Original Article

Study of the variations in apoptotic factors in hippocampus of male rats with posttraumatic stress disorder

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Abstract

Background: Post-traumatic stress disorder (PTSD) is a stress-related psychosomatic disorder caused by occurrence of a traumatic event and the hippocampus volume of the patients with Post-traumatic stress disorder decreased. However, the mechanisms that cause such damage are not well-understood. The aim of this study is to detect the expression of apoptosis-related Bax, Bcl-2, Caspase-3 and Insulin-like growth Factor-l proteins in the hippocampus region in the Predatory stress rats.

Materials and Methods: A total of 70 male wistar rats were divided into Predatory stress groups of 1d, 2d, 3d, 7d, 14d, 30d and a normal control group (N = 10). Rats were subjected to 5 min of predatory stress and then exposed to the elevated plus-maze (EPM). Serum corticosterone and Insulin-like growth factor-1 level of Hippocampus were measured by ELISA technique. The expression of Bax, Bcl-2, and Caspase-3 were detected by western blotting.

Results: Rats spent significantly more time in closed arms of the elevated plus maze (EPM) than control group after exposure to stress. Serum levels of corticosterone significantly increased at 2d-3d. The expression of hippocampal IGF-1 was significantly up-regulated at 1d-2d after stress. Both Bax and the ratio of Bax/Bcl-2 significantly peaked at Predatory stress 2d-14d. Caspase3 was significantly active among 2d-30 compared to the normal control.

Conclusion: The activation of caspase-3 in the stress groups indicates that apoptosis may be one of the reasons inducing hippocampus atrophy and play roles in the pathogenesis of PTSD. Increase in hippocampus levels of IGF-1 during early PTSD might be involved in the early molecular inhibitory mechanism of apoptosis in PTSD.

Key Words: Apoptosis, Bax, Bcl2, caspase-3, corticosterone, insulin-like growth factor-1, post-traumatic stress disorder, predator stress

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is continuous psychological disorder, taking place after facing with severe stress. In this disorder, the patient is exposed to a traumatic event, which involves death

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or a threat of death, and the patient's responses to that event are very intense fear and distress.[1] The patient shows serious disturbed behaviors and clinical disorder in his/her individual, social, and occupational performance, which would not occur before the trauma because the patient repeatedly recalls the traumatic experience.[2] Recent neuro-imaging studies have shown the volume of hippocampus in patients with PTSD decreases.[3,4] Today, there are lots of evidences on the relationship between hippocampal atrophy, though its mechanism has not been determined properly.[5]

Apoptosis is a genetically-programmed cell process, playing an important role in differentiation and tissue homeostasis.[6] Various pathways for apoptosis have been identified based on different physiological and pathological stimuli among which are the intrinsic mitochondrial and extrinsic death receptor pathways which finally end in caspase-3 and cause protein degradation and cellular integrity.[7,8] Apoptosis is one of the most important defense mechanisms protecting organisms from the pathogens, although it plays an important role in the pathogenesis of neurodegenerative disease.[9] Activation of intrinsic mitochondrial pathway due to stress and subsequent morphological variations resulting in atrophy in different areas of brain, especially hippocampus has been found in acute and chronic stress.[10-12]

Insulin-like Growth Factor 1 (IGF-1) is a polypeptide with 70 amino-acids, which chemical structure is similar to that of insulin hormone and is produced in many body tissues, especially in hippocampus.[13,14] IGF-1 produced in brain cells is obviously effective in growth of primary cells of central nervous system and also maturation of neurons.[15] IGF-1 secretion is influenced by various factors like glucocorticoids.[16] According to researchers, the expression of IGF-1 gene in some parts of adult mice brain, such as hippocampus, remarkably increased number of neurons unlike the failure in expression of the gene or the gene mutation that caused brain development delay both in animals and humans.[17] IGF-1 is known as one of the fewest inhibitors of apoptosis incidence, which prevents the reduction of the anti-apoptotic factor of cell Bcl-2, as, in the absence of IGF-1, the apoptosis was found to increase by 50% in cells.[18-20] Nowadays, the role of IGF-1 as an effective peptide in neurogenesis processes and also as treatment of some neurodegenerative diseases associated with peripheral nervous system (PNS) and central nervous system (CNS) has been known more than ever, and various applications of this factor and how it acts in many of body vital processes are the concerns of many studies in this regard.[21,22]

Based on the foregoing and considering that there are no documented results on the correlation between hippocampal atrophy and apoptosis in PSTD and the few reports on the apoptosis incidence are only about single prolong stress in animal model, the present study examined the variations in factors of apoptosis mitochondrial pathway and IGF-1 through an animal model for predator stress of this disorder, during the first procedures of this animal model of PTSD.

MATERIAL AND METHODS

In this experimental study, 70 Wistar male rats with the age of 7 weeks and weight of 160-180 g were kept in animal house of Neuroscience Department of Shahid Beheshti University of Medical Sciences under regulations of protection and research trials with laboratorial animals.

The studied animals were divided into 7 groups [each with 10 rats] called control, after stress 1st day, 2nd day, 3rd day, 7th day, 14th day, and 30th day groups, and the stress was induced using the animal model of predator stress. To do so, each rat was put in a small cage, and this cage was placed in a wooden cage with dimensions of 1 × 1.52 × 1.83 m exposed to a cat. After 5 min, the rat was taken out of the cage and put in a dark place for 5 min. According to the reference, stress induction for all groups was carried out from 8 to 10 AM at the same environmental temperature.[23,24] In order to identify anxious animals and behavioral changes, the rats were subjected to an elevated plus-maze (EPM) test apparatus for 5 min. Based on the reference, anxious animal's pauses in closed arms would be much longer than their pauses in open arms, and this was the criterion for the animal affected with PTSD in this study.[25,26]

In order to measure serum corticosterone level, the rats were quickly anesthetized with CO_2, and blood was taken after beheading the specimens. The blood coming out of rats' neck was collected, and its serum was prepared by centrifuging the blood samples for 10 min at 2000 rpm. The level of serum corticosterone was measured using ELISA test [Sandwich ELISA Kit, DRG Co.].

To examine the variations in proteins associated with apoptosis, the rats' brains were removed out of their skulls, and each hippocampus was isolated on a bar of ice. After homogenization of hippocampus in protein extraction buffer (0.1 M sodium chloride, 10 mM tris-sodium chloride, 0.1 mM EDTA, and protease inhibitor cocktail with pH=8), and centrifuging at 4°C at 1500 rpm for 45 min, the supernatant containing total protein was collected and the concentration

of the total protein was determined using Bradford method.[27] IGF-1 level of Hippocampus was measured using ELISA test (Sandwich ELISA Kit, R&D Co.).

To do western blot analysis, at first, 60 µg of the total protein of each sample was added to sodium dodecyl sulphate polyacrylamide gel and was transferred to polyvinylidene difluoride (PVDF) membrane after electrophoresis. Then, the membrane was incubated with each primary monoclonal antibody (Cell Signaling Co.) at dilution of 1:1000 against Bax, Bcl-2, caspase-3, and beta-actin [as a loading control] proteins, and then incubated with secondary antibodies (Cell Signaling Co.) at dilution of 1:10000 and finally, the bonds appeared on photographic film using Ecl-Plus kit (ECL; Amersham Pharmacia Biotech). The films were scanned and quantified by Image J software. Values of each Bax, Bcl-2, and Caspase-3 proteins were divided by the beta-actin value of the same group, and then the proportion of Bax proteins to Bcl-2 proteins was determined.

The means were compared using SPSS software by one-way ANOVA and Tukey tests at different times. Minimum significance level was $P < 0.05$.

RESULTS

The results of the behavioral experiment for different groups of rats exposed to predator stress are shown in Graph 1. In this experiment, behavioral variations, regarding the proportion of time rats spent in the closed arms to the time spent in the open arms of elevated plus-maze, in all groups under predator stress, had a significant upward trend than that in control group ($P < 0.05$). These variations reached their maximum in proportion to other groups during the second day ($P < 0.05$), then reduced to a constant level that still differed significantly from that of control group. The level of serum corticosterone significantly increased on the 2nd day and 3rd day after stress as compared to that in all groups and the control group ($P < 0.05$) [Graph 2]. The amount of IGF-1, on the first and second days after stress, had a significant upward trend than that in control group ($P < 0.05$). This amount reached its maximum on the second day as compared with that in other groups ($P < 0.05$) [Graph 3].

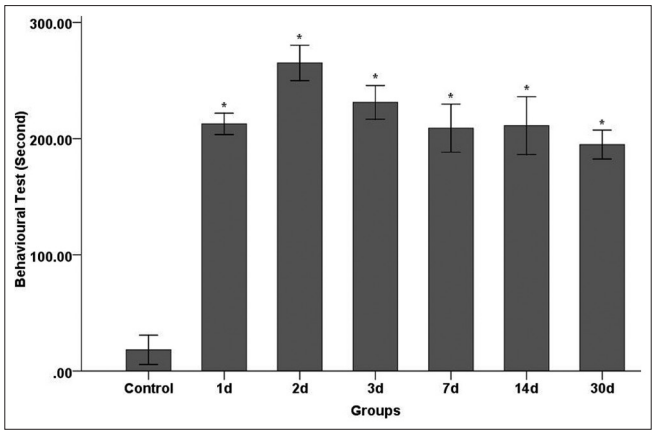
In order to study pro-apoptotic and anti-apoptotic variations in the groups under stress and control group, the amount of Bax, Bcl-2, and caspase-3 proteins in rat hippocampus was assessed using western blot analysis. The results showed that the amount of protein on the 2nd, 3rd, 7th, and 14th days after stress increased significantly as compared to that of the control group ($P < 0.05$). This amount reached its maximum on the 3rd day as compared to that in other groups ($P < 0.05$) [Graph 4 and Figure 1]. Variations in Bax protein on the 1st and 30th days were not as significant as those of control group. Variations in Bcl-2 protein, up to the 2nd day after stress, were not different from those in control group; however, a quite significant reduction was found on the 3rd and 7th days ($P < 0.05$), and then reached its normal level [Graph 5and Figure 2]. Calculation of ratio Bax/Bcl-2 as an indication of apoptosis showed this ratio in the hippocampus of the groups under stress on the 2nd, 3rd, 7th, and 14th was significant as compared with that in the control group ($P < 0.05$). This ratio reached its maximum during the 3rd day as compared with other groups ($P < 0.05$) [Graph 6].

The predator stress could significantly increase activation of caspase-3 in the 2nd to 30th groups as compared with that in control group ($P < 0.05$). This activity had a highly significant upward trend on the 3rd day ($P < 0.05$), though it followed a downward trend after 72 hours. The caspase-3 activation rate was not significant on the 1st day [Graph 7and Figure 3].

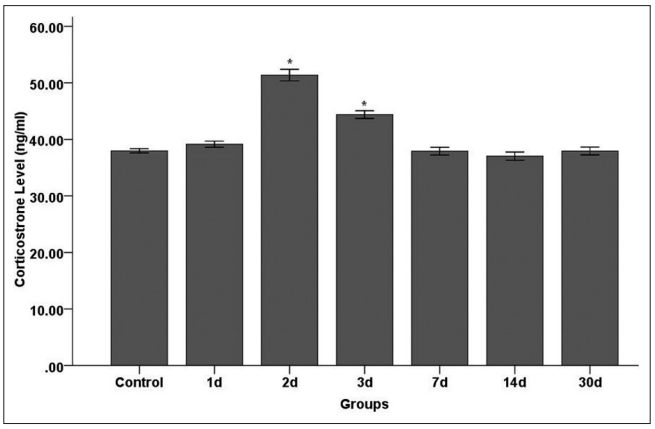
DISCUSSIONS

Studies have shown most of the victims of PTSD express social and behavioral abnormalities due to the hippocampal atrophy in these people.[28] Numerous theories have been published on the causes of atrophy; however, there are not documented evidences on the intervention of apoptosis in hippocampal atrophy.[29]

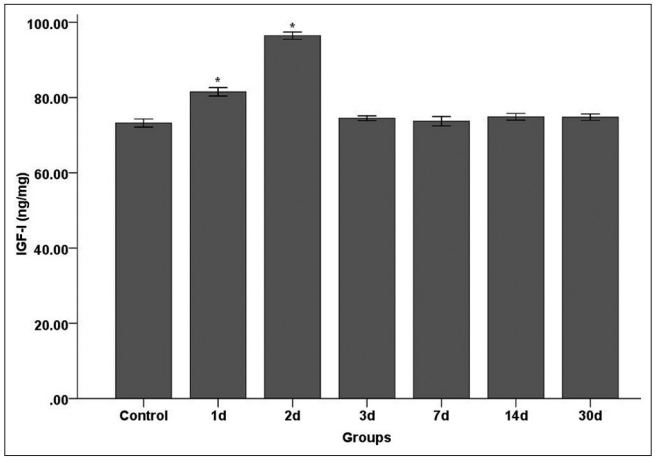
Today, because of ethical issues with the molecular studies in human models, different animal models including single prolong stress, predator stress, etc. have been suggested for studying the causes of hippocampal atrophy in PTSD, and the behavioral variation in rats on the elevated plus-maze apparatus is considered as a symptom of this disorder.[30] In the animal model of predator stress in Adamec's study and in the animal model of single prolong stress in Shi *et al*.'s study, behavioral changes in rats in all groups under stress on the elevated plus-maze apparatus were quite significant as compared with those in control group.[31,32] Shi *et al*.'s study on the rats' hippocampus, using single prolong stress model, showed the amount of apoptotic Bax, caspase-3 proteins, and the ratio of Bax/Bcl-2 in all stressed groups under 1 month increased significantly as compared with those in the control group, and the amount of anti-apoptotic Bcl-2 protein decreased significantly as compared with that in the control group.[32,33] The present study also showed significant behavioral variations on the elevated plus-maze apparatus in rats exposed to predator stress than those in the control group. Moreover, the



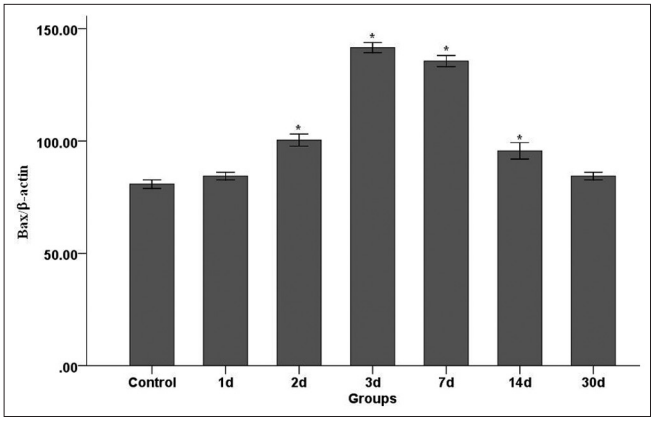
Graph 1: Comparison of behavioral pattern of rats on elevated plus-maze in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group [10 rats in each group]. Statistical difference in data obtained from the studied groups was performed using One-way ANOVA and Tukey tests. Difference at level of $P < 0.05$* is significant among all groups and control group



Graph 2: Comparison of variations in serum corticosterone level in rats in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group [10 rats in each group]. Statistical difference in data obtained from the studied groups was performed using One-way ANOVA and Tukey tests. There is a significant difference in 1st day and 2nd day groups with control group at $P < 0.05$*



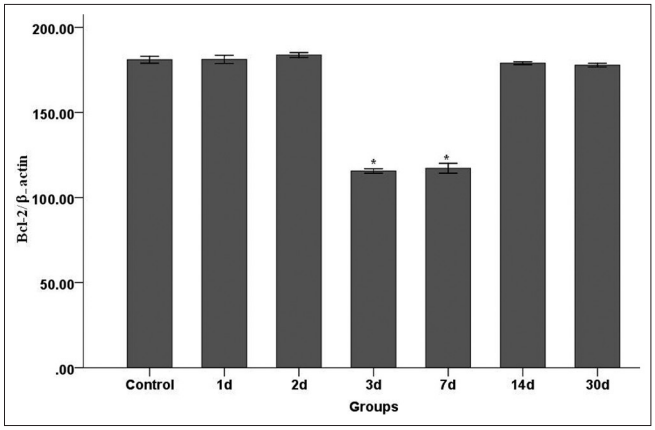
Graph 3: Comparison of variations in IGF-1 in hippocampus of the rats in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group [10 rats in each group]. Statistical difference in data obtained from the studied groups was performed using One-way ANOVA and Tukey tests. There is a significant difference in 1st day and 2nd day groups with control group at $P < 0.05$*



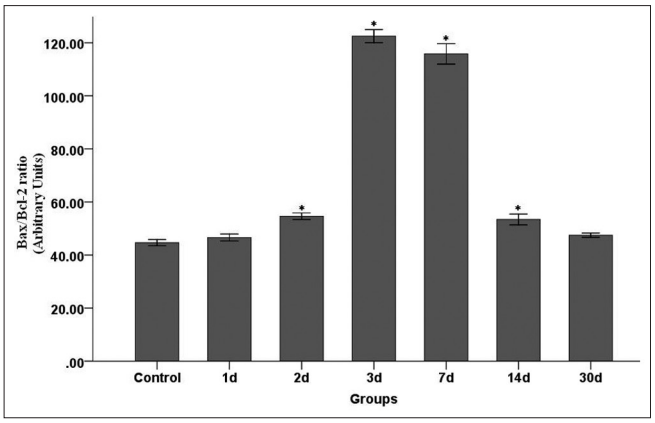
Graph 4: Comparison of variations in Bax protein in hippocampus of the rats in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group [10 rats in each group]. Statistical difference in data obtained from the studied groups was performed using One-way ANOVA and Tukey tests. There is a significant difference in 2nd day, 3rd day, 7th day, and 14th day groups with control group at $P < 0.05$*

expression rate of Bax protein in hippocampus and the ratio of Bax/Bcl-2 whose variation shows triggering of apoptosis mitochondrial pathway were significant only in the 2nd to 14th groups as compared with those in the control group. However, the expression rate of Bcl-2 was significantly reduced only in the 3rd and 7th groups as compared with that in the control group. The caspase-3 activity rate in hippocampus in the 2nd to 30th groups exposed to predator stress was not significant. Variations in factors related to apoptosis were found in the present study and in Shi *et al*.'s study; however, one of the reasons for differences in the results of these two studies might be due to the type of stress and subsequently different animal models used in these two studies.

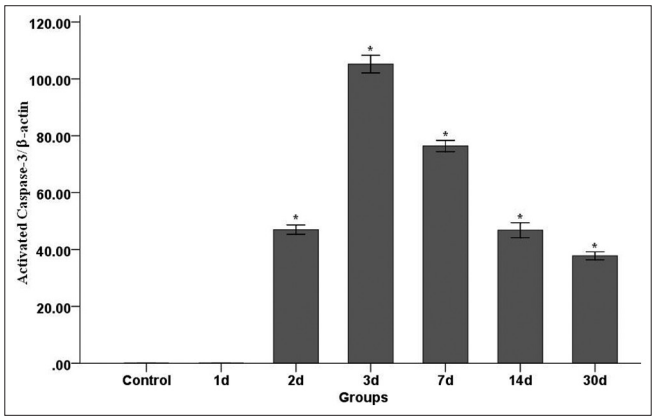
Prevention of cell programmed death may be caused by various molecular mechanisms like neurotrophins, growth factors synthesis, and expression adjustment of apoptotic regulators.[34] IGF-1 produced inside neurons acts as a protective factor, which has been found to be reduced in hippocampus in diseases related to central nervous system.[34,35] Furthermore, reduction of IGF-1 results in the increase in apoptosis in purkinje cells and anti-apoptotic effects of this factor has been found in dorsal root ganglia and human neuroblastoma cell line.[36,37] The androgenic IGF-1 exerts its anti-apoptotic effects on Bcl-2 family through preventing the increase in the ratio of intracellular Bax/Bcl-2.[38] Regarding the comparison of intracellular IGF-1 and Bcl-2 on the 1st and 2nd days after the stress in the



Graph 5: Comparison of variations in Bcl-2 protein in hippocampus of the rats in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group [10 rats in each group]. Statistical difference in data obtained from the studied groups was performed using One-way ANOVA and Tukey tests. There is a significant difference in 3rd day and 7th day groups with control group at $P < 0.05$*



Graph 6: Comparison of variations in Bax/Bcl-2 ratio in hippocampus of the rats in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group [10 rats in each group]. Statistical difference in data obtained from the studied groups was performed using One-way ANOVA and Tukey tests. There is a significant difference in 2nd day, 3rd day, 7th day, and 14th day groups with control group at $P < 0.05$*



Graph 7: Comparison of variations in caspase-3 enzyme in hippocampus of the rats in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group [10 rats in each group]. Statistical difference in data obtained from the studied groups was performed using One-way ANOVA and Tukey tests. There is a significant difference in 2nd day to 30th day groups with control group at $P < 0.05$*

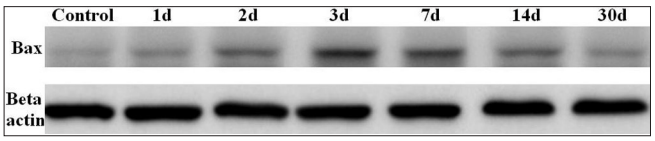


Figure 1: Immunoblot pattern of Bax and β-actin proteins in hippocampus of rats in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group

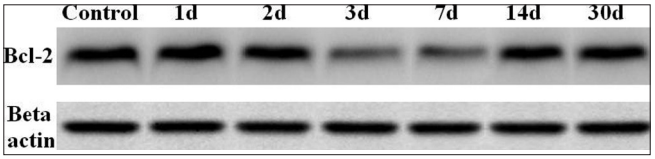


Figure 2: Immunoblot pattern of Bcl-2 and β-actin proteins in hippocampus of rats in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group

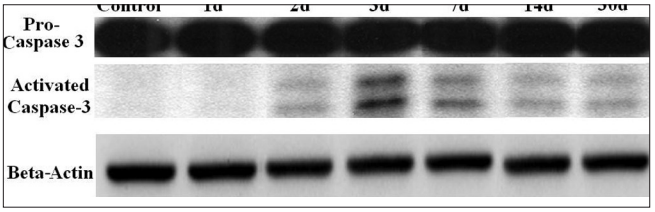


Figure 3: Immunoblot pattern of caspase-3 and β-actin proteins in hippocampus of rats in groups of 1st, 2nd, 3rd, 7th, 14th, and 30th days after exposed to predator stress and in control group

present study, IGF-1 can be considered as a highly preliminary protective factor against apoptotic effects of stress. Therefore, in this study, it seemed that the significant increase in IGF-1 of hippocampus up to the 2nd day prevented the reduction of Bcl-2, and this inhibited the quick change in ratio of intracellular Bax/Bcl-2 and consequently, the incidence of apoptosis slowed up within 48 hours after stress. However, weak activity of caspase-3 on the 30th day, despite Bax/Bcl-2 ratio's returning to its normal level, might show the activity of other apoptosis pathways besides mitochondrial pathway due to the stress.

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