroidal, and adrenal hormones have been shown to be sensitive to stress, with stress-related changes occurring in synaptic structure and dendritic structure and regulation in cerebral gyri during development and adulthood.

The plasticity induced by stress has been shown to depend on the type of stress. Frequent stress caused atrophy of dendrites in the CA3 region, whereas acute and chronic stress inhibited neurogenesis of dendrites of granular neurons in cerebral gyri (18). Chronic immobilization-induced stress induced contraction and retraction of dendrites of Betz cells in the infralimbic area of the prefrontal cortex of rats (19). Immobilization stress for 3 weeks in rats caused significant changes in apical dendrites of Betz neurons in the second and third layers of the internal prefrontal cortex and decreased the number and length of dendritic branches of these neurons (20). Swimming stress resulted in a significant reduction in apical dendrites of pyramidal neurons in the prefrontal cortex and inferalimbic area of rats (21). In contrast, the morphology of prelimbic neurons did not change in response to stress. The authors concluded that neurons of the inferalimbic area but not the prelimbic area were sensitive to even minor stress. In another study, stress reduced the numbers of pyramidal cells in the CA3 hippocampal area of pregnant rats during pregnancy but had no effect on hippocampal CA1 cells (22). Immobilization stress caused atrophy in apical dendrites of Betz cells in the fifth layer of the prefrontal cortex in rats (23). The activation of neurons and release of neurotransmitters affect the allostasis (internal stability and balance) of the brain. When someone is struggling with stress frequently, allostatic mediators cause wear and tear of the body and the brain that is called allostatic load. Examples of allostatic load include the accumulation of abdominal fat, bone mineral loss, and atrophy of nerve cells in the hippocampus (24). Research showed that stress led to atrophy and cell damage, which was followed by an increase in the release of corticosterone from the hypotalamo-pituitary-adrenal axis. The administration of stress hormones, such as corticosterone, was shown to simulate the morphological effects of stress (23). Corticosterone also resulted in atrophy of neurons in the hippocampal CA3 area and the second and third layers of the prefrontal cortex. Receiving corticosterone for 3 weeks gave rise to changes in dendrites of neurons in the amygdala and pyramidal neurons in the CA3 area of the hippocampus (25).

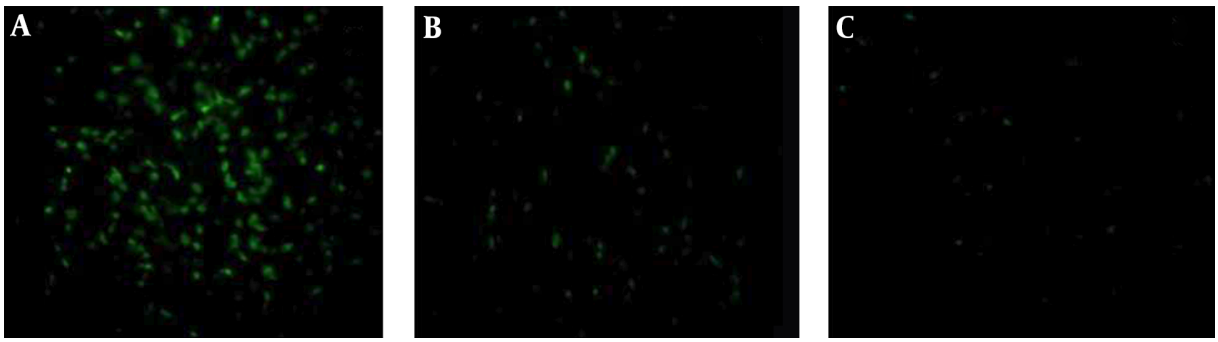
According to some research, the reduction in the size of Betz cells in the cerebral cortex may be due to the production of free radicals following exposure of animals to stress. Some researchers believe that social stress by producing kinds of reactive oxygen and combined with bilirubin leads to increased biopyrrin. Biopyrrin can also be a symptom of social stress (26). The observations of chromatolysis of Nissl bodies, nuclear condensation, and loss of neurites in the qualitative analysis in the present study

Figure 1. Microscopic View of Betz Cells



Large and small diameter Betz cells of the inner layer of the cortex in the A, control and B, stressed group. As shown, the size of the Betz cells in the stressed group was significantly decreased compared to that of the control group. Magnification is × 1000.

Figure 2. Fluorescent Microscopic Images of Apoptotic Cells



A, positive control: The microscopic slides were incubated in 1000 IU/mL of deoxyribonuclease I (Sigma) for 20 minutes at 37.5°C and 5% CO_2 in air and washed twice in PBS-PVA before TUNEL staining; B, stressed group: apoptotic cells (green yellowish color) can be observed; C, negative control: the microscopic slides were incubated in fluorescein-dUTP in the absence of TdT. Magnification is × 400.

point to a reduction in the size and number of neurons and neuronal damage, as shown in other studies. Apoptosis plays a crucial role in brain development by regulating the growth, migration, and contact of cells. Studies demonstrated that oligodendrocytes were sensitive to stress and that stress caused apoptosis during embryonic and adult life (27). The stress-induced damage led to a reduction in cell size, cell death, and apoptosis. Osada et al. showed that astrocytes and pyramidal neurons in the CA1 region underwent apoptosis in response to increases in caspase 12 after endoplasmic reticulum stress induced by ischemia (15). Stefani et al. showed that stress resulted in an accumulation of intracellular proteins by an unknown mechanism and that the latter accelerated the apoptosis process (28). In a study of the relation between oxidative stress and brain development, oxidative stress in response to DNA damage led to congenital metabolic disorders and caused apoptosis of neuronal cells (29). As the main source of energy, mitochondria are sensitive to stress-induced apoptosis, and the brain, which requires a high level of energy, is sensitive to mitochondrial damage. A previous study showed that stress-included mitochondrial damage caused apoptosis in brain cells and attributed various diseases, such as Alzheimer's, Hunting-

ton's, and Parkinson's, to such stress (30). Active apoptosis of glial tumors, which have the potential to cause death with 12 months, was shown to activate the endoplasmic reticulum stress response (31). Psychosocial stress led to damage of nerve cells in the rat, with the stress affecting interleukin and lipid peroxidation and inducing apoptosis of hippocampal cells (32). In a study of the number, size, and density of cells and the incidence of apoptosis in neurons and glial cells in the brains of people with bipolar disorder after death, oxidative stress increased the expression of apoptosis and reduced the size and number of cells (33). The results of the present study showed that chronic sequential stress had negative effects on rat cerebral cortex by reducing the size and number of Betz cells and increasing the incidence of apoptosis. However, more studies are needed to confirm these results.

Acknowledgments

We would like to thank the research deputy of Qazvin University of Medical Sciences for financial support and the staff of the histotechnique laboratory for technical support.

Footnotes

Authors' Contribution: Farzad Rajaei planned and supervised all the experiments; Toofan Sabernia performed the experiments; and Hossein Piri acted as thesis advisor.

Funding/Support: This research was financially supported by the cellular and molecular research centre of Qazvin University of Medical Sciences.

References

1. Selye H. Forty years of stress research: principal remaining problems and misconceptions. *Can Med Assoc J.* 1976;**115**(1):53–6. [PubMed: 1277062].
2. McEwen BS. Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *Eur J Pharmacol.* 2008;**583**(2-3):174–85. doi: 10.1016/j.ejphar.2007.11.071. [PubMed: 18282566].
3. Woolverton WL, Ator NA, Beardsley PM, Carroll ME. Effects of environmental conditions on the psychological well-being of primates: a review of the literature. *Life Sci.* 1989;**44**(14):901–17.
4. Schreck CB, Contreras-Sanchez W, Fitzpatrick MS. Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture.* 2001;**197**(1):3–24.
5. Chigurupati S, Son TG, Hyun DH, Lathia JD, Mughal MR, Savell J, et al. Lifelong running reduces oxidative stress and degenerative changes in the testes of mice. *J Endocrinol.* 2008;**199**(2):333–41. doi: 10.1677/JOE-08-0306. [PubMed: 18701639].
6. Fuchs E, Flugge G, Czeh B. Remodeling of neuronal networks by stress. *Front Biosci.* 2006;**11**:2746–58. [PubMed: 16720347].
7. Gao J, Wang H, Liu Y, Li YY, Chen C, Liu LM, et al. Glutamate and GABA imbalance promotes neuronal apoptosis in hippocampus after stress. *Med Sci Monit.* 2014;**20**:499–512. doi: 10.12659/MSM.890589. [PubMed: 24675061].
8. Rajaei F, Otoi T. Effect of cryoprotectants on DNA fragmentation in porcine blastocysts. *J Reprod Infertil.* 2007;**5**:37–43.
9. Ishida H, Mitsui K, Nukaya H, Matsumoto K, Tsuji K. Study of active substances involved in skin dysfunction induced by crowding stress. I. Effect of crowding and isolation on some physiological variables, skin function and skin blood perfusion in hairless mice. *Biol Pharm Bull.* 2003;**26**(2):170–81. [PubMed: 12576676].
10. Perez-Cruz C, Simon M, Flugge G, Fuchs E, Czeh B. Diurnal rhythm and stress regulate dendritic architecture and spine density of pyramidal neurons in the rat infralimbic cortex. *Behav Brain Res.* 2009;**205**(2):406–13. doi: 10.1016/j.bbr.2009.07.021. [PubMed: 19643147].
11. Shansky RM, Morrison JH. Stress-induced dendritic remodeling in the medial prefrontal cortex: effects of circuit, hormones and rest. *Brain Res.* 2009;**1293**:108–13. doi: 10.1016/j.brainres.2009.03.062. [PubMed: 19361488].
12. Mescher AL. Junqueira's basic histology: text and atlas. Mcgraw-hill; 2013.
13. Reagan LP, Magarinos AM, McEwen BS. Neurological changes induced by stress in streptozotocin diabetic rats. *Ann N Y Acad Sci.* 1999;**893**:126–37. [PubMed: 10672234].
14. Marti O, Armario A. Anterior pituitary response to stress: time-related changes and adaptation. *Int J Dev Neurosci.* 1998;**16**(3-4):241–60. [PubMed: 9785121].
15. Osada N, Kosuge Y, Ishige K, Ito Y. Characterization of neuronal and astroglial responses to ER stress in the hippocampal CA1 area in mice following transient forebrain ischemia. *Neurochem Int.* 2010;**57**(1):1–7. doi: 10.1016/j.neuint.2010.03.017. [PubMed: 20362024].
16. Mirescu C, Gould E. Stress and adult neurogenesis. *Hippocampus.* 2006;**16**(3):233–8. doi: 10.1002/hipo.20155. [PubMed: 16411244].
17. Lambert KG, Buckelew SK, Staffiso-Sandoz G, Gaffga S, Carpenter W, Fisher J, et al. Activity-stress induces atrophy of apical dendrites of hippocampal pyramidal neurons in male rats. *Physiol Behav.* 1998;**65**(1):43–9. [PubMed: 9811363].
18. McEwen BS, Magarinos AM. Stress and hippocampal plasticity: implications for the pathophysiology of affective disorders. *Hum Psychopharmacol.* 2001;**16**(S1):S7–S19. doi: 10.1002/hup.266. [PubMed: 12404531].
19. Goldwater DS, Pavlides C, Hunter RG, Bloss EB, Hof PR, McEwen BS, et al. Structural and functional alterations to rat medial prefrontal cortex following chronic restraint stress and recovery. *Neuroscience.* 2009;**164**(2):798–808. doi: 10.1016/j.neuroscience.2009.08.053. [PubMed: 19723561].
20. Cook SC, Wellman CL. Chronic stress alters dendritic morphology in rat medial prefrontal cortex. *J Neurobiol.* 2004;**60**(2):236–48. doi: 10.1002/neu.20025. [PubMed: 15266654].
21. Izquierdo A, Wellman CL, Holmes A. Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice. *J Neurosci.* 2006;**26**(21):5733–8. doi: 10.1523/JNEUROSCI.0474-06.2006. [PubMed: 16723530].
22. Pawluski JL, Valenca A, Santos AI, Costa-Nunes JP, Steinbusch HW, Strekalova T. Pregnancy or stress decrease complexity of CA3 pyramidal neurons in the hippocampus of adult female rats. *Neuroscience.* 2012;**227**:201–10. doi: 10.1016/j.neuroscience.2012.09.059. [PubMed: 23036618].
23. Liu RJ, Aghajanian GK. Stress blunts serotonin- and hypocretin-evoked EPSCs in prefrontal cortex: role of corticosterone-mediated apical dendritic atrophy. *Proc Natl Acad Sci U S A.* 2008;**105**(1):359–64. doi: 10.1073/pnas.0706679105. [PubMed: 18172209].
24. McEwen BS. Sex, stress and the hippocampus: allostasis, allostatic load and the aging process. *Neurobiol Aging.* 2002;**23**(5):921–39. [PubMed: 12392796].

25. Morales-Medina JC, Sanchez F, Flores G, Dumont Y, Quirion R. Morphological reorganization after repeated corticosterone administration in the hippocampus, nucleus accumbens and amygdala in the rat. *J Chem Neuroanat.* 2009;**38**(4):266–72. doi: 10.1016/j.jchemneu.2009.05.009. [PubMed: 19505571].
26. Miyashita T, Yamaguchi T, Motoyama K, Unno K, Nakano Y, Shimoi K. Social stress increases biopyrrins, oxidative metabolites of bilirubin, in mouse urine. *Biochem Biophys Res Commun.* 2006;**349**(2):775–80. doi: 10.1016/j.bbrc.2006.08.098. [PubMed: 16949032].
27. Butts BD, Houde C, Mehmet H. Maturation-dependent sensitivity of oligodendrocyte lineage cells to apoptosis: implications for normal development and disease. *Cell Death Differ.* 2008;**15**(7):1178–86. doi: 10.1038/cdd.2008.70. [PubMed: 18483490].
28. Stefani IC, Wright D, Polizzi KM, Kontoravdi C. The role of ER stress-induced apoptosis in neurodegeneration. *Curr Alzheimer Res.* 2012;**9**(3):373–87. [PubMed: 22299619].
29. Hayashi M, Miyata R, Tanuma N. Oxidative stress in developmental brain disorders. *Adv Exp Med Biol.* 2012;**724**:278–90. doi: 10.1007/978-1-4614-0653-2_21. [PubMed: 22411250].
30. Federico A, Cardaioli E, Da Pozzo P, Formichi P, Gallus GN, Radi E. Mitochondria, oxidative stress and neurodegeneration. *J Neurol Sci.* 2012;**322**(1-2):254–62. doi: 10.1016/j.jns.2012.05.030. [PubMed: 22669122].
31. Johnson GG, White MC, Grimaldi M. Stressed to death: targeting endoplasmic reticulum stress response induced apoptosis in gliomas. *Curr Pharm Des.* 2011;**17**(3):284–92. [PubMed: 21348829].
32. Kubera M, Obuchowicz E, Goehler L, Brzeszcz J, Maes M. In animal models, psychosocial stress-induced (neuro)inflammation, apoptosis and reduced neurogenesis are associated to the onset of depression. *Prog Neuropsychopharmacol Biol Psychiatry.* 2011;**35**(3):744–59. doi: 10.1016/j.pnpbp.2010.08.026. [PubMed: 20828592].
33. Gigante AD, Young LT, Yatham LN, Andreazza AC, Nery FG, Grinberg LT, et al. Morphometric post-mortem studies in bipolar disorder: possible association with oxidative stress and apoptosis. *Int J Neuropsychopharmacol.* 2011;**14**(8):1075–89. doi: 10.1017/S146114571000146X. [PubMed: 21205433].