

The effect of maternal forced exercise on offspring pain perception, motor activity and anxiety disorder: the role of 5-HT₂ and D₂ receptors and *CREB* gene expression

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The effect of maternal forced exercise on central disorders in offsprings has been shown but the mechanism is still unclear. In this study, the role of 5-HT₂ and D₂ receptors in neuroprotective effects of maternal forced exercise on offspring neurodevelopment and neurobehavioral symptoms is evaluated. Sixty pregnant rats were trained by forced exercise and some behavioral and molecular aspects in their offspring were evaluated in presence of 5-HT₂ and D₂ receptors agonists and antagonists. The results showed that maternal forced exercise causes increase of pain tolerability and increase latency of pain perception in offspring in hot plate test, writhing test and tail flick test. Also maternal forced exercise causes decrease of depression and anxiety like behavior in offsprings. On the other hand, treatment of mothers by forced ex-


ercise in combination with 5-HT₂ and D₂ receptor antagonists inhibited the protective effects of forced exercise and cause disturbance in pain perception and tolerability and increase depression and anxiety in offsprings. Also expression of cyclic AMP response element binding protein (CREB) was changed in all experimental groups. In conclusion, our data suggested that maternal forced exercise causes neurobehavioral protective effect on offsprings and this effect might probably be mediated by 5-HT₂ and D₂ receptors and activation of *CREB* gene expression.

Keywords: Maternal forced exercise, Pain perception, Motor activity, Anxiety, Depression

INTRODUCTION

Several studies demonstrated that exercise lowers the stress and anxiety and increases endorphin secretion in brain (Salmon, 2001). Physical activity improves symptoms of anxiety in healthy population and patients; chronic forced exercise shows anxiolytic-like effects in some experiments (Lawlor and Hopker, 2001). It has also been shown that exercise can counteract depression and can increase the cognitive activity (Kim and Seo, 2013; Kramer et al., 2006; Tomporowski, 2003). Chronic exercise in mice resulted in antidepressant-like behavioral changes, that may involve a brain-derived neurotrophic factor (BDNF) related mechanism similar to what is hypothesized for antidepressant drug treatment (Cotman and Berchtold, 2002; Park et al., 2013). Exercise in-

creases synthesis and release of dopamine, stimulates neuroplasticity and promotes feelings of well-being (Cotman and Berchtold, 2002; Motaghinejad et al., 2014b; Ojoawo et al., 2017). Many previous studies have shown that maternal exercise enhances neurogenesis in rat's pups but the exact mechanism of this effect remains unclear (Lee et al., 2006; Kim et al., 2007; Kim, 2013). Maternal treadmill running during pregnancy has beneficial effects on short-term memory, hippocampal cell survival and the expression of BDNF mRNAs in rat pups (Aksu et al., 2012; Kim et al., 2017; Uysal et al., 2011). On the other hand, many neurotransmitters and neuromodulators can have effect on neurogenesis in fetus during development (Salmon, 2001). Some other studies have shown that dopamine and serotonin increase the neurogenesis and activate the neurogenesis pathways via activation of

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D₂ and 5-HT₂ receptors respectively (Benninghoff et al., 2012; Gaspar et al., 2003; Whitaker-Azmitia, 2001). Moreover, research studies have shown that cyclic AMP response element binding protein (CREB) acts as major transcription factor in brain neurodevelopment and neurogenesis (Cho et al., 2013; Dworkin and Mantamadiotis, 2010; Gass and Riva, 2007; Merz et al., 2011). Therefore, the aim of the present study is the evaluation of the influence of two neurotransmitters, dopamine and serotonin, and their receptors, D₂ and 5-HT₂, on maternal forced exercise protective effect on offspring neurodevelopment and neurobehavioral signs; such as pain perception, anxiety, depression and also the involvement of CREB in this type of development was evaluated.

MATERIALS AND METHODS

Animal

Sixty-eight pregnant Wistar rats, weighting 250 g, were used for all the experiments. They were divided randomly into 10 groups as mentioned bellow. After delivery, their offspring's were kept in the light controlled room under a 12-hr light and dark cycle with temperature of 22°C ± 2°C. Food and water were available ad libitum. Before the tests starts, the animals were allowed to adapt themselves to the laboratory for at least 2 hr and they were used only once. All experimental procedures followed the Guidelines on Ethical Standards for investigation of experimental pain in animals (Motaghinejad et al., 2014b; Motaghinejad et al., 2015a; Noori et al., 2014; Shojaii et al., 2015).

Drug

Haloperidol, trazodone, bromocriptine, and ketanserin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Experimental design of pregnant rats

All pregnant animals were divided randomly into 10 groups (6 rats in each group).

- As the negative control, group 1 received normal saline (0.2 mL/rat, intraperitoneally, once daily) during their pregnancy.
- Group 2 were trained with forced exercise only during their pregnancy.
- Group 3 were treated with haloperidol (5 mg/kg, intraperitoneally, once daily) in combination with forced exercise during their pregnancy.
- Group 4 were treated with bromocriptine (10 mg/kg, intraperitoneally, once daily) in combination with forced exercise during their pregnancy.

- Group 5 were treated with O-4310 (10 mg/kg, intraperitoneally, once daily) in combination with forced exercise during pregnancy
- Group 6 were treated with trazodone (5 mg/kg, intraperitoneally, once daily) in combination with forced exercise during their pregnancy.
- Group 7 were treated with haloperidol (5 mg/kg, intraperitoneally, once daily) during their pregnancy.
- Group 8 were treated with bromocriptine (10 mg/kg, intraperitoneally, once daily) during their pregnancy.
- Group 9 were treated with trazodone (5 mg/kg, intraperitoneally, once daily) during their pregnancy.
- Group 10 were treated with O-4310 (10 mg/kg, intraperitoneally, once daily) during their pregnancy.

Treadmill forced exercise protocol

Rats were allowed to run on a motor-driven leveled treadmill (Model T408E, Diagnostic & Research Instruments Co., Taoyuan, Taiwan). The animals of groups 2, 3, and 4 were trained with treadmill for 30 min/day, 5 days per week. The training speed was 10 m per minute (for the first week) and increased to 20 and 30 m per minute in the second and the third week respectively. The slope and the intensity of the exercise were settled as 0° at the first 10 min, 5° for the second 10 min and 15° for the last 25 min (Motaghinejad et al., 2014b; Motaghinejad et al., 2015b).

Behavioral and molecular assays in offspring

After the treatment of pregnant rats with the mentioned drugs and the forced exercise, 10 adolescent rats were randomly selected from each group (one offspring rat from each treated mother) and some behavioral and molecular assays were done on offsprings.

Writhing test

This test is based on the induction of pain by intraperitoneal injection of 10 mL/kg acetic acid (0.8%). The measurement of severity of the pain was done by counting the number of abdominal contractions known as writhing. The total number of writhings recorded by camera during 30 min after injection of acetic acid and was counted afterwards. In addition, the onset of the first writhing was recorded as latency time (Motaghinejad et al., 2014a; Shojaii et al., 2015).

Tail flick test

This test was done in all animals before the treatments. In this test, radiant heat (Tail-flick apparatus model P-131, Pouyaye Ar-

maghan Co., Tehran, Iran) was applied for assessment of nociception and acute responses in rats. Five millimeters of the tail was submitted to noxious heat ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$). To avoid damaging the tail, if a response did not occur, trial was automatically terminated at 12 sec (cutoff time) (Motaghinejad et al., 2014a; Shojaii et al., 2015).

Hot plate test

In this test, analgesic activity was measured with a thermostatically heated surface (Hot plate apparatus model P-162, Pouyaye Armaghan Co.) which is maintained at the temperature of $55^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The time of reaction was the time that the animal was left on the hot plate until the time the animal licks its hands or jumps up. The onset of the first licking reaction and the duration of the tolerance on the hot plate were recorded as the latency time (Motaghinejad et al., 2014a; Shojaii et al., 2015).

Open field test

Open field test (OFT) as a standard test was used for the assessment of anxiety and locomotor activity in rodents. To perform this test, an apparatus (Open field apparatus model P-222, Pouyaye Armaghan Co.) which its bottom was divided into 16 equally spaced squares bordered by opaque high walls of 65.90 cm was used. All parts of bottom were dyed black except for the 6 mm broad white lines which divided the ground into 16 squares. During the experiment the whole room except for the open field was kept dark, and the apparatus was illuminated with a 100-W bulb which was focused on the field at the height of about 110 cm from the ground. For the assessment of anxiety and locomotor activity, each animal was positioned in the center of the test apparatus for a maximum period of 5 min to monitor the following behaviors:

- Ambulation distance: the distance in which the rat crosses the grid lines
- Center square entries: the number of times that the rat crosses one of the central red lines with all its four paws and enters into the central square.
- Center square duration: the time that the rat spends in the central square.
- Rearing: the number of times in which the rat stand on its hind legs in the maze (Motaghinejad et al., 2015b).

Elevated plus maze

Elevated plus maze (EPM) is another test which is used for the anxiety of the experimental animals. EPM equipment (Elevated plus maze apparatus model P-141, Pouyaye Armaghan Co.) in-

cludes two opposite arms (60 cm \times 20 cm), which are joined with a central square (10 cm \times 10 cm), forming a plus sign. Two arms are kept open, while the other arms are enclosed with 40 cm elevated wall. All parts of apparatus are being set in 50-cm height above the ground. All subjects were situated individually in the center of the maze in front of one of the enclosed arms. The time that the animal spent on the open arms were recorded during 5 min for each rat. The time rats spent in open arm was considered as nondepressive behavior (Motaghinejad et al., 2015c).

Quantitative real-time reverse transcription-polymerase chain reaction studies

After behavioral assay, hippocampus was isolated and total RNA was extracted by using Trizole reagent (Biobasic, Canada) and then treated with DNase I, RNase-Free (Thermo Scientific, Fermentas, Waltham, MA, USA) according to the manufacturer's recommendations. Briefly, in the presence of 0.5- μL oligod T primer and Random 6mers, and reverse transcription at 37°C , 15 min in 2 μL of 5x PrimeScript buffer (PrimeScriptTM RT reagent kit, Takara, Tokyo, Japan), the total RNA (0.5 μg) were denatured. Inactivation of the enzyme (85°C , 5 sec) was followed by glyceraldehyde-3-phosphatedehydrogenase (GAPDH) polymerase chain reaction to assess the quality of the template DNA.

The reverse transcription-polymerase chain reaction was carried out with SYBR Premix Ex TaqTM II (TliR-NaseH Plus), Bulk (Takara) kit. The setup of cycling parameters were 95°C for 30 sec, 42 cycles at 95°C for 10 sec and 60°C for 32 sec (Dorak, 2007).

The primers for rats GAPDH and CREB (Cinna Gen, Iran):

- GAPDH: Sense: AGACAGCCGCATCTTCTTGT;
Antisense: CCGTTCACACCGACCTTCA
- CREB: sense: CAGACAACCAGCAGAGTGGA;
Antisense: CTGGACTGTCTGCCATTG

Samples were analyzed on a real-time qPCR cyclor (CFX96, Biorad, Hercules, CA, USA) and the relative mRNA expression of the CREB was normalized to GAPDH expression using the simplified comparative threshold cycle $2^{-\Delta\Delta\text{Ct}}$ method (Dorak, 2007).

Hematoxylin and eosin staining

For determination of the changes in hippocampal cell density, its isolated tissues were fixed in formalin solution for 48 hr after being washed in physiologic serum. They were dehydrated through graded concentrations of ethanol, embedded in paraffin wax, sectioned in 5- μm thickness, and stained with Mayer's hematoxylin and eosin (H&E) (Kumar et al., 2014).

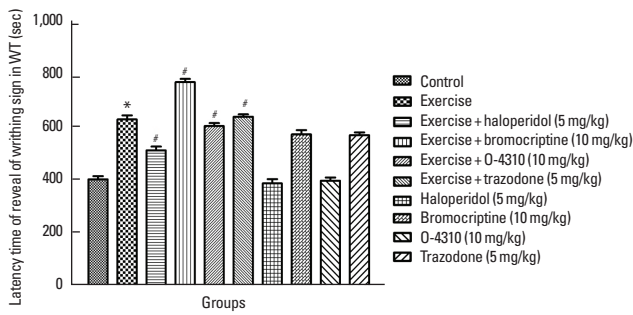


Fig. 1. Latency time for expression of abdominal contraction in writhing test (WT) in the control group and which their mother treated by exercise or its combination with haloperidol, bromocriptine, trazodone or O-4310 and groups under treatment by haloperidol, bromocriptine, trazodone or O-4310 alone. Values are presented as mean \pm standard error of the mean ($n=8$). * $P<0.05$, significant difference from control group. # $P<0.05$, Significant difference from group which their mother were treated by exercise.

Neuropathological examination

Pathological damage of hippocampal cells in dentate gyrus (DG) region was examined with standard H&E histological staining of brain paraffinized sections (5 μ m). The morphological changes and the granular cell density were observed in the DG sector by counting the number of surviving neurons per mm length of the pyramidal layer (Hosseinzadeh et al., 2015; Liu et al., 2006).

Statistical analysis

All data expressed as the mean \pm standard error of the mean. The differences between positive and negative control groups were analyzed by the unpaired Student *t*-test. The differences between the treatment groups were compared with one-way analysis of variance and then, with a post hoc test of Bonferroni test for group-by-group. The statistically significant value was taken as $P<0.05$.

RESULTS

The effect of maternal exercise and the role of D₂ and 5-HT₂ receptors on offsprings' pain response in WT

Figs. 1 and 2 indicate the effects of maternal forced exercise on offsprings in writhing test. The maternal forced exercise significantly increased the latency time of writhing sign compared to the control group ($P<0.05$) (Fig. 1). The treatment of mothers with forced exercise in combination with haloperidol or bromocriptine caused respectively decrease and increase in latency time of writhing sign in comparison with the group which their mothers were treated with the exercise only ($P<0.05$) (Fig. 1). The treatment of mothers with forced exercise in combination with trazodone or O-4310 respectively increased and decreased

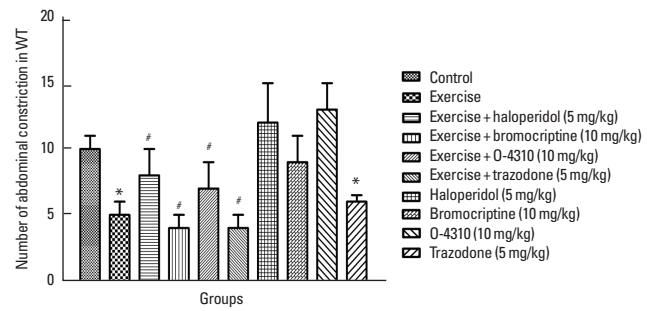


Fig. 2. Number of abdominal contraction in writhing test (WT) in the control group which their mother treated by exercise or its combination with haloperidol, bromocriptine, trazodone or O-4310 and groups under treatment by haloperidol, bromocriptine, trazodone or O-4310 alone. Values are presented as mean \pm standard error of the mean ($n=8$). * $P<0.05$, significant difference from control group. # $P<0.05$, Significant difference from group which their mother were treated by exercise.

the latency time of writhing sign in comparison with the group whose mothers were treated with exercise only ($P<0.05$) (Fig. 1). Also, the maternal treatment with bromocriptine or trazodone caused the increase of latency time but the treatment with haloperidol or O-4310 decreased it comparing to the control group but these changes were not statistically significant (Fig. 1). On the other hand, the maternal forced exercise caused significant decrease of number of writhing in comparison to the control group ($P<0.05$) (Fig. 2). Treatment of mothers by forced exercise in combination with trazodone or bromocriptine significantly increased the number of writhing in comparison with the group which their mothers were treated with exercise only ($P<0.05$) (Fig. 2). The treatment of mothers with forced exercise in combination with haloperidol or O-4310 significantly decreased the number of writhing in comparison to the group whose mothers were treated with the exercise only ($P<0.05$) (Fig. 2).

The treatment of mothers with haloperidol or O-4310 significantly increased the writhing number but the treatment with bromocriptine or trazodone significantly decreased the writhing test in comparison with the control group in which this decrease was significant in trazodone treated group by $P<0.05$ but it was not statistically significant in haloperidol, O-4310 and bromocriptine treated groups (Fig. 2).

The effect of maternal forced exercise and the role of D₂ and 5-HT₂ receptors on offsprings' pain response in TFT

The result of present study indicates that maternal forced exercise significantly increased the latency time in tail flick test (TFT) reaction comparing to the control group ($P<0.05$) (Fig. 3). In addition, the treatment of mothers by forced exercise in combina-

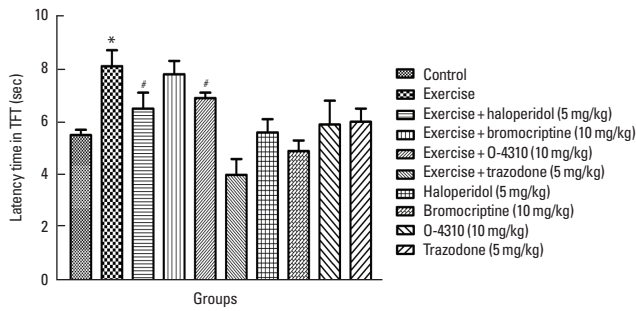


Fig. 3. Latency time of tail flick test (TFT) reaction in the control group and groups which their mother treated by exercise or its combination with haloperidol, bromocriptine, trazodone or O-4310 and groups under treatment by haloperidol, bromocriptine, trazodone or O-4310 alone. Values are presented as mean ± standard error of the mean (n=8). **P*<0.05, significant difference from control group. #*P*<0.05, Significant difference from group which their mother were treated by exercise.

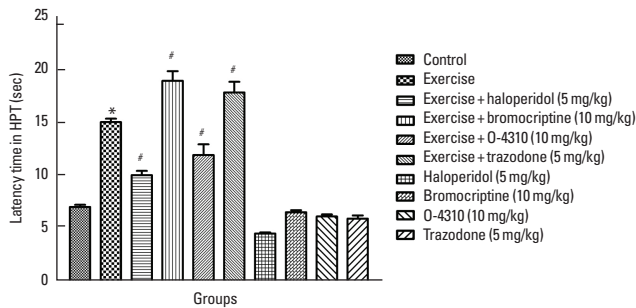


Fig. 4. Latency time of hot plate test (HPT) reaction in the control group and which their mother treated by exercise or its combination with haloperidol, bromocriptine, trazodone or O-4310 and groups under treatment by haloperidol, bromocriptine, trazodone or O-4310 alone. Values are presented as mean ± standard error of the mean (n=8). **P*<0.05, significant difference from control group. #*P*<0.05, Significant difference from group which their mother were treated by exercise.

tion with haloperidol or O-4310 caused decrease in latency time in comparison with the group whose mothers were treated with the exercise only (*P*<0.05) (Fig. 3), but treatment of mothers with the forced exercise in combination with bromocriptine or trazodone has not shown any significant differences in comparison to the group which has been treated with exercise only. The maternal treatment with haloperidol or O-4310 caused the decrease of tail flick latency time while the treatment with bromocriptine or trazodone increased the latency time which was not significant in comparison with the control group (Fig. 3).

The effect of maternal exercise and the role of D₂ and 5-HT₂ receptors on offsprings' pain response in HPT

The results of hot plate test (HPT) indicate the effect of maternal forced exercise on responses of offsprings to pain stimulus (Fig.

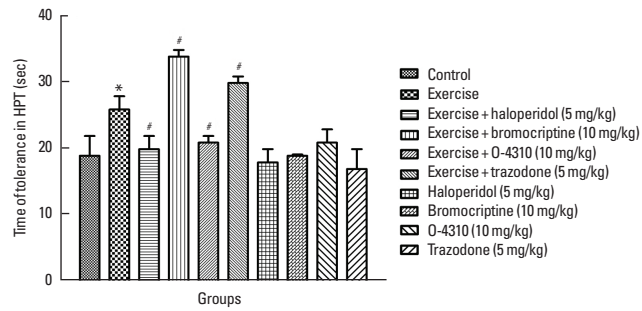


Fig. 5. Hot plate test (HPT) time tolerance in the control group and which their mother treated by exercise or its combination with haloperidol, bromocriptine, trazodone or O-4310 and groups under treatment by haloperidol, bromocriptine, trazodone or O-4310 alone. Values are presented as mean ± standard error of the mean (n=8). **P*<0.05, significant difference from control group. #*P*<0.05, Significant difference from group which their mother were treated by exercise.

4). The maternal exercise did not change the latency time of HPT comparing to the control group. However, the treatment of mothers by forced exercise in combination with haloperidol or O-4310 caused the decrease in latency time of HPT and treatments of mothers by forced exercise in combination with bromocriptine or trazodone caused an increase in latency time of HPT, all these changes was statistically significant in comparison with the group whose mothers were treated with the exercise only (*P*<0.05) (Fig. 4). Also the maternal treatment by haloperidol or O-4310 caused the decrease in latency time of HPT while the treatment with bromocriptine or trazodone increased the latency time in offsprings, both of these changes were not significant (Fig. 4).

Fig. 5 indicates that the maternal exercise increased the HPT time tolerance in comparison to the control group (*P*<0.05) (Fig. 5). The maternal forced exercise in combination with haloperidol or O-4310 caused a decrease and the combination treatment with bromocriptine or trazodone increased the tolerance in HPT comparing to the group whose mothers were treated with the exercise only (*P*<0.05) (Fig. 5). In addition, the maternal treatment by haloperidol, O-4310, bropmocriptine or trazodone did not change the time of tolerance in HPT in offsprings as compared to the control group (Fig. 5).

The effect of maternal exercise and the role of D₂ and 5-HT₂ receptors on offsprings' OFT behavior assay

As it is shown in Table 1, the group whose mother were trained with the forced exercise has more frequency of central square entries, more time spent in the central region of the OFT and also more ambulation number and rearing in comparison with the control group (*P*<0.05) (Table 1). The treatment of mothers with

Table 1. Effect of maternal exercise and role of D₂ and 5-HT₂ receptors in offspring open field exploratory and anxiety like behavior (n=8)

Group	Ambulation distance	Time spent in central square	Central square entries	Number of rearing
Control	402 ± 20	30 ± 2	8 ± 2	9 ± 2
Exercise	489 ± 14*	101 ± 8*	25 ± 3*	19 ± 1*
Exercise + haloperidol (5 mg/kg)	419 ± 12 [#]	75 ± 9 [#]	19 ± 2 [#]	16 ± 2 [#]
Exercise + bromocriptine (10 mg/kg)	518 ± 11 [#]	131 ± 9 [#]	39 ± 2 [#]	22 ± 3 [#]
Exercise + trazodone (5 mg/kg)	436 ± 15 [#]	67 ± 11 [#]	21 ± 11 [#]	17 ± 3 [#]
Exercise + O-4310 (10 mg/kg)	500 ± 10 [#]	115 ± 10 [#]	36 ± 3 [#]	21 ± 3 [#]
Haloperidol (5 mg/kg)	385 ± 19	28 ± 8	7 ± 1	9 ± 3
Bromocriptine (10 mg/kg)	410 ± 13	41 ± 6	10 ± 4	11 ± 4
O-4310 (10 mg/kg)	373 ± 15	20 ± 3	8 ± 4	8 ± 5
Trazodone (5 mg/kg)	411 ± 9	40 ± 6	9 ± 2	10 ± 4

Values are presented as mean ± standard error of the mean.

**P* < 0.05, significant difference from control group. [#]*P* < 0.05, Significant difference from group which their mother were treated by exercise.

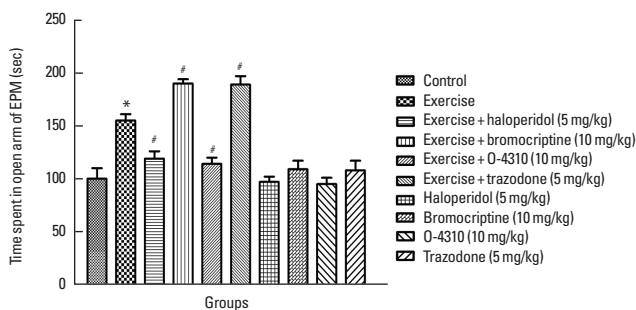


Fig. 6. Duration of time spent in open arms (sec) in elevated plus maze (EPM) test in the control group which their mother treated by exercise or its combination with Haloperidol, bromocriptine, trazodone or O-4310 and groups under treatment by haloperidol, bromocriptine, trazodone or O-4310 alone. Values are presented as mean ± standard error of the mean (n=8). **P* < 0.05, significant difference from control group. [#]*P* < 0.05, Significant difference from group which their mother were treated by exercise.

forced exercise in combination with haloperidol or O-4310 caused the decrease of central square entries, the duration of time spent in the central region and the ambulation number and rearing in comparison with the group whose mothers were treated with exercise only (*P* < 0.05) (Table 1). The treatment of mothers with trazodone or bromocriptine in combination with forced exercise increased the mentioned behavior comparing to the group whose mothers were treated with the exercise only (*P* < 0.05) (Table 1). The maternal treatment with haloperidol or O-4310 decreased the mentioned behavior but bromocriptine or trazodone increased OFT behaviors in offsprings, but these results were not statistical-significant (Table 1).

The effect of maternal exercise and the role of D₂ and 5-HT₂ receptors on offsprings' anxiety like behavior in EPM

Fig. 6 indicates the effect of maternal forced exercise on EPM in

offsprings. The maternal exercise significantly increased the duration spent in open arm in EPM comparing to the control group of offsprings (*P* < 0.05) (Fig. 6). Also, the treatment of mothers with forced exercise in combination with haloperidol or O-4310 caused decrease in time spent in open arm in EPM while bromocriptine or trazodone increased this time in comparison to the group whose mothers were treated with exercise only (*P* < 0.05) (Fig. 6). The group whose mothers were treated with haloperidol or O-4310 spent less time in open arm in EPM, and the group whose mother were treated with bromocriptine or trazodone spent more time in open arm in EPM in comparison to the control group (*P* < 0.05) (Fig. 6).

The alteration in CREB gene expression in offsprings

Maternal exercise significantly increased the expression of CREB in offsprings as compared to the control group (*P* < 0.05) (Fig. 7). Groups whose mother received haloperidol or ketanserin in combination with exercise showed significant decrease in offsprings' CREB gene expression. In contrast, in group whose mothers were treated with bromocriptine or trazodone, the expression of CREB gene was increased. All these changes were significant in comparison with the offsprings of mothers that were treated only with exercise (*P* < 0.05) (Fig. 7). The maternal treatment by bromocriptine, haloperidol and trazodone caused changes in CREB gene expression in offsprings but these changes were not statistically significant (Fig. 7).

The alteration in cell density and morphology of DG in offsprings

The maternal exercise significantly increased the number of the granular cells in DG in offsprings' hippocampus as compared to

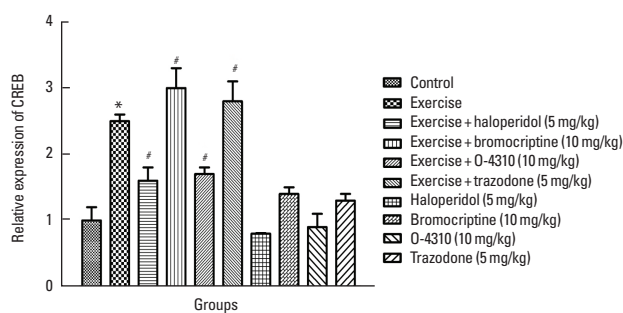


Fig. 7. Alteration of expression of cyclic AMP response element binding protein (CREB) in the control group and groups which their mother treated by exercise or its combination with haloperidol, bromocriptine, trazodone or O-4310 and groups under treatment by haloperidol, bromocriptine, trazodone or O-4310 alone. Values are presented as mean \pm standard error of the mean (n=8). * $P < 0.05$, significant difference from control group. # $P < 0.05$, Significant difference from group which their mother were treated by exercise.

Table 2. Effect of maternal exercise and role of D_2 and 5-HT₂ receptors in offspring hippocampus neuronal cell counting (number/mm) (n=8)

Group	Neuronal density (number/mm)
Control	502.14 \pm 4.9
Exercise	629.11 \pm 5.1*
Exercise + haloperidol (5 mg/kg)	582.0 \pm 9.5#
Exercise + bromocriptine (10 mg/kg)	632.1 \pm 3.4
Exercise + trazodone (5 mg/kg)	624.3 \pm 8.3
Exercise + O-4310 (10 mg/kg)	587.6 \pm 9.1#
Haloperidol (5 mg/kg)	498.5 \pm 7.4
Bromocriptine (10 mg/kg)	485.8 \pm 8.1
O-4310 (10 mg/kg)	491.6 \pm 8.8
Trazodone (5 mg/kg)	481.3 \pm 6.2

Values are presented as mean \pm standard error of the mean. * $P < 0.05$, significant difference from control group. # $P < 0.05$, Significant difference from group which their mother were treated by exercise.

the control group ($P < 0.05$) (Table 2). A quantitative comparison of the granular cells in DG density in the group whose mothers was treated with the exercise in combination with haloperidol or O-4310 showed the decrease in granular cells' density in DG in comparison to the group whose mothers were treated with the exercise only ($P < 0.05$) (Table 2). However, the mothers treated with the exercise in combination with bromocriptine or trazodone showed an increase which was not significantly different in comparison to the group whose mothers were treated with the exercise only. The maternal treatment with bromocriptine, haloperidol, trazodone and O-4310 decreased granular cells' density in DG in comparison to the control group and this was not statistically significant (Table 2). As it is shown in Fig. 8, the forced exercise in comparison to the control group caused the increase of DG cell layers. There was a dramatic loss of DG cell layers in group whose

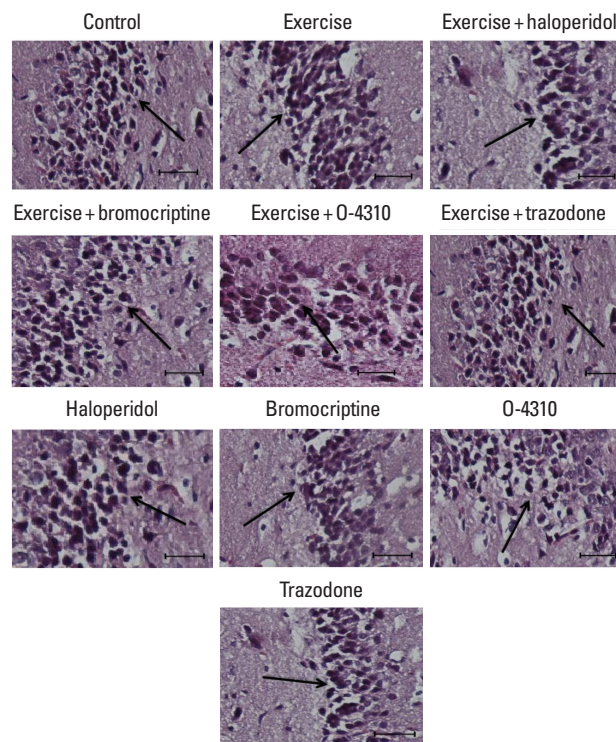


Fig. 8. Hematoxylin and eosin staining of the shows dentate gyrus (DG) cell layer of the hippocampus in the control group and groups which their mother treated by exercise or its combination with haloperidol, bromocriptine, trazodone or O-4310 and groups under treatment by haloperidol, bromocriptine, trazodone or O-4310 alone (magnification, $\times 400$). Scale bar represents 100 μ m. Arrow shows cell layers in all groups.

mother treated with the exercise in combination with haloperidol or O-4310 ($P < 0.05$), while the exercise in combination with bromocriptine or trazodone did not have any effect on DG cell layers in offsprings. The maternal treatment with bromocriptine, haloperidol, trazodone and O-4310 could change DG cell layers, but this was not statistically significant (Fig. 8).

The correlation between hippocampal CREB expression and gentate gyrus cell density

The Pearson correlation analysis also demonstrated a significant correlation between hippocampal DG cell density and CREB mRNA expression in all experimental groups ($r = 0.9744$, $P < 0.001$) (Fig. 9).

DISCUSSION

The present study indicated that the maternal forced exercise could modulate pain perception, anxiety, depression and CREB gene expression in pups. The maternal forced exercise caused some

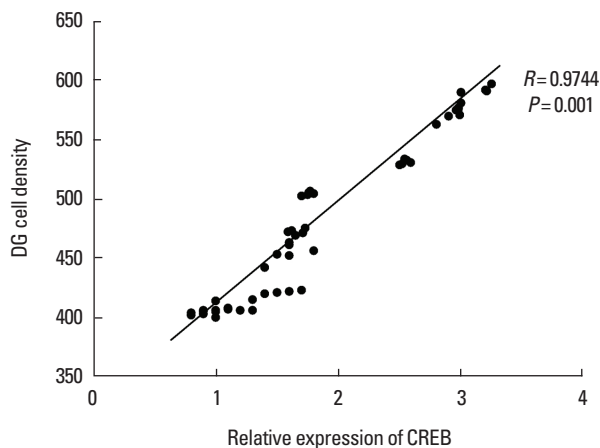


Fig. 9. Correlation analysis between the cyclic AMP response element binding protein (CREB) expression and dentate gyrus (DG) cell density in all groups. $r=0.9744$, $P<0.001$.

behavioral alterations in FST (swimming), EPM (open arm entry), and OFT (central area entry, central area duration, ambulation, and rearing) of their offsprings. Moreover, the maternal forced exercise could diminish the pain perception in pups, and that probably was modulated with D_2 (dopaminergic) receptor and 5-HT₁ (serotonergic) receptor which consequently caused the alteration of *CREB* gene expression level. Previous studies have revealed that physical activity lowers stress and anxiety levels and releases endorphins into the brain. Exercise can counteract drug abuse withdrawal symptoms with the attenuation of depression, the reduction of anxiety and help the patient to feel better. Several research studies have demonstrated that exercise can manage this recovery process. Physical activity can increase the synthesis and the release of dopamine, stimulate neuroplasticity and promote the feelings of well-being. Chronic exercise in mice results in antidepressant-like behavioral changes that may involve a BDNF related mechanism similar to the hypothesis for antidepressant drug treatment (Aksu et al., 2012; Lee et al., 2006).

Also the present study showed that the maternal forced exercise decreased the number of writhings in offsprings. This study showed that the effect was modulated by dopamine D_2 and serotonin 5-HT₂ receptors. The treatment with haloperidol or ketanserin inhibited the protective effect of maternal forced exercise on the abdominal pain in writhing test. Treatment of mothers with forced exercise, trazodone and bromocriptine enabled the protective effects of forced exercise on offsprings pain management. Many previous studies demonstrated that the maternal physical activity could develop the neural regeneration in offsprings (Herring et al., 2012) and based on the present study, one kind of neu-

ral development was the pain perception and the increase in pain tolerability (Galdino et al., 2010). On the other hand, previous researches have shown that D_2 and 5-HT₂ receptors can modulate the pain and these receptors play an important role in neuronal regeneration (Borta and Höglinger, 2007; Whitaker-Azmitia, 2001). They have also shown that D_2 and 5-HT₂ agonists have analgesic effect suggesting that the receptors can modulate pain perception pathways in brain. Based on this concept we can argue that forced exercise have effect on offsprings' pain tolerability probably through D_2 and 5-HT₂ receptors (Treister et al., 2011; Wood, 2008).

Offsprings of mothers that were treated with haloperidol considerably increased the writhing number in comparison to the control group but the treatment with bromocriptine or trazodone significantly decreased the writhing number in comparison to the control group. The decrease was significant in trazodone treated group. The results can be discussed on the basis of previous studies which showed that bromocriptine or trazodone has neuroprotective effect and this probably could be the reason for brain development (Jensen et al., 2008; Huey et al., 2006).

The present study showed that maternal forced exercise increases the latency time in writhing test in offsprings and the treatment of mothers by haloperidol in combination with forced exercise decreases the latency time in writhing test. Moreover, in mothers treated with forced exercise and bromocriptine or trazodone, the latency time was increased in writhing test. Physical activity could cause inhibition of inflammatory and neuropathic pain in offsprings, previous studies have shown that physical activity in mothers cause modulation of neurohormone and potentiate immunological function in children and attenuates immunologic disorder in offsprings (Gleeson, 2007; Vega et al., 2015). It was approved that D_2 and 5-HT₂ receptors have important role in inflammatory pain. Also, agonists on these receptors can inhibit the expression of inflammatory and acetic acid induced pain in animal models (Shojaii et al., 2015; Zakaria et al., 2005). In present study, pups whose mothers were treated with haloperidol, trazodone caused decrease in latency time while in pups whose mother treated with bromocriptine caused the increase of latency time. Based on this study, there might be a link between maternal forced exercise and D_2 and 5-HT₂ receptors' involvement in brain pathways of inflammatory pain perception.

In TFT, for evaluation of maternal forced exercise on spinal pain perception, this research showed that forced exercise in mothers caused an increase in latency time of TFT and this effect was probably mediated through 5-HT₂ and D_2 receptors. The results

showed forced exercise in combination with haloperidol caused the decrease in the latency time in comparison to the group whose mothers were treated with exercise only.

Many previous studies showed that physical activity increase the tolerance against pain stimulant especially in spinal pain. Moreover, there is growing number of evidence supporting the role of dopamine and serotonin in spinal pain perception (Berger et al., 2009; Potvin et al., 2009; Treister et al., 2011). The present study showed that protective role of forced exercise in management of spinal pain perception in tail flick was mediated by dopamine and serotonin in which 5-HT₂ and D₂ receptors were involved.

This research indicated that maternal forced exercise might cause increase of the hot plate tolerance time in offsprings. On the other hand, the present study suggested that this effect might be mediated by dopamine D₂ receptor and serotonin 5-HT₂ receptor since treatment by haloperidol inhibited the effect of maternal forced exercise and abolished the tolerance. Also, treatment of mothers by bromocriptine and trazodone increased the tolerance of offsprings to pain stimulus in HPT. The maternal treatment by haloperidol decreased the duration of tolerance and treatment with bromocriptine or trazodone increased the time of tolerance in the HPT in offsprings in comparison to the control group. Accordingly, in HPT the latency time in offspring of mothers that were treated with forced exercise only were increased while haloperidol could inhibit this effect and trazodone or bromocriptine potentiated the effect of forced exercise. The mother's treatment with ketanserin or haloperidol also decreased the latency time and trazodone or bromocriptine increased it in pups. About the effect of forced exercise in pain management similar to other researches, this study indicated that maternal activity could potentiate offspring tolerance and endogenous analgesic pathways in HPT model. Many previous studies have demonstrated that maternal physical activity can increase the number of receptors for bioamines in brain and spinal cord, so this increase could be responsible for various protective effects of physical activity in brain disorders (Foley and Fleshner, 2008; Lan et al., 2014; Ma et al., 2008). This research confirmed the concept which maternal forced exercise had effects on the pain perception of pups in HPT was and this probably is mediated by D₂ and 5-HT₂ receptors and it could show the role of serotonin and dopamine in forced exercise neuroprotection in pain perception pathway synaptic plasticity.

In OFT, This research indicated that pups of mothers treated with forced exercise had more frequency of central square entries, and spent more time in the central region of the OFT, more ambulation and rearing number in this test. Treatment of mothers by

haloperidol in combination with forced exercise abolished the effect of forced exercise on this behavior which suggests that antidepressant and anxiolytic effect of exercise might have been mediated by D₂ and/or 5-HT₂ receptors. Treatment of mothers by trazodone or bromocriptine in combination with forced exercise increased the frequency of central square entries, time spent in the central region, ambulation and rearing number in pups. Pups whose mothers were treated with haloperidol showed attenuation and those whom their mothers were treated with bromocriptine or trazodone showed improvement in OFT behaviors in comparison to the control group. Forced exercise in mothers can modulate motor activity in mothers as well as pups (Choi et al., 2013; Dayi et al., 2012). Many previous studies demonstrated that deficiency in dopamine and serotonin cause motor dysfunction and the present study confirmed this concept. Blockade of D₂ or 5-HT₂ receptors in mothers causes motor activity dysfunction in offsprings which is shown in OFT (Robinson and Bucci, 2012). On the other hand, forced exercise in mothers has neuroprotective effect and potentiates the plasticity of neurons which is responsible for motor activity (Robinson and Bucci, 2012). D₂ or 5-HT₂ receptors probably mediate the effect of forced exercise since their antagonist inhibits this effect of forced exercise while their agonist has potentiated the effect in OFT. The maternal forced exercise causes increase in time spent by pups in open arm in EPM as compared to the control group in offsprings. On the other hand, haloperidol inhibited this effect of forced exercise, but bromocriptine and trazodone potentiate the anxiolytic effect of exercise and increase the time spent in open arm in EPM. Also, treatment of mothers by bromocriptine, trazodone and haloperidol cause changes in anxiety like behavior in open arm in EPM in their offsprings. Many studies have shown that depressive and anxiety like behaviors in offsprings of mothers who had a regular exercise during their pregnancy is less than the pups whose mothers had no physical activity during their pregnancy (Akhavan et al., 2008; Mello et al., 2009; Uysal et al., 2011). This study confirmed this concept and indicated that anxiolytic and antidepressant effect of maternal exercise on offsprings were mediated by D₂ or 5-HT₂ receptors which are probably involved in protective effect of maternal forced exercise.

Maternal forced exercise increases the *CREB* gene expression as compared to the control group in offsprings. On the other hand, haloperidol inhibits this effect of forced exercise, but bromocriptine and trazodone potentiate the effect of maternal forced exercise on *CREB* gene expression in pups. Also, treatment of mothers with bromocriptine or trazodone increase *CREB* gene expression while haloperidol or ketanserin decrease it in offsprings. Many

previous studies have indicated that CREB as transcription factor has main protective roles against some neurodegenerative conditions such as Parkinson, Alzheimer, and seizure. On the other hand, many studies have demonstrated that physical activity can increase gene expression and protect the brain against oxidative agents (Barco and Marie, 2011; Saura and Valero, 2011). This research suggested that maternal forced exercise increased *CREB* gene expression and probably its protective effect in offspring such as anxiolytic, antidepressant and tolerability to pain were mediated by increase in *CREB* gene expression in pups. Also, the present study showed that the effect of forced exercise in *CREB* gene expression was mediated by D₂ or 5-HT₂ receptors, in which combination treatment of mothers by forced exercise with D₂ or 5-HT₂ receptor agonists potentiated this transcription factor expression while D₂ or 5-HT₂ receptor antagonists abolished the effects of forced exercise on offsprings. This study indicated that maternal forced exercise caused the increase of cell density in DG region of hippocampus in offsprings and forced exercise increased DG cell layer in children. Previous study have shown that maternal exercise caused development of offsprings brain especially hippocampus (Kim et al., 2007). The treatment of mothers with forced exercise in combination with haloperidol or ketanserin, decreased the cell density and caused dramatic loss in cell layer of DG and suggest that D₂ or 5-HT₂ receptor has significant role in protective effect of maternal forced exercise on brain cell density. Many previous studies have demonstrated that D₂ or 5-HT₂ receptor has important role in brain development during growth in mothers' uterus (Akhavan et al., 2008; Mamo et al., 2004). Histo-morphological findings of the present research suggested that some part of protective maternal physical activity was mediated by D₂ or 5-HT₂ receptors. Also this study showed that there was correlation between cell density in DG region and *CREB* gene expression. It suggested that there was a great correlation between maternal forced exercise and *CREB* gene expression, as one of main transcription factors involved in neuroprotection in brain (Oury et al., 2010). The findings of this correlation between forced exercises induced elevation in cell density in DG region and neurodevelopment is a novel achievement. This research confirmed previous results showed that CREB has strategic role in many degenerative diseases such as neurodegenerative disorders with defect in expression of this transcription factor (Li et al., 2009). The data obtained in present study supported the hypothesis that the maternal physical activity has neuroprotective properties in offsprings such as reduction of anxiety and depression and increase in pain tolerability. Also, this research showed that these neuropro-

tective effects of maternal forced exercise might probably be mediated by D₂ and/or 5-HT₂ receptors and *CREB* gene expression as main downstream transcription factor of these receptors.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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