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Title: Therapeutic Effect of Exercise on Anxiety and Bowel Oxidative Stress in the Maternal Separation Animal Model

Running Title: Effects of exercise on the bowel oxidative stress in MS animal model

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Highlights
- Maternal separation is a valid animal model of brain–gut axis dysfunction in rats;
- Behavioral abnormalities were associated to mitochondrial dysfunction;
- The negative impact of maternal separation on the bowel mitochondrial function and animals’ behavior reduced following the voluntary exercise.

Plain Language Summary
According to evidence, early-life stress, mood disorders, and some medical conditions such as irritable bowel syndrome (IBS) are correlated; IBS is among the commonest disabling diseases. This gastrointestinal illness is addressed by episodes of abdominal pain and bowel bloating. Unfortunately, the effect of antidepressant and anxiolytic agents as well as other drug therapies on IBS treatment is still unclear. Using a rat-model evaluation, current study findings showed that the negative impacts of MS on anxiety and mitochondrial dysfunction of the gut can remarkably be controlled via running wheel (RW) exercise with no side effects.
Abstract

**Purpose of the study:** According to evidence, early-life stress (ELS), mood disorders, and medical comorbidities—i.e., irritable bowel syndrome (IBS)—are correlated; however, the direct contribution of ELS to IBS manifestations is less understood. The current study aimed at evaluating the effect of voluntary exercise on mitochondrial dysfunction of the bowel fibroblasts, following confirmation of anxiety behavior. **Methods:** For this purpose, postnatal day (PND) rats underwent maternal separation (MS), a valid animal model of the brain–gut axis dysfunction, within the days 2-14; three hours daily. On the day 21, animals were divided to four groups including: Control, Running wheel (RW) exercise, Maternal Separation (MS), and MS+RW groups; the groups were housed in separate cages (four rats per cage) until the onset of intervention. On the day 60, the elevated plus maze was used to assess anxiety-like behaviors; the level of oxidative stress biomarkers—i.e., ROS (reactive oxygen species), GSH, as well as ATP was measured in order to determine the gut mitochondrial function. **Results:** Findings revealed that ELS affected the gut energy metabolism in rates; the negative effects of MS on anxiety and the gut mitochondrial dysfunction decreased via RW exercise during adolescence. **Conclusion:** Overall, anxiety behaviors and ROS production, leading to increase in the level of GSH and ATP, improved after RW exercise; this plays an important role in the function of colon secretory mitochondria. According to the positive effects of RW exercise on mitochondrial dysfunction in an ELS animal model, a potential relationship was found between brain and gut in rats.

**Keyword:** Maternal Separation (MS); Anxiety, Exercise, Brain, Gut; Oxidative Stress
Introduction:

Early-life stress (ELS) and stress-related psychiatric disorders are correlated in today communities, in spite of its disabling nature and prevalent status (O’Mahony, Hyland, Dinan, & Cryan, 2011). Merging lines of research indicate that mood disorders are accompanied by other comorbid disorders in the periphery organs such as heart and gut (Barrett et al., 2012; Clarke, Cryan, Dinan, & Quigley, 2012; Eutamene et al., 2007; Klooker et al., 2009). Irritable bowel syndrome (IBS) is a common gastrointestinal illness addressed by episodes of abdominal pain, bloating; in common sense, it is the brain-gut axis disorder (Zhao & Qian, 2014). It has been estimated that the worldwide prevalence of 10–15% is the most important reason for patients being visited by gastroenterologists. It is important to note that prevalence of IBS is enhanced proportionate to MS during childhood (Lowman, Drossman, Cramer, & McKee, 1987).

According to evidence, the nature and severity of symptoms, physiological complications, and psychiatric disorders play a remarkable role in IBS treatment. Unfortunately, effect of antidepressant and anxiolytic agents as well as other drug therapies on IBS treatment is still unclear (Ford, Talley, Schoenfeld, Quigley, & Moayyedi, 2008). Some studies have suggested physical exercise as a novel and more efficacious agents without side effect to treat both IBS and anxiety (Carek et al., 2011; Herring et al., 2010).

As already known, physical exercise increases the average life-span and improve health in humans (Radak, Chung, & Goto, 2008). Besides, other data have proven that exercise ameliorates negative impact of maternal separation stress on behavior and incidence of oxidative stress in adult male rats (Radak, Taylor, Ohno, & Goto, 2001). An animal model study on male rats showed that RW
exercise can descend depressive-like behaviors, and reduce the expression of immune genes in the hippocampus (Sadeghi, Peeri, & Hosseini, 2016).

Evidence indicates that there are many mechanisms through which ELS induces its negative effects on the development of brain and formation of behavioral abnormalities (Herring et al., 2010). Recent studies indicated the correlation of oxidative stress and mitochondrial dysfunction with pathophysiology of ELS-induced disorders (Taft & Keefer, 2016). Also, based on preclinical data, psychopathologies such as depressive- and anxiety-like behaviors are irritated following the experimental induction of colitis indicating the bidirectional relationship between gut and brain (Bercik et al., 2010). Unfortunately, there is little research about the effects of ELS, exercise and their interaction on the bowel mitochondrial function and anxiety-like behaviors. Therefore, the current study aimed at examining whether 1) The rats with ELS induced by MS can develop anxiety-like behaviors, 2) a correlation is between behavioral changes and the gut mitochondrial function; 3) the ELS consequences on gut mitochondrial function are controlled by RW exercise. For this purpose, gut fibroblasts, as an important component of the bowel for isolation of mitochondria were selected.
2. Materials and Methods

2.1. Animals

Twenty pregnant albino Wistar rats (gestation day 1) were purchased from the Pasteur Institute of Iran (Tehran, Iran) and kept under the standard laboratory conditions (22±2°C temperature, 50%±10% humidity, 12:12-hour light–dark cycle, and standard rodents food and water ad libitum). All the experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication # 80-23) as well as institutional guidelines for animal care and use (issued by Department of Exercise Physiology, Central Tehran Branch, Islamic Azad University, Tehran, Iran).

2.2. Maternal stress (MS) paradigm

Twenty pregnant rats were used in this study. 6 male offspring were obtained for experiments and subsequently introduced to the MS paradigm based on the previously-published protocol (Amini-Khoei et al., 2017; Daniels, Marais, Stein, & Russell, 2012). The birthday was considered as the postnatal day 0 (PND 0) and then, within the PNDs 2 to 14, the pups experienced maternal separation everyday for 180 minutes, beginning at 09:00 a.m. On PND 14, they were returned to their mothers. Four male rats were assigned to each group in a cage until testing on PNDs 60-62. All rats were healthy with no signs of sickness or death during the experiments.

2.3. Design of the Experiment

The rats were equally assigned to four groups 10-sample groups after the acclimation period; the groups were as follows: 1) the Control (which had no access to the RW), 2) RW group, 3) MS
group, and 4) MS+RW treatment. After completion of the RW intervention on PND 60, behavioral tests were performed on rats (n=6-7 in each group). Also, on PND 60, the rats in the experimental groups (n=3-4 in each group) were beheaded under mild anesthesia and the bowel was removed on an ice surface for biochemical assay.

2.4. RW exercise protocols:

On PND 21, RW exercise protocol was performed on rats selected randomly (n= 10 per group). To do this, after one week period of acclimation to RW apparatus, MS rats underwent RW based on Miladi-Gorji investigation protocol (Miladi-Gorji, Rashidy-Pour, & Fathollahi, 2012). Each exercise cage that housed a pair of MS rats was separated with perforated Plexiglas from the neighbor cage to avoid animals’ isolation and facilitate communication; an RW apparatus was embedded in each cage. Similar conditions were provided for the control group, except for access to the RW. The Plexiglas wheels were 105 cm in circumference and 10 cm in width, with 5-g freely rotation (Novidan Tab, Iran). The rotation of the wheel was monitored hourly by a magnetic switch connected to and a counter placed outside the cage. There was no time limitation for using the RW apparatus and rats were kept under the same conditions for 32 days until PND 60; their running distance per day was recorded in kilometers.

2.5.1. Elevated plus maze

On RW exercise final day, behavioral tests were performed in rats (Pellow & File, 1986). The EPM relies on the natural fear of mice of open, unprotected, and elevated areas (Suo et al., 2013). The black Plexiglas-made EMP had two open (50×10 cm) and two closed (50×10×40 cm) cross-shared arms connected by a central platform (10 × 10 cm). The apparatus was located 60 cm from the ground with dim light. First, the rats were placed in the central zone facing the open arm and
gave them a 5-minute chance to explore the maze as described previously (Sadeghi et al., 2016). A video camera was embedded above the EMP to record the sessions; the amount of time spent on the open arms plus the open arm entries were scored. To eliminate residual odors of the previous rat, the maze was cleaned between the tests with 70% ethanol.

2.5.2. Force Swimming Test (FST)

On the final day of RW exercise treatment, behavioral tests were performed in rats according to the protocol of Amini-Khoei et al., 2017. The passive behavior of rats (immobility time) was monitored, while they were forced to swim individually in a 50×20 cm (height×diameter) tall glass tank that 30 cm of it was water (23±1°C). Immobility is referred to when the animal floats in the water and makes only those movements necessary to keep its head above the surface.

2.6. Mitochondrial isolation from bowel fibroblasts:

After completion of behavioral and treatment with exercise, animals were kept at fasting state for 24 hours and then were anesthetized. After opening the intestinal cavity, the bowel was removed immediately and washed with PBS. The obtained tissue was quickly transferred to DMEM medium and after gently cutting was transferred to the Falcon Tube, and collagenase (IV) was added to medium (6-7 min). Then, the medium was filtered and again DMEM was added to the tube and placed in stiller so that bowel cells could be isolated uniformly (10 min). Then, the centrifuge was conducted at 400 rpm and the supernatant was removed and it was conducted again at 1000 g, and the resulting sediment was suspended again in the DMEM medium. The resulting sediment contained bowel fibroblasts. In the second stage, the extraction of mitochondrial fractions from fibroblasts was performed on the centrifuge at 2500 g for 10 minutes; then the supernatant was collected into a Falcon tube, and centrifuged at 8000 g (10 min), and the dark pallet tube
containing the mitochondria was dispersed in terms of test in the desired buffer based on each test (Vicario et al., 2012; Wieckowski, Giorgi, Lebiedzinska, Duszynski, & Pinton, 2009). To ensure the purity of the obtained mitochondrial suspension, MTT test was conducted to confirm the results based on reports from other studies (Shaki et al., 2012).

2.7. ATP assay

ATP assay was performed by ATP assay kit using colorimetric assay according to the protocol recommended by the manufacturer (abcam-83355). It is based on the phosphorylation of glycerol in order to generate a product which can be measured by Elisa-reader at 570 nm based on calibration standard curve.

2.8. Measurement of ROS production

DCFH-DA was employed to measure the mitochondrial ROS production. 5 μl of 10 μM DCFH-DA was added to the supernatant which composed of respiratory buffer (Sonei et al., 2017). The amount of DCF, fluorescent final product, was determined respectively at excitation-emission wavelengths of 485 and 525 nm (Amiri et al., 2016; Sonei et al., 2017).

2.9. Reduced glutathione (GSH) level assay:

Glutathione (GSH) level was measured by a spectrophotometer (UV-1601 PC, Shimadzu, Japan) at 412 nm using DTNB reagent following the emergence of yellow color based on calibration standard curve (Hosseini, Shaki, Ghazi-Khansari, & Pourahmad, 2014; Shaki et al., 2012).

2.10. Statistical analysis
All data were expressed as mean ± standard deviation. One-way ANOVA followed by post hoc Tuckey tests was employed to make comparison between the groups. The level of significance was set to <0.05.

3. Results

Anxiety-like behavior was assessed in the studied rats using EPM test. In this test, reduced frequency of the open arm entry and increased time spent in the closed arms indicate higher levels of anxiety. Using statistical analysis, it was determined that MS stress significantly causes anxiety-like behaviors. In other words, the number of entry to the open arm of apparatus in MS groups [\( F(3,27) = 10.275, \, df=3, \, P<0.001, \) Fig.1] was lower than that of the control group. Also, the time spent in the open arm was decreased in MS groups in comparison to control groups [\( F(3,27) = 17.930, \, df=3, \, P<0.001, \) Fig.1]. Besides, data showed that MS+RW groups exhibited a significant increasing in the open arm entry and spent the time spent in the open arms compared to the MS groups, suggesting significant reduction in the anxiety-like behaviors (Fig.1).

Increased immobility time in FST was considered a behavioral despair that is a symptom of human depression (Amiri et al., 2016). One-way ANOVA yielded a significant difference in the FST [\( F(3, 27) =38.462, \, P<0.001 \)]. According to Figure 1B, immobility time of MS rats was significantly higher than that of the controls (***P <0.001). Besides, the immobility time was shorter in the RW-treated MS rats compared with the controls (###P <0.001).

As shown in Table.1, ROS level increased time-dependently in the MS group compared with the control group after 15 minutes [\( F (3,11) = 47.896, \, df=3, \, P <0.001 \)] and 60 min [\( F (3,11) = 32.641, \, df=3, \, P <0.001 \)], which indicates involvement of oxidative stress in isolated mitochondria obtained.
from bowel fibroblast. The ROS production rate had no significant elevations in MS+RW rats after a 60-minute exposure compared to the controls (P >0.05).

According to Figure 2, a significant decrease was observed in GSH level of the MS rats in comparison with the controls (P <0.05). However, in the rat groups that received RW exercise, GSH level had a significant elevation than the MS group \[ F (3,11) = 22.941, df=3, P<0.001 \] was observed.

the ATP level significantly decreased in the MS rats compared to the controls \[ F (3,11) = 13.5, df=3, P<0.001 \]. However, the rat groups that received RW exercise showed a significant increase at ATP level compared to the MS groups (Fig.3).
4. Discussion

Maternal separation stress in rodents is a non-pharmacological method which is able to induce behavioral changes such as depression, anxiety and inducing of visceral hypersensitivity to rectal distention (Carek et al., 2011; Sadeghi et al., 2016). The current study findings confirmed experiencing MS-induced anxiety- and depressive-like behaviors and mitochondrial dysfunction in bowel fibroblast, indicating that oxidative stress and antioxidant imbalance contribute to the negative effects of ELS on gut energy metabolism. Besides, RW exercise could reverse the ELS-induced anxiogenic behavior and abnormalities in the gut mitochondrial function.

Several lines of clinical and preclinical research have suggested that early life stress and trauma are able to alter GI motility, disrupt intestinal epithelial barrier, and activate gut mucosal inflammatory responses during adulthood (Cryan & Holmes, 2005; Nicholl et al., 2008; O'Mahony et al., 2009; O'Malley, Julio-Pieper, Gibney, Dinan, & Cryan, 2010). According to clinical data, 50%-60% of patients with IBS also have psychiatric disorders such as depression and anxiety (Vicario et al., 2012). Indeed, according to the current study, MS-treated group had anxiety-like behaviors which were characterized by spending shorter time on the open arms and less frequency of the open arms in the EPM. Besides, the data confirmed that RW exercise ameliorated anxiety-like behaviors in rats.

Vicario et al., indicated that the reversible mitochondrial damage and upregulated corticotropin-releasing factor receptor type-1 are induced in the gut following the incidence of chronic social stress, which can cause IBS-like gut dysfunction (Vicario et al., 2012). Also, it was shown that physical activity and exercise induce widespread neurobiological adaptations, and are able to reduce anxiety-like behavior, enhance neurogenesis via promotion of IGF1 and BDNF activity (Droste et al., 2003; Schoenfeld, Rada, Pieruzzini, Hsueh, & Gould, 2013), and reduce ACTH.
hormones level and corticosterone (Hare, Beierle, Toufexis, Hammack, & Falls, 2014; Wang, Chen, Lin, JiHong, & Chen, 2014). A previously-done study has shown the expression of NR2A subunit of NMDA receptors increased following MS in the hippocampus which is crucial in the incidence of depression and anxiety-like behaviors. Besides, negative impacts of MS on behavior and mitochondrial function was controlled via RW in adult rats (Fattahi, Peeri, Azarbayjani, & Hosseini, 2017). Therefore, it is supposed that MS is able to enhance glutamatergic signaling which causes anxiety-like behaviors and mitochondrial dysfunction in bowel similar to depressive-like behaviors.

Accordingly, recent evidence indicates that dysfunction in the mitochondrial bioenergetics plays a part in the pathophysiology of psychiatric disorder; i.e., depression and anxiety via different routes such as activation of oxidative stress or inflammatory pathways, activation of NMDA receptor and altering the plasticity of neurons (Brown & Bal-Price, 2003; Gardner & Boles, 2011; Klinedinst & Regenold, 2015; Morava & Kozicz, 2013). The study showed induction of mitochondrial dysfunction and oxidative stress in the bowel fibroblasts of MS adult rats. (Vicario et al., 2012). The results of ROS production showed that the rate of ROS production was higher in MS rats compared with the controls in the mitochondria obtained from bowel, suggesting that ROS overproduction in mitochondria is induced following the early-life maternal separation, that is associated with mitochondria dysfunction and oxidative stress in the cells of the bowel (Table.1).

In line with these results, the findings of the study indicated that MS exposure causes a significant decrease in GSH level which confirms increased ROS production. Also, data of the study confirms that MS has negative impact on GSH level as the main antioxidant system, and adversely affects mitochondrial permeability transition pore opening. ATP levels in MS rats were decreased...
compared to the control groups, suggesting that early life can disrupt the metabolism of energy in the bowel mitochondria isolated from fibroblast in adult rats.

Our The present investigation showed significant reduction of ROS production on the isolated mitochondria obtained from bowel tissue following treatment with RW exercise (Bachur et al., 2007; Salim et al., 2010). It seems that improving antioxidant system following RW exercise and decreasing of superoxide radical production or ROS production has a protective role against MS-induced changes. Also, the study findings were consistent with other studies indicating exercise is able to increase GSH level (Bachur et al., 2007; Hovanloo, Hedayati, Ebrahimi, & Abednazari, 2011; Oh-Ishi et al., 1994; Rezin et al., 2009; Sonei et al., 2017). The inconsistency between the results is due to difference in the antioxidant and oxidant scavenger pattern or difference in intensity and duration of exercise. In terms of recommendations, since finding indicated the induction of mitochondrial dysfunction in the bowel fibroblast by ELS-derived stress, it appears that the impact of exercise on anxiety is affected by intensity and duration of exercise, resting time after exercise and severity of psychiatric disorder.

In summary, it was shown in the study that experiencing stress due to early-life maternal separation is a risk factor for medical comorbidities; i.e., bowel complications in adulthood, but RW exercise during adolescence has anxiolytic and protective effects on the negative outcomes of ELS on mitochondrial function of the bowel.
Ethical Considerations
All institutional and national guidelines for the care and use of laboratory animals were considered.

Funding
There was no funding for the study.

Conflict of interest
Authors declared no conflict of interest.

Acknowledgements
None

References:


Table 1. Effect of RW exercises on ROS formation: Reactive oxygen species were measured by fluorescent dye DCFH-DA. The change in fluorescence was determined at 485 nm for excitation and at 525 nm for emission after 15 and 60 min incubation, using fluoremetry.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ROS formation (%)</th>
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<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>60 min</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4±2</td>
<td>13±4</td>
<td></td>
</tr>
<tr>
<td>RW</td>
<td>4±2</td>
<td>8±3</td>
<td></td>
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<tr>
<td>Maternal Separation (MS)</td>
<td>32±6***</td>
<td>50±9***</td>
<td></td>
</tr>
<tr>
<td>MS+RW</td>
<td>5±2###</td>
<td>19±5###</td>
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Figure 1A. Effect of RW exercise on anxiogenic-like behaviors: Effect of chronic adolescent RW exercise on the spent time of open arm, open arm entry and Time spent in center of EPM. Values are expressed as the mean ± SD and were analyzed using one-way ANOVA followed by tukey’s post-test analysis (n=7-8 in all groups). ***P<0.001 compared with the control group; and ###P<0.001 compared with the MS groups.
Figure 1B. Effect of RW exercise on depressive-like behaviors: Effect of chronic adolescent RW exercise on immobility time on OFT. Values are expressed as the mean ± SD and were analyzed using one-way ANOVA followed by tukey’s post-test analysis (n=7-8 in all groups). ***P<0.001 compared with the control group; and ###P<0.001 compared with the MS groups.

Figure 2. Effect of RW exercises on GSH level: Values are expressed as Mean±SD. *** P<0.001 compared with control group and ###P <0.001 compared with MS group.
Figure 3. The effect of MS in childhood on mitochondrial ATP level: Values are expressed as Mean ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with control group and # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ compared with MS group.