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The TrkB agonist 7,8-dihydroxyflavone improves sensory-motor performance and reduces lipid peroxidation in old mice

Hatice Keser[1], Şeniz Doğramacı[2], Elif Şahin[2], Neslihan Sağlam[2], Mehmet Erdem[2], Ahmet Alver[2] and Selcen Aydın-Abidin[1]

[1] *Department of Biophysics, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey*
[2] *Department of Medical Biochemistry, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey*

Abstract. 7,8-Dihydroxyflavone (7,8-DHF) is a natural flavonoid compound that act as Trk-B agonist. 7,8-DHF is also a potent antioxidant. When applied systematically, 7,8-DHF can pass through blood-brain barrier and exhibit potential therapeutic effects in several animal models of neurodegenerative disorders. This study investigates the remedial effects of 7,8-DHF on behavioral impairments and biochemical changes associated with aging with a species emphasis on cortex. For this purpose three experimental groups were formed which are young control group, old group and old-DHF groups. 5 mg/kg 7,8-DHF was administered intraperitoneally to old-DHF group for 3 weeks. We assessed the hang wire and adhesive removal performances of mice. Also, oxidative stress, neuroinflammation and synaptic protein levels in the cortex were measured. We observed that chronic administration of 7,8-DHF improved behavioral performance of old mice. Besides, 7,8-DHF reversed MDA level which was increased in old control animals. However, 3 weeks application of 7,8-DHF failed to recover the levels of neuroinflammation markers (TNF-α and IL-6) and synaptic proteins (PSD-95 and Synaptophysin) which were reduced in old group. These findings demonstrate that improvement of age-dependent behavioral impairments and MDA levels by 7,8-DHF could be attributed to its antioxidant actions.

Key words: Aging — 7,8-Dihydroxyflavone — Oxidative stress — Synaptic proteins — Mice cortex

Introduction

Aging is a physiological process and in aging brain neuroinflammation and oxidative stress increases, synaptic structures and overall functions are disrupted (Bao et al. 2014). Brain aging is shown to be a natural predisposition for some neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Keller 2006; Lin and Beal 2006). Although the exact mechanism of aging is not known, one theory is related to oxidative stress and neuroinflammation. It has been proposed that neuroinflammation and oxidative stress is a major cause of aging process (Harper et al. 2004). Due to its high content of lipids and less antioxidant defense mechanisms, the brain is more vulnerable to oxidative stress (Floyd 1999). Oxidative stress products such as superoxide and free radicals attack cellular components and damage functioning of cells (Pak et al. 2003). In addition, recent studies have shown that chronic inflammation is an important risk factor for aging and related diseases (Chung et al. 2006). Proinflammatory agents such as interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) have been associated with cellular damage and neuronal loss (Heneka et al. 2015).

Brain derived neurotrophic factor (BDNF) is a small dimeric and a basic protein, which has essential regulatory roles in the central nervous system (Mattson et al. 2004). BDNF plays a crucial role in neuronal development, survival, maintenance and differentiation of neurons (McAllister et al. 1999). BDNF is important also in learning and memory and BDNF levels has been shown to be crucial for the synaptic function in hippocampus (Tyler et al. 2002) and various cortical regions (Abidin et al. 2006, 2019). It has been known that BDNF levels alter with increasing age (Webster et al. 2006). BDNF expression decreased in normal aging and also in Parkinson's disease, Alzheimer's disease and depression

Correspondence to: Selcen Aydın-Abidin, Department of Biophysics, Faculty of Medicine, Karadeniz Technical University, 61080 Trabzon, Turkey
E-mail: selcenabidin@yahoo.com

(Holsinger et al. 2000; Yasutake et al. 2006; Komulainen et al. 2008). Besides the major roles as neuromodulator, BDNF has been shown to have protective effects against oxidative stress (Valvassori et al. 2015; Abidin et al. 2018).

7-8, dihydroxyflavone (7,8-DHF) which is a potent agonist of Trk-B and mimics the effects of BDNF, has been identified in 2010 and found to cross blood brain barrier when injected systematically (Jang et al. 2010). Since its discovery, it has been demonstrated that 7,8-DHF has remedial effects on symptoms of neurological diseases and disorders in animal models including Parkinson's disease (Sconce et al. 2015), Alzheimer's disease (Zhang et al. 2014), amyotrophic lateral sclerosis (Korkmaz et al. 2014), Huntington's disease (Jiang et al. 2013), stroke (Wang et al. 2014), depression and Rett syndrome (Liu et al. 2010), and traumatic brain injury (Agrawal et al. 2015). Andero and colleques showed that 7,8-DHF application enhanced acquisition and extinction of fear in mice (Andero et al. 2011). It was observed that 7,8-DHF improved stress and aging associated cognitive impairment and plasticity in hippocampus (Andero et al. 2012; Zeng et al. 2012). These studies focused on hippocampus and the effect of 7,8-DHF on aging cortex is yet to be defined. Hence, in the present study, we investigated the potential effects of 7,8-DHF on age-related changes in cortex. Also, in the search of a mechanistic correlate, we measured the neuroinflammation markers, synaptic protein levels and oxidative stress in young, old and DHF-treated old mice.

Materials and Methods

Animals

This study was approved by Karadeniz Technical University Animal Care and Ethics Committee. Mice used in the study were provided by Karadeniz Technical University Surgical Application and Research Center and all experimental procedures were performed in this center. A total of 27 male C57BL/6 strain mice were divided into three groups as young control (5 months old, $n = 10$), old control (18 months old, $n = 10$) and old-DHF (18 months old, $n = 7$). 7,8-DHF (Sigma-Aldrich) was administered intraperitoneally to old-DHF group at a dose of 5 mg/kg in a vehicle of 17% dimethylsulfoxide in phosphate-buffered saline (DMSO) (Andero et al. 2011) for 3 weeks. 17% DMSO alone was injected to young and old control groups for the same time. At the end of administration two different behavioral tests were performed to test cortical sensory-motor functions.

Behavioral tests

Behavioral tests were done at 10:00–12:00 o'clock. The experiments were recorded by using a video camera connected to a PC and analyzed offline. Hang wire and adhesive removal tests were conducted as these tests were related to somatosensory performance and closely related to the cortical function (Abe et al. 2009; Bouet et al. 2009).

Hang wire

Hang wire test was used to evaluate grip strength, balance and endurance in mice after chronic administration of 7,8-DHF to mice. The mouse was placed on a stretched wire with a 0.5 cm thick. The mouse had to carry its body weight with its forelimbs. A pillow was placed under 35 cm of the mice to prevent dangerous fall of the mice. Mice used their forelimbs to hang on the wire. The time until the mouse fell down was recorded. If the mouse didn't fall down in 120 s, it was taken by the experimenter. The protocol for each mice consisted of two repeats both of which also consisted of 2 trials (in total 4 trials). There were 1 h intervals between two repeats and 2–3 min delay between two trials. The averages of 4 trials were calculated for each animal and used for statistical analysis (Li et al. 2004; Abe et al. 2009). In hang wire test, holding time of the mice to wire was measured in seconds.

Adhesive removal

Adhesive removal test is another behavioral test that is used to assess sensorimotor deficits in mice. The healthy mouse feels the presence of a foreign material in its pow and then tries to get rid of it as earliest times. The delays in the first touching to the object and removal of object reflect sensorimotor deficits. A 3×3 mm square, red adhesive tape strips were stuck to the front paws of the mice. The mouse tended to feel this foreign object and get rid of it. The time to touch one of the tapes with nose or mouth and the time to remove each tape were used for statistical comparisons. The tests were recorded with a maximum test time of 3 min (Bouet et al. 2009; Fleming et al. 2013). All scoring and analysis were confirmed by offline evaluation of video recordings. The test for each mouse was repeated for three times and there were 2–3 min intervals between repeats. In adhesive removal test too, the performances were given in seconds.

One day after the last behavioral test, the mice were sacrificed and the brains were rapidly removed. The cortical tissues were dissected and stored at –80°C until the biochemical analysis.

Enzyme-linked immunosorbent assay (ELISA)

The cortical concentrations of Synaptophysin protein (SYP) and postsynaptic scaffolding protein postsynaptic density 95 kD (PSD-95) and the levels of neuroinflammation markers IL-6 and TNF-α were determined by ELISA. Left cortex of each mouse was processed for ELISA. Lysis buffer (1 mg/

tissue weight) contained: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 7.7 mM Na_2HPO_4, 1% Triton X-100, 5 mg/ml aprotinin, 5 mg/ml leupeptin m, pH 7.4. Then the cortical tissue was homogenized by an ultrasonic cell disrupter (Sonics vibracell, Newtown, CT, USA). For 20 min, the suspension was centrifuged at 20,000 × *g*. The supernatant was used for ELISA tests. TNF-α, IL-6, SYP and PSD-95 levels were determined by using enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, Catalog No: E-EL-M0049, E-EL-M0044, E-EL-M1105 and USCN, Catalog No: SEG168Mu, respectively) according to the manufacturer's guide. Samples and standards absorbance were measured at 450 nm by using tunable microplate reader (VERSA max, Molecular Devices, Sunnyvale, CA, USA). Results were expressed as ng/ml.

Tissue malondialdehyde (MDA) measurement

A 50 to 60 mg piece of right cortex was homogenized in 2 ml ice-cold buffer 1.15% KCl solution containing 0.5 ml/l Triton X-100. MDA concentrations were measured from homogenates according to method by Mihara and Uchiyama (1978). Tetramethoxypropane was used as standard solution. MDA levels were expressed as nmol/ml *per* gram of wet tissue.

Measurements of tissue antioxidant enzyme activities

Superoxide dismutase (SOD) and catalase (CAT) activities were measured from tissue samples of right cortex. Tissues were homogenized in 2 ml ice-cold Tris-HCl buffer (50 mmol/l, pH = 7.4). SOD activities were measured by the method of Sun et al. (1988). This method essentially is based on reduction of nitroblue tetrazolium by xanthine/xanthine oxidase system. SOD activity was given in U/mg protein. CAT activities were determined by the method of Goth (1991). This method utilizes H_2O_2 and ammonium molybdate stable complex. The yellow complex was measured at 405 nm. CAT activities were expressed as kU/mg protein. For determination of protein concentrations, the tissue lysates were used. Bicinchoninic acid protein assay kit was used (Merck Millipore, Darmstadt, Germany) and results were expressed as mg/ml.

Statistical analysis

Data were presented as means ± SEM. When data were normally distributed, significant differences between groups were analyzed by one-way ANOVA using GraphPad Prism software. For multiple comparisons Tukey's *post hoc* test was used. If data did not pass normality tests then non parametric Kruskal Wallis Test and Dunn's multiple comparison tests were used for comparison of group differences. $p < 0.05$ was accepted as significant.

Results

Effects of DHF on behavioral tests

Analyzing the results of hang wire test, Kruskal-Wallis comparison revealed significant differences between groups. According to Dunn's multiple comparison *post hoc* test, the young mice hold the wire (84.65 ± 9.47 s) significantly longer than old mice (10.22 ± 2.02 s) ($p < 0.0001$). 7,8-DHF injected mice in old-DHF group (43.40 ± 7.3 s) hold the wire significantly longer than old control mice ($p < 0.05$) (Fig, 1A).

In adhesive removal test, the contact time to the tape and first and second removal times were measured in seconds. One way ANOVA test of contact time revealed no significant differences between groups. Contact times to the tape were 6.1 ± 0.85 s, 4.65 ± 0.46 s, 4.57 ± 0.75 s in young, old and old-DHF group, respectively. However, one way ANOVA test revealed significant differences in first and second removal

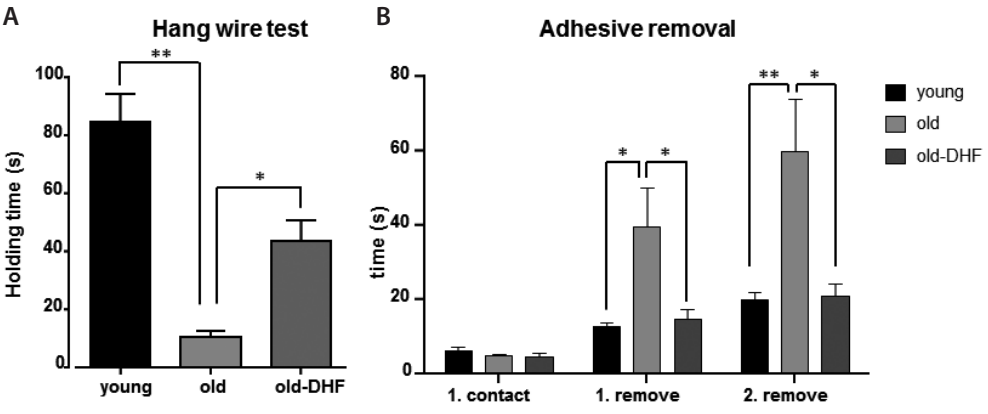


Figure 1. Effects of 7,8-DHF on behavioral experiments. **A.** Performance of old group was lower in hang wire test. Administration of 7,8-DHF improved the performance. **B**. Adhesive removal test. First removal and second removal of tape times were significantly higher in old group than young group. 3 weeks of application of 7,8-DHF considerably shortened the removal times (* $p < 0.05$, ** $p < 0.005$; $n = 10$ for young and old-DHF group, $n = 7$ for old group).

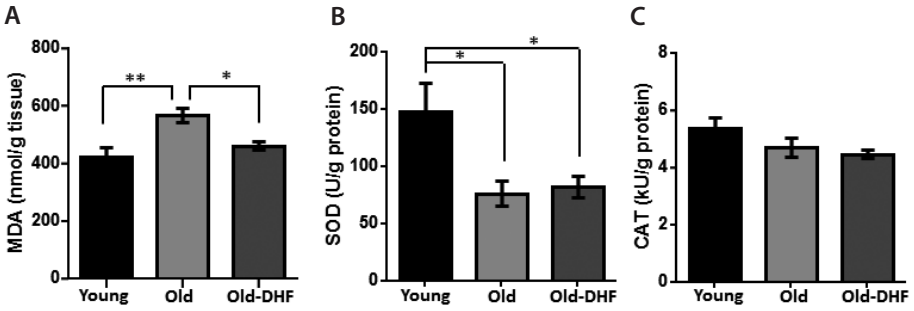
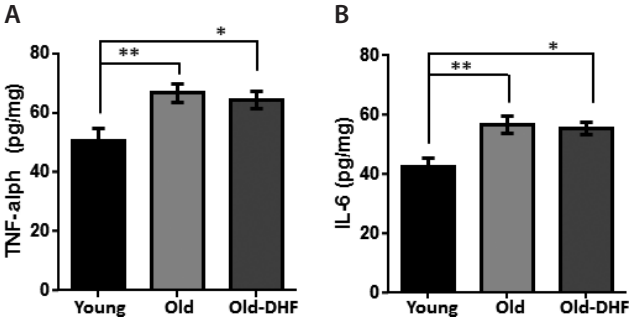


Figure 2. Effects of 7,8-DHF on oxidative stress. **A.** MDA level was found to be higher in old mice than young animals whereas chronic administration of 7,8-DHF reversed MDA level to young mice's level. **B.** SOD activity is less in old mice than young mice. Administration of DHF did not have any effect on SOD activity. **C.** There were no significant differences in CAT activities (* $p < 0.05$, ** $p < 0.005$; $n = 10$ for young and old-DHF group, $n = 7$ for old group).

of tape times between groups (F(2.48) = 6.601, $p = 0.0029$). Tukey's *post hoc* tests showed significant differences that first removal ($p < 0.05$) and second removal ($p < 0.005$) of tape times was significantly higher in old group than young group. In young group, first and second removal times were 12.80 ± 0.97 s and 19.75 ± 2.1 s, respectively. In old group, removal times were 39.67 ± 10.53 s and 59.78 ± 14.35 s. Administration of 7,8-DHF significantly improved the removal times (14.46 ± 2.79 s and 20.92 ± 3.22 s) ($p < 0.05$). In old-DHF group the removal times decreased compared to old group (Fig. 1B).

Effects of DHF on oxidative stress in aged mice

To investigate the effects of 7,8-DHF on oxidative stress in aged mice we analyzed MDA level, SOD and CAT activity in cortex of mice brain. One way ANOVA revealed a significant differences in MDA levels between groups (F(2.25) = 9.215; $p = 0.001$). As shown in Figure 2A, MDA level was found to be higher in old mice (570.2 ± 24.54 nmol/g) than young animals (423.9 ± 31.08 nmol/g) ($p = 0.0009$, according to Tukey's *post hoc* test); whereas chronic administration of 7,8-DHF reversed MDA level (461.7 ± 15.62 nmol/g) to young mice's level ($p = 0.0199$). SOD activity of different groups was shown in Figure 2B. There were significant differences in SOD activity between groups (F(2.24) = 5.232; $p = 0.013$). Aging decreased SOD activity in mice. SOD activity is lesser in old (76.30 ± 10.56 U/g) than young mice (147.8 ± 24.81 U/g) ($p = 0.0175$, Tukey's *post hoc* test). Administration of 7,8-DHF did not have any effect on SOD activity (82.04 ± 9.59 U/g). CAT activities were 5.37 ± 0.38 kU/g, 4.7 ± 0.32 kU/g and 4.46 ± 0.13 kU/g in young, old and old-DHF groups, respectively. There were no significant differences in CAT activities (F(2.23) = 2.107; $p = 0.1444$) (Fig. 2C).



Figures 3. Effects of 7,8-DHF on neuroinflammation. TNF-α (**A**) and IL-6β (**B**) levels in the cortex of the old mice were significantly higher than those of the young mice but 7,8-DHF did not suppress IL-6β and TNF-α levels in the old mice (* $p < 0.05$, ** $p < 0.005$; $n = 10$ for young and old-DHF group, $n = 7$ for old group).

Effects of DHF on neuroinflammation in aged mice

To investigate the effects of 7,8-DHF on inflammatory responses, levels of interleukins (IL-6β) and tumor necrosis factors (TNF-α) were determined. IL-6 levels were 42.30 ± 3.26 pg/mg, 56.69 ± 3.08 pg/mg and 55.51 ± 2.09 pg/mg in young, old and old-DHF groups, respectively. According to one way ANOVA tests, IL-6β (F(2.24) = 7.354; $p = 0.0032$) and TNF-α levels (F(2.24) = 6.62; $p = 0.0051$) are significantly different between groups. Figure 3 shows that IL-6β ($p = 0.0047$) levels in the cortex of the old mice were significantly higher than those of the young mice but 7,8-DHF did not suppress IL-6β. Moreover TNF-α ($p = 0.0061$) levels were also increased in old group (66.82 ± 3.01 pg/mg) significantly compared to young group (50.83 ± 4.02 pg/mg). In old-DHF group, TNF-α level (64.54 ± 2.9 pg/mg) was not different compared to old group.

Effects of DHF on synaptic proteins in aged mice

The levels of synaptic proteins were decreased in old mice. One way ANOVA showed there is a significant differences in PSD-95 levels between groups (F(82.24) = 4.363; $p = 0.0242$). Tukey's test showed that PSD-95 levels were reduced in old mice (4.2 ± 0.27 ng/mg) compared to young ones (5.61

± 0.47 ng/mg) (p = 0.0185). SYP level in old-DHF group was 4.99 ± 0.11 ng/mg which is higher than in old group but it is not significant (F(2.24) = 1.151; p = 0.333). Besides, we did not find any significant differences in SYP levels between groups. SYP levels were 0.72 ± 0.07 ng/mg, 0.60 ± 0.046 ng/mg and 0.66 ± 0.04 ng/mg in young, old and old-DHF groups, respectively. Although 7,8-DHF application increased the levels of synaptic protein, the differences were statistically insignificant (Fig. 4).

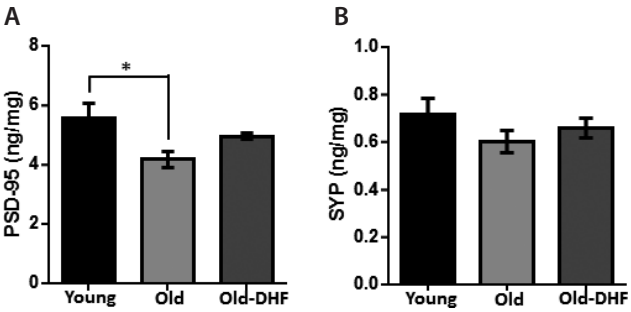


Figure 4. Effects of 7,8-DHF on Synaptic Proteins. **A.** PSD-95 levels were augmented in old group. However, administration of 7,8-DHF did not rescue PSD-95 level. **B.** There were no significant differences in synaptophysin level between groups (* p < 0.05, n = 10 for young and old-DHF group, n = 7 for old group). SYP, Synaptophysin; PSD-95, postsynaptic scaffolding protein postsynaptic density 95 kD.

Discussion

Aging is a complex process in which both structural and functional changes occur in the brain (Hedden and Gabrieli 2004). In fact, aging is considered to be an important risk factor for various pathological conditions, including impaired behavioral performance, memory and cognition (Petersen 2004). Many studies suggest that the development of these pathologies may be affected by changes in expression of inflammatory markers (Rancán et al. 2017), increase in oxidative stress (Dröge and Schipper 2007) and reduction in synaptic plasticity (Bishop et al. 2010).

In this study we evaluated the beneficial effects of 7,8-DHF on age-induced changes in the cortex. We observed that aging worsened the performance of mice in behavioral tests which are basically related to cortical function (Seidler et al. 2010; Farias et al. 2019). During aging process, the expression of synaptic proteins decreases. The number of small synapse expressing PSD-95 (Ma et al. 2014; Xu et al. 2018) and the expression of SYP (Rancán et al. 2017; Ma et al. 2019) were reduced. In another study it has been shown that there was a decline in synaptophysin expression in old human subjects (Hatanpää et al. 1999). SYP and PSD-95 are markers of pre- and postsynapse, respectively. SYP, presynaptic vesicle protein, is a synaptic density marker and influences the release of neurotransmitters (Donovan et al. 2002) that induces the regeneration of additional neurons. PSD95 is the post synaptic marker which interacts with NMDA receptors to participate in the information storage process. PSD95 expression has an important role in regulating the potency of synaptic activity (Gardoni 2008). In agreement with these mentioned studies we also observed a decline in PSD-95 and SYP in elderly mice.

Another cause of synapse loss in old age may be neuroinflammation. Accordingly in this study, a significant increase in the amount of proinflammatory cytokines, TNF-α and IL-6 was observed in the cortex of elderly mice. High amount of neuroinflammation could have hazardous effects on synaptic proteins and other proteins. The increased neuroinflammation in old animals has also been shown in other studies (Buchanan et al. 2008; Rancán et al. 2017; Zhao et al. 2018). In general, in the brain, neuronal inflammation and oxidative stress are related to each other (Durany et al. 1999; Floyd and Hensley 2002).

In this study, we have seen that there was an increment in lipid peroxidation in elderly mice. In old group of mice, MDA levels were significantly higher than younger ones. These findings of old mice were in line with previous studies (Montine et al. 2002; Petursdottir et al. 2007; Butterfield and Mattson 2020). Previously, the relation between cortical function and lipid peroxidation were reported in several studies. Elevated cortical lipid peroxidation levels were in correlation with evoked responses (Yaras et al. 2003; Yargıçoğlu et al. 2004). In line with these findings, in our study we observed a close relation between behavioral performances related to cortical functions and lipid peroxidation. Both are improved by 7,8-DHF application.

7,8-DHF is a small flavonoid that binds to Trk-B receptor and mimics BDNF. 7,8-DHF can efficiently pass through blood-brain barrier and enters the brain (Jang et al. 2010; Choi et al. 2010). This property of 7,8-DHF makes this molecule a therapeutic agent that can be used in place of BDNF. Here in our study, we have shown that chronic administration of 7,8-DHF to 18 months old mice reversed the impairment of behavioral performances in given tasks. In a previous study, chronic application of 7,8-DHF improved spatial learning and memory in old rats (Zeng et al. 2012). Similar to our findings, 7,8-DHF improved the behavioral outcomes in various animal models. It has been reported that 7,8-DHF rescued hippocampus-dependent memory deficits in Alzheimer's disease mice model (Devi and Ohno 2012). 7,8-DHF has been shown to improve motor function and prolongs survival in Huntington's disease (Jiang et al. 2013). In fact, 7,8-DHF has been shown to have a therapeutic effect when administered chronically in animal models such as Parkinson (Nie et al. 2019), Alzheimer's (Zhang et

al. 2014), and depression models (Liu et al. 2013). These remedial action of 7,8-DHF in behavior performance might be attributed to its powerful antioxidant activity as evidenced by a significant decrease in MDA level in cortical areas in old mice. We suggest that 7,8-DHF improved the behavioral performances by its antioxidant action in our study. Although the antioxidant enzyme activities, SOD and CAT, were not affected by drug administration, the lipid peroxidation was reduced. In a recent study made with neuronal cells, 7,8-DHF protected high glucose-damaged cell against oxidative stress (Cho et al. 2019). Powerful antioxidant action of 7,8-DHF has been demonstrated in several studies against in scopolamine-induced oxidative stress (Chen et al. 2014), glutamate-induced toxicity (Chen et al. 2011), cerebral ischemia injury (Wang et al. 2014). All these studies are in line with our findings. In the present study CAT is not affected by age or 7,8-DHF application. The literature about the relation between aging and CAT is not in agreement. In the literature it has been reported that catalase activity is reduced by aged mice brain (Ahn et al. 2016). Another study suggested that catalase activity is increased in the cortex by age (Falone et al. 2012). However, there are also reports showing that catalase activity is not altered in various brain regions of the mice by age (Sohal et al. 1994; Hussain et al. 1995).

The anti-inflammatory action of 7,8-DHF has been shown in cells lines (Park et al. 2012, 2014; Choi et al. 2017). However, in this study we could not observe anti-inflammatory action of 7,8-DHF. It did not affect the levels of TNF-α and IL-6 markers in the cortical areas of old mice.

Contrary to studies showing that 7,8-DHF increases synaptic proteins in elderly rats (Zeng et al. 2012; Zhang et al. 2014), we could not observe a healing effect of 7,8-DHF on synaptic proteins in this study. It has been previously demonstrated that the decrease in activity of the BDNF-TrkB signaling pathway in elderly rats is associated with synaptic dysfunction and loss of synapse (Zeng et al. 2011). Thus, chronic administration of 7,8-DHF would be expected to improve synaptic proteins and increase the expressions. There are certain differences between the studies above and our experiments. Besides the differences in animal species, the application of the 7,8-DHF in our study is shorter. In this study, we applied 7,8-DHF for 3 weeks, which significantly less than in other studies in which the drug was administered for 4 months. From this point, we can argue that the duration of application is critical and 3 weeks of application were not sufficient to reverse the changes in inflammatory pathways and synaptic protein renewal.

Conclusion

In conclusion, these findings reflect that chronic application of small molecule 7,8-DHF improved age-related impairments of sensorimotor performances. In correlation, 7,8-DHF also reduced the MDA levels which may be attributed to its antioxidant action.

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