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### Assessment of oxidative stress parameters of brain-derived neurotrophic factor heterozygous mice in acute stress model

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ARTICLEINFO	ABSTRACT
<b>Article type:</b> Original article	<ul> <li>Objective(s): Exposing to stress may be associated with increased production of reactive oxygen species (ROS). Therefore, high level of oxidative stress may eventually give rise to accumulation of oxidative damage and development of numerous neurodegenerative diseases. It has been presented that brain-derived neurotrophic factor (BDNF) supports neurons against various neurodegenerative conditions. Lately, there has been growing evidence that changes in the cerebral neurotrophic support and especially in the BDNF expression and its engagement with ROS might be important in various disorders and neurodegenerative diseases. Hence, we aimed to investigate protective effects of BDNF against stress-induced oxidative damage.</li> <li>Materials and Methods: Five- to six-month-old male wild-type and BDNF knock-down mice were used in this study. Activities of catalase (CAT) and superoxide dismutase (SOD) enzymes, and the amount of malondialdehyde (MDA) were assessed in the cerebral homogenates of studied groups in response to acute restraint stress.</li> <li>Results: Exposing to acute physiological stress led to significant elevation in the markers of oxidative stress in the cerebral groups.</li> <li>Conclusion: As BDNF-deficient mice were observed to be more susceptible to stress-induced oxidative damage, it can be suggested that there is a direct interplay between oxidative stress indicators and BDNF levels in the brain.</li> </ul>
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#### Introduction

Recent studies have suggested that exposure to physiological or psychological stress is related to the formation of reactive oxygen species (ROS), which induce the accumulation of oxidative damage to biomolecules in the brain, ultimately causing many neurodegenerative diseases (1-3). The brain is particularly vulnerable to oxidative process due to its decreased level of antioxidants and increased level of ROS (1). Oxidative stress occurs when an unbalance is developed between production of ROS and defensive cellular antioxidant activity. In human disease, this balance of oxidant-antioxidant is shifted for the benefit of the reactive species, thus oxidative damage levels increase (4). This event has a substantial contribution to tissue injury, and provides appropriate circumstances for examining the effects of various cell protecting factors and antioxidants.

Brain-derived neurotrophic factor (BDNF) has a fundamental role in the survival of neuronal cells,

adjustment of brain synaptic plasticity, and neural integrity and connectivity (5-7). Having a crucial function in neuronal processes, BDNF has been intensively studied over the last decade. Changes in the levels and activities of BDNF may contribute to impaired neuronal development, neuroplasticity and synaptic connectivity, leading to a number of neurodegenerative disorders (8-11). Moreover, BDNF and its interaction with ROS may be crucial for several symptoms of neurodegenerative and neuropsychiatric abnormalities. In parallel with its roles in maintaining neuronal plasticity and integrity, several studies provide pilot data supporting a role for BDNF in stress and stress-related disorders (12-16). Furthermore, decreased expression of BDNF is implicated in the sensitivity to stress and enhanced stress responses (17, 18). Findings have shown that BDNF heterozygous mice are more vulnerable to stress than control mice, displaying behavioural desperation after mild handling stress (19). However, the possible interaction between BDNF

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and oxidative stress in response to harmful stimuli or stressful condition that induce oxidative stress has not yet been demonstrated. Therefore, a very fundamental question is still to be answered: If the BDNF expression levels and oxidative stress biomarkers are in a causative relation, does a decrease in BDNF concentration in the brain causes a change in the oxidative stress status under normal and stressful circumstances? In order to highlight this issue, in the present study, we compared the superoxide dismutase (SOD) and catalase (CAT) antioxidant enzyme activities, and malondialdehyde (MDA) levels, as a sign of lipid peroxidation in the brain tissue of BDNF heterozygous and wild-type mice in response to acute restraint stress.

#### Materials and Methods

#### Animals

All the animal protocols were approved by the Local Institutional Animal Care and Use Committee of the Faculty of Medicine, Karadeniz Technical University, Turkey. Male mice at age of 5 to 6 month old (n=8 for each experimental group) were used in this study. The transgenic mouse model was characterized by lacking one of the BDNF coding alleles and first established by Korte *et al* (20). Wild-type littermates were used as controls. The existence of the transgene was verified by polymerase chain reaction (PCR) from tail tissue (21). Both control (WT) and BDNF heterozygous (BDNF (+/-)) mice were divided into unstressed and stressed groups. Mice in the stressed groups were subjected to immobilization stress for 2 hr.

#### Acute restraint stress protocol

The animals in stress-treatment groups were individually held in well-ventilated 50-ml polypropylene centrifuge tube for 2 hr. The tube was large enough to restrain a mouse, allowing it to move its extremities and head, but not to move back and forth. Control mice were left in the home cages.

#### Corticosterone assay

For measurement of corticosterone levels, trunk blood was collected immediately after the immobilization stress test, and serum was then separated by centrifugation and stored at -80 °C. The Corticosterone ELISA kit (Cayman Chemical Company, USA) was used according to the manufacturer's instructions to measure corticosterone concentration.

# Sample collection and preparation of tissue homogenates

Mice were sacrificed by cervical dislocation and the brains were quickly removed. The brain tissues containing only cerebral cortex were collected, and then 100 mg tissue was immediately homogenized. The tissue homogenate was centrifuged at 3000 rpm for 10 min and the supernatant was utilized for different estimations. Protein amount in brain homogenates was measured according to the method of Bradford (22).

#### Malondialdehyde assay

MDA levels in brain samples were measured by the method of Uchiyama and Mihara (23). This method depends on the formation of MDA as an indicator of lipid peroxidation, which reacts with thiobarbituric acid producing thiobarbituric acid reactive substances (TBARS), measured spectrophotometrically at 532 nm.

#### Superoxide dismutase activity assay

Activity of SOD enzyme was evaluated by the method of Sun *et al* (24). The analysis of SOD was based upon the principle in which xanthine reacts with xanthine oxidase to produce superoxide radicals. The SOD activity is measured by the level of suppression of this reaction. Results were expressed as U/mg protein.

#### Catalase activity assay

Catalase enzyme activity was evaluated using a spectrophotometric test based on the yellow complex with molybdate and hydrogen peroxide, which was described in detail by Goth (25).

#### Statistical analyses

Statistical significance was determined using oneway ANOVA, following by *post hoc* Tukey test. The data are expressed as mean  $\pm$  standart error (SE). Results were acknowledged statistically significant at *P*-value < 0.05.

#### Results

Stress exposure markedly increased serum corticosterone levels compared with unstressed control groups (P < 0.001, Figure 1). BDNF (+/-)-stressed mice had higher serum corticosterone concentration (16867.29 ± 350.88 pg/ml) than WT-stressed ones (14167.46 ± 433.16 pg/ml) (P < 0.001).



**Figure 1.** Effect of acute immobilization stress on serum corticosterone concentration of control (WT) and BDNF heterozygous (BDNF (+/-)) mice. Data represent the mean  $\pm$  SE for 8 animals in each group. \* *P* <0.001 vs. WT group and # *P* <0.001 vs. WT-Stress group.



**Figure 2.** Effect of acute restraint stress on MDA levels in the cerebral cortex of control (WT) and BDNF transgenic (BDNF (+/-)) mice. Data represent the mean  $\pm$  SE for 8 animals in each group. \* *P* <0.05, \*\* *P* <0.05 vs. WT group and # *P* <0.05 vs. WT-Stress group.

Lipid peroxidation can be used as an indicator of oxidative damage in cells and tissues (26). In the present study, we examined the level of lipid peroxidation in brain extracts to evaluate differences in oxidative stress triggered by immobilization stress. Our results showed that there was no significant difference in MDA content between unstressed control and BDNF heterozygous groups  $(12.75 \pm 0.61 \,\mu\text{M/mg} \text{ tissue and } 13.86 \pm 0.98 \,\mu\text{M/mg})$ tissue, respectively) (Figure 2). On the other hand, MDA values were significantly increased in the groups exposed to acute stress (16.82  $\pm$  1.01  $\mu$ M/mg tissue for WT-Stress group and 20.88  $\pm$  1.2  $\mu$ M/mg tissue for BDNF (+/-)-Stress group) compared with the control group. Furthermore, MDA levels of BDNF (+/-)-Stress group were found to be significantly higher than WT-Stress group (P < 0.05, Figure 2).

The SOD and CAT enzymes are important components for the cellular defense system against free radicals. SOD, which catalyzes the breakdown of superoxide into hydrogen peroxide and molecular oxygen, is an important antioxidant defence in almost all living cells exposed to oxygen (1, 27). Again, CAT plays fundamental role in the cellular defence against oxidative stress by catalyzing the decomposition of hydrogen peroxide to water and oxygen (1, 28). Although there was a slight increase in SOD activity of wild-type-stressed animals, no significant change was detected in enzymatic antioxidant SOD activity among studied groups (Figure 3).

There was not a statistical significance in CAT activity between wild-type and BDNF heterozygous mice;  $16.62 \pm 0.35$  U/mg protein and  $17.46 \pm 1.03$  U/mg protein, respectively (Figure 4). Immobilization stress remarkably elevated brain levels of CAT enzyme activity with respect to the control group ( $23.72 \pm 1.16$  U/mg protein for WT-Stress group and  $20.15 \pm 0.15$  U/mg protein for BDNF (+/-)-Stress group). However, the increase in CAT activity of BDNF (+/-)-Stress group was significantly lower compared to the WT-Stress group (P < 0.05, Figure 4).



**Figure 3.** Effects of acute restraint stress on SOD activity in the brain cortexes of control (WT) and BDNF heterozygous (BDNF (+/-)) mice. The results are expressed as the mean  $\pm$  SE for 8 animals in each group.

#### Discussion

The target of this research was to scrutinize the effect of acute immobilization stress on oxidative stress-associated changes such as stress-related determinants, lipid peroxidative activity and enzymatic antioxidant systems in BDNF-deficient mice. In the brain cortex of this animal model, the expression of BDNF levels was shown to be reduced by nearly 50% (29). The results of our study indicated that there is no significant difference in oxidative stress biomarkers between BDNF heterozygous and wild-type cerebral cortexes of mice that did not undergo stress. This finding suggests the possibility that the levels of BDNF expression in heterozygotes are sufficient to maintain oxidative homeostasis under non-stress conditions. Moreover, it is also conceivable that lack of change in oxidative markers may be due to signaling by other growth factors that compensate the effects of reduced BDNF under normal physiological conditions.



**Figure 4.** Effects of acute restraint stress on CAT activity in the brain cortex of control (WT) and BDNF transgenic (BDNF (+/-)) mice. The results are expressed as the mean  $\pm$  SE for 8 animals in each group. \* *P* <0.05, \*\* *P* <0.001 vs. WT group and # *P* <0.05 vs. WT-Stress group

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The increased serum corticosterone level in stressed group is in accordance with previous studies (30-33) showing that serum corticosterone is an important indicator of stress. BDNF (+/-)-stressed mice had higher serum corticosterone concentration than WT-stressed ones, which indicated that the lack of BDNF enhanced stress response. It is reported that exposure to glucocorticoids or stress may cause oxidative injury in various tissues (34).

Restraint/immobilization stress was demonstrated to be a good model for psychological (escape reaction) and physical (muscle work) stressmeditated alterations in the balance of oxidantantioxidant in brain tissue (30, 35, 36). Under oxidative stress conditions, polyunsaturated fatty acids can undergo lipid peroxidation to form complex cytotoxic reactive aldehyde species that may lead to abnormal neuronal functions through their ability to react with biomolecules (1, 37, 38). In the present study, exposure of mice to acute restraint stress enhanced brain lipid peroxidation levels compared with the control group. Increase in lipid peroxidation was more evident in the mice with BDNF deficiency. Parallel to our findings, it has been demonstrated that oxidative stress may rise up where BDNF is found to be decreased. It has been previously shown that there is a substantial negative correlation among serum TBARS and BDNF levels in subjects with acute mania (39). Contrary to these results, a positive correlation between serum TBARS and BDNF was observed in chronically mediated patients with schizophrenia (40).

The antioxidant enzyme system acts as the first line of antioxidant defense. Combination of SOD and CAT anti-oxidative effects is supposedly sufficient to remove superoxide anions and hydrogen peroxide and protect cells against reactive hydroxyl radicals (1, 41). In our study, the activity of the key enzyme CAT was upregulated significantly in response to stress. However, the increment in antioxidative CAT enzyme activity was less pronounced in the brain of BDNF heterozygous mice with respect to the stressed wild-type animals, indicating that the ability to scavenge free radicals was diminished. In other words, these changes suggest the possibility that normal wild-type mice have a better stress tolerance than BDNF heterozygous mice. Although there was a slight increase in SOD activity of wild-type-stressed animals, we failed to find any significant alteration in enzymatic antioxidant SOD activity among studied The possible impairing effect groups. of corticosterone on brain antioxidant capacities may be causal factor in the unchanged SOD activity (30). Recently, Zhang et al reported a significant reduction in BDNF serum levels as well as in plasma antioxidant SOD enzyme activity, and a significant increase in plasma MDA levels of subjects with chronic schizophrenia. Moreover, the CAT activity has been shown to be unchanged (42). However, it is still uncertain whether peripheral BDNF levels and oxidative stress markers reflect similar changes in the central nervous system. In contrast, some other investigators did not find any difference in SOD activity between patients and controls (43); or even increased antioxidant defense in schizophrenia (44). In our study, the elevation of antioxidant CAT enzyme activity suggests that acute immobilization stress could have increased ROS production in brain. This result brings up the prospect that hydrogen peroxide level is higher in the brain of immobilization-stressed group than in the control animals.

Finally, according to our results, endogenous defense mechanisms against ROS were less effective to suppress the oxidative damage in BDNF-deficient group exposed to stress. Taken all together, our findings suggest the possibility that the neuroprotective effect of BDNF may in particular involve the suppression of oxidative impairment. While we have demonstrated that BDNF protects against oxidative damage induced by immobilization stress in mice, the exact mechanism of action of BDNF is still largely unknown. Hence, further studies are needed to assess the effects of BDNF on the expression of stress-related proteins such as p38, p53, and heat shock proteins (HSP) to support our findings.

#### Conclusion

The results of this study allow us to hypothesize that there is direct interaction between oxidative stress biomarkers and BDNF, as downregulation of BDNF increased sensitivity to oxidative damage under stressful circumstances.

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The authors declare that the research was performed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

#### References

1. Hong IS, Lee HY, Kim HP. Anti-oxidative effects of Rooibos tea (Aspalathus linearis) on immobilizationinduced oxidative stress in rat brain. PLoS One 2014; 21 9:e87061.

- 2. Kelly GS. Nutritional and botanical interventions to assist with the adaptation to stress. Altern Med Rev 1999; 4:249-265.
- 3. Liu J, Mori A. Stress, aging, and brain oxidative damage. Neurochem Res 1999; 24:1479-1497.
- 4. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a

review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol 2009; 7:65-74.

5. Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 2003; 72:609-642.

6. Numakawa T, Suzuki S, Kumamaru E, Adachi N, Richards M, Kunugi H. BDNF function and intracellular signaling in neurons. Histol Histopathol 2010; 25:237-258.

7. Vedunova MV, Mishchenko TA, Mitroshina EV, Mukhina IV. TrkB-mediated neuroprotective and antihypoxic properties of brain-derived neurotrophic factor. Oxid Med Cell Longev 2015; 2015:453901.

8. Numakawa T, Matsumoto T, Numakawa Y, Richards M, Yamawaki S, Kunugi H. Protective action of neurotrophic factors and estrogen against oxidative stress-mediated neurodegeneration. J Toxicol 2011; 2011:405194.

9. Ferrer I, Goutan E, Marín C, Rey MJ, Ribalta T. Brain-derived neurotrophic factor in Huntington disease. Brain Res 2000; 866:257-261.

10. Gines S, Seong IS, Fossale E, Ivanova E, Trettel F, Gusella JF, *et al.* Specific progressive cAMP reduction implicates energy deficit in presymptomatic Huntington's disease knock-in mice. Hum Mol Genet 2003; 12:497-508.

11. Counts SE, Mufson EJ. Noradrenaline activation of neurotrophic pathways protects against neuronal amyloid toxicity. J Neurochem 2010; 113:649-660.

12. Giese M, Unternaehrer E, Brand S, Calabrese P, Holsboer-Trachsler E, Eckert A. The interplay of stress and sleep impacts BDNF level. PLoS One 2013; 16 8(10):e76050.

13. Aydemir O, Deveci A. BDNF measurement in stress-related mood disorders: a review of clinical studies. Turk Psikiyatri Derg 2009; 20:385-391.

14. Duman RS. Pathophysiology of depression: the concept of synaptic plasticity. Eur Psychiatry 2002; 17:306S-310S.

15. Pae CU, Chiesa A, Porcelli S, Han C, Patkar AA, Lee SJ, *et al.* Influence of BDNF variants on diagnosis and response to treatment in patients with major depression, bipolar disorder and schizophrenia. Neuropsychobiology 2011; 65:1-11.

16. Haghighi M, Salehi I, Erfani P, Jahangard L, Bajoghli H, Holsboer-Trachsler E, *et al.* Additional ECT increases BDNF-levels in patients suffering from major depressive disorders compared to patients treated with citalopram only. J Psychiatr Res 2013; 47:908-915.

17. Hosang GM, Shiles C, Tansey KE, McGuffin P, Uher R. Interaction between stress and the BDNF Val66Met polymorphism in depression: a systematic review and meta-analysis. BMC Med 2014; 16 12:7.

18. Tsuru J, Tanaka Y, Ishitobi Y, Maruyama Y, Inoue A, Kawano A, *et al*. Association of BDNF Val66Met polymorphism with HPA and SAM axis reactivity to psychological and physical stress. Neuropsychiatr Dis Treat 2014; 11:2123-2133.

19. Burke TF, Advani T, Adachi M, Monteggia LM, Hensler JG. Sensitivity of hippocampal 5-HT1A receptors to mild stress in BDNF-deficient mice. Int J Neuropsychopharmacol 2013; 16:631-645. 20. Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc Natl Acad Sci U S A 1995; 92:8856-8860.

21. Abidin I, Eysel UT, Lessmann V, Mittmann T. Impaired GABAergic inhibition in the visual cortex of brain-derived neurotrophic factor heterozygous knockout mice. J Physiol 2008; 586:1885-1901.

22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-254.

23. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978; 86:271-278.

24. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem 1988; 34:497-500.

25. Goth L. A simple method for determination of serum catalase activity and revision of reference range. Clin Chim Acta 1991; 196:143-151.

26. Palmieri B, Sblendorio V. Oxidative stress tests: overview on reliability and use. Part II Eur Rev Med Pharmacol Sci 2007; 11:383-399.

27. Landis GN, Tower J. Superoxide dismutase evolution and life span regulation. Mech Ageing Dev 2005; 126:365-379.

28. Al-Abrash AS, Al-Quobaili FA, Al-Akhras GN. Catalase evaluation in different human diseases associated with oxidative stress. Saudi Med J 2000; 21:826-830.

29. Abidin I, Köhler T, Weiler E, Zoidl G, Eysel UT, Lessmann V, *et al.* Reduced presynaptic efficiency of excitatory synaptic transmission impairs LTP in the visual cortex of BDNF-heterozygous mice. Eur J Neurosci 2006; 24:3519-3531.

30. Sahin E, Gümüşlü S. Immobilization stress in rat tissues: alterations in protein oxidation, lipid peroxidation and antioxidant defense system. Comp Biochem Physiol C Toxicol Pharmacol 2007; 144:342-347.

31. Laugero KD, Moberg GP. Energetic response to repeated restraint stress in rapidly growing mice. Am J Physiol Endocrinol Metab 2000; 279:33-43.

32. Lehmann J, Russig H, Feldon J, Pryce CR. Effect of a single maternal separation at different pup ages of the corticosterone stress response in adult and aged rats. Pharmacol Biochem Behav 2002; 73:141-145.

33. Djordjevic J, Cvijic G, Davidovic V. Different activation of ACTH and corticosterone release in response to various stressors in rats. Physiol Res 2003; 52:67-72.

34. Fontella FU, Siqueira IR, Vasconcellos AP, Tabajara AS, Netto CA, Dalmaz C. Repeated restraint stress induces oxidative damage in rat hippocampus. Neurochem Res. 2005; 30:105-111.

35. Singh LK, Rang X, Alexacos N, Netaumen R. Theoharides, acute immobilization stress triggers skin mast cell degranulation via corticotropin releasing hormone neurotension and substance link to neurogenic skin disorders. Brain Behav Immunol 1993; 3:225-239.

36. Ramanova TP, Karpel GG, Brill GF, Markow KM. Mechanism of disorders of the cerebral blood supply

during stress in spontaneously hypertensive rats. Pathol Fiziol Exp Ter 1994; 3:5-8.

37. Maestre I, Jordan J, Calvo S, Reig JA, Cena V, Soria B, *et al.* Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the beta-cell line INS-1. Endocrinology 2003; 144:335-345.

38. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991; 11:81-128.

39. Kapczinski F, Frey BN, Andreazza AC, Kauer-Sant'Anna M, Cunha AB, Post RM. Increased oxidative stress as a mechanism for decreased BDNF levels in acute manic episodes. Rev Bras Psiquiatr 2008; 30:243-245.

40. Gama CS, Berk M, Andreazza AC, Kapczinski F, Belmonte-de-Abreu P. Serum levels of brain-derived neurotrophic factor and thiobarbituric acid reactive

substances in chronically medicated schizophrenic patients: a positive correlation. Rev Bras Psiquiatr 2008; 30:337-340.

41. Posmyk MM, Bailly C, Szafranska K, Janas KM, Corbineau F. Antioxidant enzymes and isoflavonoids in chilled soybean (Glycine max (L.) Merr.) seedlings. J Plant Physiol 2005; 162:403-412.

42. Zhang XY, Chen DC, Tan YL, Tan Shu-ping, Wang ZR, Yang FD, *et al*. The interplay between BDNF and oxidative stress in chronic schizophrenia. Psychoneuroendocrinology 2015; 51:201-208.

43. Yao JK, Reddy R, McElhinny LG, van Kammen DP. Reduced status of plasma total antioxidant capacity in schizophrenia. Schizophr Res 1998; 32:1-8.

44. Zhang XY, Zhou DF, Zhang PY, Wu GY, Su JM, Cao LY. A double-blind, placebo-controlled trial of extract of Ginkgo biloba added to haloperidol in treatment-resistant patients with schizophrenia. J Clin Psychiatry 2001; 62:878-883.