Can Psychobiotics Administration Influence Behavioral Responses and Physiological Stress in Healthy Rats?

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Abstract

Background: Bidirectional communication between gut and brain function is well demonstrated. Evidence is accumulating to suggest that psychobiotics [prebiotics, probiotics or synbiotics)] have beneficial effects of on psychological distress in disease states. However, their role in healthy status remains relatively unclear.

Aims: Therefore, we aimed to clarify if psychobiotics (*L.plantarum*, inulin or their combination) can influence behavioral responses and physiological stress in healthy rats.

Methods: Twenty-eight male Wistar rats were divided into four groups. Then, psychobiotics were administered to intervention groups for 8 weeks. Behavioral tests were performed at the end of the intervention.

Results: Our finding indicated that unlike inulin, the administration of *L.plantarum* and synbiotic could ameliorate depression and anxiety-like behavior and cognitive performance. Serum and brain oxidative stress markers were significantly improved by synbiotic consumption. Also, intake of *L.plantarum* resulted in the decreased oxidative stress in the hippocampus and amygdala. In addition, a significant increase in hippocampal serotonin and BDNF concentration was observed after symbiotic and *L.plantarum* intake. Furthermore, there is a strong correlation between serum and brain markers with behavioral performances.

Conclusion: Our study suggests that administration of psychobiotics therapy may be associated with prevent or ameliorate psychological disorders.

Background

In the past decade, numerous studies have revealed the bidirectional relationship between the gut microbiome and brain [1]. As shown, gut disorders and diseases can affect the central nervous system (CNS), especially behavioral response such as cognition, anxiety, and depression-like behaviors [2, 3]. Therefore, gut microbiota can be a new interesting target to change behaviors performance and brain functions. Probiotics and prebiotics are two nutritional supplements with multiple effects in health and disease [4, 5]. There is a controversy about the synergistic effect of probiotics and prebiotics [6-8];

Psychobiotics are probiotics, prebiotics or synbiotics which have beneficial psychological effects [9, 10]. It was found that intake of probiotics or prebiotics can improve depression, anxiety, and memory in some diseases [9-12]. Unfortunately, findings are mostly reported in disease states in animals and human study. While Psychobiotics may have preventive and/or ameliorative effects on cognitive and behavioral functions [13-15].

Oxidative stress is reported to underlie the pathogenesis of neuropsychological disorders [16, 17]. Given the widespread roles of reactive oxygen species (ROS) in neurological disorders, there has been a long

effort to develop antioxidant treatments. Recent studies have shown that the use of probiotic or prebiotic could increase the activity of antioxidant enzymes [8, 18, 19].

Brain-derived neurotrophic factor (BDNF) is one of the major neurotrophins of the CNS with important roles in survival, maintenance, growth and differentiation of neurons [20]. Serotonin is also a vital neurotransmitter that plays a crucial role in cognitive and behavioral mechanisms [21]. Though BDNF and serotonin are two apparently separate signaling systems in several brain functions, they have synergistic effects [22]. Evidence indicated that the use of some probiotics and prebiotics could improve BDNF and serotonin levels in different brain regions [9, 10, 23]. The hippocampus, amygdala, and prefrontal cortex (PFC) are the most important regions of the brain in the creation and regulation of the behavioral process. Moreover, these regions have important interactions with each other in the regulation of neurological mechanisms [24, 25].

In our previous works on diabetic rats, the beneficial effects of inulin and *L.plantarum* and their synbiotic in the regulation of CNS function were reported [8, 9]. Therefore, we hypothesized whether the use of psychobiotics can have beneficial psychological effects in healthy rats. Therefore, the present study was aimed to investigate the effects of separate and concurrent supplementation of *L.plantarum* and inulin on serum and brain oxidative stress markers, BDNF, and serotonin levels as well as behavioral performance in healthy rats

Materials And Methods

Animals and diet

Twenty-eight healthy male Wistar rats (240 ± 20 g) at 6 ± 2 weeks of age were purchased from Tabriz University of Medical Sciences (TBZMED) Laboratory Animal Center (Tabriz, Iran). All experiments were approved by the TBZMED Animal Experimentation Ethics Committee in accordance with the guidelines of the Principles of Laboratory Animal Care (NIH Publication, revised 1986). This study was carried out in compliance with the ARRIVE guidelines. The animals were housed in polycarbonate cages and maintained under standard laboratory conditions ($21\text{--}24^\circ\text{C}$, 40-60% humidity and 12:12 h light/dark cycle) and allowed free access to food and water. Initially, the rats were kept with standard laboratory chow diet for 7 days to adapt to their new condition. Then, they were randomly assigned to 4 groups (7 rats per group), as below: HSh, healthy sham; HLI, healthy rats treated by the *L.plantarum* and inulin; HI, healthy rats treated by inulin; HL, healthy rats treated by the *L.plantarum*.

Preparation of supplementation

Preparation of the supplements and the intervention method has been described elsewhere [9]. Briefly, *L. plantarum* ATCC 8014 was obtained from TBZMED Biotechnology Research Center (Tabriz, Iran). Ten mL was inoculated in MRS (Man-Rogosa-Sharpe broth) broth and cultured in aerobic conditions at 37°C for 48 h in phosphate buffered saline (PBS). Fresh bacterial suspensions were prepared at a concentration of 10^7 colony-forming units (CFU)/mL. Gastric gavage was performed every 24 h for each rat. The inulin

content of the rat diet was calculated based on 5% of the daily food weight, and was dissolved in drinking water.

Behavioral tests

Behavioral tests were performed in a standard room. For compatibility with room conditions, the animals were placed into the room for 2h prior to the experiment.

Elevated plus maze *Elevated plus maze* (EPM) was used to assess anxiety-like behavior. In brief, the apparatus of a black wooden maze consisted of a central platform with two open and two enclosed arms. The rats were observed for 5 minutes, using the video-computerized tracking system, just after placing them on the center of the platform (facing the open-arm). The time elapsed in the open or closed arms represented the amount of anxiety in the rat. Also, the total distance was measured as an index of locomotor activity. **Forced swimming test** To evaluate depression-like behavior, the **forced swimming test** (FST) was performed. The rats were introduced into a transparent plexiglass cylinder, filled with water at 23–25°C up to a height of 50 cm from the base. The duration of each struggling and immobility was recorded from the start to the end for 5 minutes. Immobility, defined as the absence of all motions with the exception of movements required to keep the rat's head above the water, was calculated by subtracting the scrambling duration from the total test time.

Morris Water Maze

Morris water maze (MWM) test, designed for estimation of spatial learning and memory, consists of a circular pool made of black PVC with 70 cm high walls and a diameter of 150 cm and filled to a depth of 40 cm with 25°C water. The trial started by randomly placing the rat in water facing the wall of the pool at one of the four starting points. During the learning phase, the animals were trained to locate on the platform in 60 s and allowed to stay there for 15s in four consecutive days (the first day with the visible platform and the rest with the hidden ones). Finally, the latency time to reach the platform was measured.

Preparation of blood and tissue samples

The rats were anesthetized with pentobarbital sodium (65 mg/ kgBW, sigma, intraperitoneal injection). Then, about 5 mL of blood was taken from the cardiac puncture and centrifuged at 3000 rpm at 4°C for 20 min; sera were stored at -80 °C for subsequent analysis. After sacrificing the anesthetized rats, the brain was instantly removed from the cranium and the three desired regions (the hippocampus, amygdala, and PFC) were dissected out on an ice-cold plate and stored at -80°C. Brain slices were homogenized and the supernatant was separated after a 20-minute centrifugation at 6,000 rpm at 4°C. The Bradford method was used to make it compatible with the data [26].

Biochemical assays

SOD, MDA, GPx, and TAC were assayed both in the whole blood and brain of the rats to determine oxidative stress markers as well as antioxidant status. Briefly, xanthine and xanthine oxidase were used

to generate the superoxide radicals. The SOD level was measured by the degree of inhibition of this reaction (by 505 nm on a spectrophotometer) [27]. MDA activity was measured through the analysis of the reaction of MDA with thiobarbituric acid (TBA), which forms an MDA-TBA adduct absorbed strongly at 535 nm [28]. GPx activity was measured by the Paglia and Valentine method [29], using cumene hydroperoxide as a substrate. Nicotinamide adenine dinucleotide phosphate)NADPH(disappearance was monitored by a spectrophotometer at 340 nm. The TAC assay relies on the ability of antioxidants in the sample scavenge ABTS radical [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], produced by peroxidase and H₂O₂ (making blue green color). Suppression of this color was measured by spectrophotometer at 600 nm [30]. The levels of BDNF and serotonin concentrations in the serum and supernatants of all three regions were determined, according to the recommended manufacturer's guidelines, using enzyme-linked immunosorbent assay (ELISA) kits.

Statistical analysis

All data were presented as means $\pm$ standard error of the mean (SEM). Statistical analysis was performed with a one-way analysis of variance (ANOVA); when ANOVA showed a significant difference, the post-hoc Tukey's test was applied to demonstrate the differences. Data were analyzed using SPSS software (version 22). *P*-values less than 0.05 were considered statistically significant.

Results

Effects of *L.plantarum* and inulin on markers of oxidative stress

Treatment with synbiotics led to increased levels of SOD in the serum (*P*=0.005), amygdala (*P*=0.032), hippocampus (*P*=0.008), and PFC (*P*=0.024), compared to HSh group. Moreover, supplementation with synbiotics led to MDA reduction in the serum (*P*=0.015), amygdala (*P*=0.037), and hippocampus (*P*=0.003) in DLI group, but this change was not significant in the PFC (Figure. 1). In addition, hippocampus levels of GPX (*P*=0.025) and TAC (*P*=0.003) increased significantly in DLI group, compared to the HSh group. Probiotic supplementation only reduced the MDA levels in the hippocampus (*P*=0.017). But it had no significant effect on other oxidative stress parameters (TAC, SOD, and GPx) either in the serum or the amygdala and PFC. Eight-week administration of inulin did not significantly affect serum and brain oxidative status of the healthy rats (Figure. 1).

Effects of *L.plantarum* and inulin on serotonin and BDNF

Compared to HSh group, synbiotic administration increased levels of BDNF (*P*=0.011) and serotonin (*P*=0.012) in the hippocampus. Moreover, it elevated BDNF concentration in the PFC (*P*=0.015) and serotonin in the amygdala (*P*=0.043). Treatment with *L.plantarum* led to increased levels of BDNF (*P*=0.035) and serotonin (*P*=0.040) only in the hippocampus, but did not show significant effects on serum, PFC, and amygdala (Figure. 2). Supplementation with inulin did not produce significant changes in either serum or brain BDNF and serotonin levels. The post hoc analysis also revealed no significant differences among HLI, HL, and HI groups in terms of serotonin and BDNF concentration (Figure. 2).

Effects of *L.plantarum* and inulin on anxiety and depression

Compared with the HSh group, the rats in HLI and HL group spent a longer duration of time in the open arms ($P=0.005$ and $P=0.011$, respectively) and a shorter duration of time in the closed arms ($P=0.017$ and $P=0.036$, respectively) of the maze, an indicator of anxiety improvement (Figure. 3). Compared with the HSh group, HLI and HL group had more OAT (open arms duration/total time $\times 100$) ($P=0.005$ and $P=0.011$, respectively) and less CAT (close arms duration/total time $\times 100$) ($P=0.017$ and $P=0.036$, respectively). Intervention groups (HL, HI, and HLI) showed no significant differences in distance moved in the central area of the maze, as compared to HSh group. Also, measurement of the total motion to assess locomotor activity showed no significant difference among the HLI, HL, and HI groups, compared to the HSh group. One-way repeated measures ANOVA revealed that probiotic and synbiotic treatment led to a remarkable reduction of immobilization time ($P=0.041$ and $P=0.013$, respectively) in FST, compared to the HSh group (Figure. 3).

Effects of *L.plantarum* and inulin on learning and memory

The intervention groups displayed an identical performance in locating the hidden platform in the first to the third day of learning phase, compared to the HSh group ($P=0.032$). But on the fourth day of learning phase, the escape latency decreased in HL ($P=0.021$) and HLI ($P=0.014$) groups (Figure. 4). In the spatial probe test performed on day five, the swimming time spent within the target quadrant by the HLI group was increased, in comparison to HSh group ($P=0.032$). Whilst the HL and HI group did not have a significant difference in the time elapsed within the target quadrant, as compared with the HSh group. Also, Post hoc analysis confirmed no statistical variation among the performance of the intervention groups in the MWM (Figure. 4).

Discussion

There is growing evidence that the administration of psychobiotics may have protective and ameliorative effects on behavioral disorders. The present study was conducted to provide a new insight into the effects of psychobiotics on psychological behaviors in three major brain regions that play important roles in the regulation of behaviors. According to our findings, the administration of *L.plantarum* and synbiotic could alleviate depression and anxiety-like behavior and enhance learning, whereas inulin intake could not significantly improve behavioral responses. However, serum and brain oxidative stress markers were significantly declined by synbiotic consumption. Also, intake of *L.plantarum* resulted in the decreased oxidative stress markers in the hippocampus and amygdala. In addition, hippocampal serotonin and BDNF concentrations were significantly elevated following symbiotic and *L.plantarum* intake. Furthermore, there are strong correlations between serum and brain parameters with behavioral responses (Figure. 5).

Administration of synbiotic resulted in ameliorated oxidative status in the serum, hippocampus, and amygdala. Furthermore, *L.plantarum* intake could significantly improve oxidative stress markers in the amygdala and hippocampus (Figure. 1). Oxidative stress can be derived from a variety of sources that

observed even in normal conditions [31]. As mentioned earlier, oxidative stress can damage the CNS function and behavioral process [16, 17]. Recently, oxidative stress indices, known as risk factors for some diseases, appear to be prospective biomarkers for early prediction of healthy people [32]. In our previous works, we demonstrated that consumption of synbiotic, *L.plantarum*, and inulin in diabetic rats could improve antioxidant levels in the serum, hypothalamus, and amygdala; Antioxidant enzymes could be a potential target for the prevention of memory deterioration. Liu *et al.* [33] found that SOD and catalase protect cognitive functions from damages. D'souza *et al.* [19] showed that probiotic supplements are effective antioxidants and may be beneficial for combating the adverse effects of ROS via reducing inflammation and increasing antioxidant enzymes such as SOD and GPx. Huang *et al.* [34] demonstrated that *L.plantarum* K68 (10^9 CFU/ mL) intake could increase the activity of SOD, catalase, and GPx, resulting in improved hyperglycemia, IR, and hyperlipidemia in rats with insulin resistance. In contrary, Davari *et al.* [12] indicated that daily consumption of a mixture of probiotics (*L.acidophilus*, *B.lactis*, and *L.fermentum*) for 56 days could not significantly ameliorate oxidative stress markers in healthy rats. In their study, the number of probiotics (CFU/mL) for intervention was not mentioned and each rat was kept in a separate cage (one animal per cage). While in our study evry four rats were housed in a cage. Moreover, our results demonstrated that there is a positive correlation between serum and brain regions oxidative stress markers with anxiety and cognition performances (Figure.5). According to the evidence, it can be concluded that the species and strain of probiotics could beneficial effects on oxidative stress status.

In this work, we demonstrated that a significant increase in hippocampal serotonin and BDNF concentration was observed after symbiotic and *L.plantarum* intake. As well as, administration of symbiotic could increase PFC BDNF and amygdala serotonin concentrations (Figure. 2). BDNF and serotonin are extensively distributed in the CNS and affect various brain functions such as survival, maintenance, growth, differentiation of neurons, and finally, physiological behaviors [20, 21]. It has been shown that the levels of these parameters decrease in anxiety and depression-like behaviors and other disorders that lead to cognitive impairment [22]. In a study, it was shown that any change in the levels of neurotrophins in the hippocampus and amygdala of mice is strongly correlated with anxiety [35]. Likewise, our previous work showed a strong correlation between the increase of MDA and the reduction of the amygdala BDNF and serotonin levels in the diabetic rats [9]. Salim *et al.* [36] demonstrated that oxidative stress leads to a decrease in the amygdala and hippocampal BDNF concentration. Moreover, Shankaran *et al.* [37] indicated that induction of oxidative stress leads to depletion of brain serotonin level in the striatum and hippocampus which can be improved by vitamin E and vitamin C intake. Probably one of the mechanisms to improve the antioxidant capacity in the brain is via increased serotonin concentration; however, its clear mechanism is unknown [38]. Hence, boosting the antioxidant system could protect against brain damage as well as prevent behavioral disorders. Psychobiotics could prevent nerve oxidative damage via increasing antioxidative enzyme levels. Consistent with our finding, in a study conducted by Toldy *et al.* [39], nettle consumption as an antioxidant could not increase BDNF and nerve growth factor (NGF), despite reducing oxidative stress in both cerebellum and frontal lobes.

Therefore, other mechanisms may also be involved with changes in the levels of nerve parameters such as BDNF.

In another work, Burokas *et al.* [10] reported that administration of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) combination (FOS+GOS) in chronic stress could increase expression of gamma-aminobutyric acid B1 (GABA_{B1}), GABA_{B2}, BDNF and in the hippocampus of mice. In addition, after FOS+GOS intake, elevation of serotonin level in the PFC was observed. In their study, FOS+GOS consumption through regulated HPA activity (plasma and hypothalamic corticosterone), improved dysbiosis, and increased short-chain fatty acids (SCFAs) concentrations could ameliorate behavioral responses. In addition, the combination of the two prebiotics was more effective than their separate use. In our study, inulin was not probably able to significantly alter the gut microbial composition. On the other hand, it seems that the prebiotic intake may be more helpful in pathological conditions [8, 9].

Our results on behavior responses indicated that the synbiotic and *L.plantarum* consumption had significant effects on learning, memory, depression, and anxiety in the healthy rats (Figure. 3, 4). Also, a strong correlation was found between the oxidative stress markers with serotonin and BDNF concentration in different regions of the brain with behavioral tests (Figure. 5). The beneficial effects of psychobiotics on behavioral disorders have been reported in several diseases, as well [9, 40, 41]. But studies about healthy individuals or animals are confined. Allen *et al.* [14] demonstrated that administration of *B.longum* 1714 (10^9 CFU/mL) resulted in modulation of stress and amelioration of memory performance in addition to cortisol reduction in healthy volunteers. In another study Jeong *et al.* [42] indicated that, compared to the control group, elderly rats treated with *L.plantarum* C29 (2×10^9 CFU/mL) for 8 weeks had a significant increase in the expression of hippocampal BDNF and cAMP response element binding protein (CREB) genes as well as improvement in cognitive behavior via inhibiting NF- κ B signaling pathway. Our findings also indicated that there is a positive correlation between memory and learning with PFC and hippocampal BDNF (Figure.5). In addition, in this study, the improvement of anxiety like-behavior was consistent with increasing levels of serotonin in the amygdala and hippocampus (Figure.5). In another research by Takada *et al.* [43], it was demonstrated that administration of *L.casei* could regulate stress in both healthy subjects and rats. They showed that *L.casei* intake declined levels of corticotropin-releasing factor (CRF) and cortisol in the hypothalamus of rats which probably affected the vagus nerve signaling to the brain and decreased activity of the HPA axis. Furthermore, Bravo *et al.* [44] indicated that *L.rhamnosus* (10^9 CFU/mL) consumption resulted in reduced anxiety (EPM) and depression (FST) as well as decreased levels of mRNA expression of GABA_{B1b} in the hippocampus, amygdala, and locus coeruleus, compared with healthy control mice.

According to the evidence as well as our previous works [9, 14, 42, 44], several mechanisms have been proposed in relation to the effects of psychobiotics on behavioral responses through intestinal microbial changes. First, as a result of improved microbial composition, lipopolysaccharide (LPS) production decrease and immune and inflammatory responses are reduced, subsequently leading to alleviation of ROS production [43, 45]. Secondly, the regulation of HPA hyperactivity in most studies has a direct

correlation with the anxiety and depression. The most important factor involved in this pathway is cortisol, directly linked to increased neuropsychological disorders [43, 46]. Third, the effect of psychobiotics on the gut- vagus nerve- brain axis which results in improved CNS neurotrophins (like BDNF) and neurotransmitters (like serotonin) [9, 47]. Although valuable findings were obtained in the present study, there were some limitations. We could not assess changes of the microbial population which could be very helpful. Our results could be more comprehensive if the microbial composition of the rats were also examined. Finally, supplementation with other probiotics and prebiotics in different doses is proposed, as varied results might be produced.

Conclusion

In the current study, it was demonstrated that unlike inulin, the intake of *L.plantarum* and synbiotic could improve antioxidant enzyme, brain BDNF, and serotonin concentration as well as the cognitive and behavioral performance of healthy rats after 8 weeks. The effects of the supplements on three different brain regions of healthy rats were indicated. Also, it was found that most of the changes occurred in the hippocampus, compared to the amygdala and PFC, following the intervention. Our finding well demonstrated that there is a strong correlation between serum and brain parameters levels with behavioral responses. These findings provide further evidence for beneficial effects of psychobiotics and delineate the significance of a new therapeutic agent for the prevention and amelioration of behavioral disorders in healthy states. Further studies are warranted to clarify the mechanisms of psychobiotics on the gut-brain axis.

Declarations

Acknowledgment

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not Applicable.

Authors' contributions

MM, MSA, SH and GA designed the study, carried out the study, data analyses, performed the statistical analyses and prepared the first draft of the manuscript. KBV and VH conceived the study and edited the manuscript. MM commented on study design, data analyses, inference of the results, and critically edited the manuscript. All authors read and approved the final manuscript.

Conflict of interest

There is nothing to declare.

Consent for publication

All authors agreed to the submission and approved the final version of the manuscript.

Ethical approval

All experiments were approved by the TBZMED Animal Experimentation Ethics Committee in accordance with the guidelines of the Principles of Laboratory Animal Care (NIH Publication, revised 1986).

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Figures

Figure 1

Effects of *L. plantarum* and inulin treatment on oxidative stress markers of the healthy control and intervention groups of the rats (n=7 per group). SOD, GPx, TAC, and MDA concentration in the (A-D) serum, (E-H) amygdala, (I-L) PFC, and (M-P) hippocampus. HSh, healthy sham; HLI, healthy rats treated by *L. plantarum* and inulin; HI, healthy rats treated by inulin; HL, healthy rats treated by *L. plantarum*; One-way analysis of variance, followed by post hoc Tukey's test, was used. All values are expressed as means $\pm$ SEM. *P<0.05 compared with the control group.

Figure 2

Effects of *L.plantarum* and inulin treatment on serotonin and BDNF levels of the healthy control and intervention groups of the rats (n=7 per group). Serotonin and BDNF concentration in (A, B) serum, (C, D) amygdala, (E, F) hippocampus, and (G, H) PFC. HSh, healthy sham; HLI, healthy rats treated by *L.plantarum* and inulin; HI, healthy rats treated by inulin; HL, healthy rats treated by *L.plantarum*. One-way

analysis of variance, followed by post hoc Tukey's test, was used. All values are expressed as means $\pm$ SEM. * $P < 0.05$ compared with the control group.

Figure 3

Effects of *L.plantarum* and inulin treatment on anxiety and depression-like behaviors of the rats ($n=287$ per group). Bars show time (seconds) spent in the (A) open arms and (B) OAT (open arms duration/total time $\times 100$) during a 5-min elevated plus maze test (EPM). (C) The total number of closed arm entrance was measured to assess locomotor activity. (D) Duration of immobility in the FST. HSh, healthy sham; HLI, healthy rats treated by *L.plantarum* and inulin; HI, healthy rats treated by inulin; HL, healthy rats treated by *L.plantarum*. One-way analysis of variance, followed by post hoc Tukey's test, was used. All values are presented as means $\pm$ SEM. * $P < 0.05$ compared with the control group.

Figure 4

Effects of *L.plantarum* and inulin treatment on learning and memory in the Morris water maze (MWM) (n=7 per group). (A) Duration of time in which animals remained in the target quadrant (B) Time elapsed by rats to locate the platform. (C) Heat map of MWM in different treatment groups. HSh, healthy sham; HLI, healthy rats treated by *L.plantarum* and inulin; HI, healthy rats treated by inulin; HL, healthy rats

treated by *L.plantarum*. One-way analysis of variance, followed by post hoc Tukey's test, was used. All values are presented as means $\pm$ SEM. * $P<0.05$ compared with the control group.

Figure 5

Correlations of oxidative stress and neurological parameters in serum and brain regions with behavioral tests (A-J). Correlations were computed by Pearson correlation coefficient test. Data were expressed as means $\pm$ SEM. $P<0.05$ was regarded as statistically significant.