Maternal separation can affect the reproductive system by inflammasome activation in female mice

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Objective The aim of this study is to investigate effect of maternal separation stress on the ovarian function in adult female mice.

Methods In this study, maternal separation in pups was performed during postnatal days 2–14. The histological alterations in ovarian tissue, reactive oxygen species (ROS) production (using 2',7'-dichlorofluorescin diacetate assay), gene expression of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1, TLR4, BAX, BCL-2 and TNF-α (using RT-PCR), protein levels of ATP, GPx, interleukin (IL)-1β and IL-18 (using enzyme-linked immunosorbent assay). Also, protein expression of caspase-3 and NLRP3 (using immunocytochemistry) were evaluated.

Results This showed that maternal separation decreased percentage of primordial follicles while increased percentage of secondary and Graafian follicles. In addition, maternal separation increased ROS production and decreased ATP and GPx concentrations. Furthermore, maternal separation significantly affected expression of cytokines and genes involved in inflammation and apoptosis including NLRP3, ASC, caspase-1, TLR4, TNF-α, IL-1β, IL-18, BAX and BCL2. Findings also showed that stress-induced maternal separation significantly increased percentage of caspase-3 and NLRP3 positive cells. We concluded that maternal separation stress has harmful effects on ovarian tissue.

Conclusion It seems that these harmful effects probably occur through increase of ROS production and impact on mitochondrial function, inflammatory process and apoptosis pathways.

Keywords maternal separation stress, inflammasome, inflammation, oxidative stress, apoptosis, reproductive system

Introduction

The early childhood is a sensitive age period in which development of brain can be affected harmfully by stressful events. The maternal separation model, as a model mimicking early-life stress, has been broadly studied to evaluate the effect of early-life stress on brain development and associated biological networks. Exposure to this stressful event can affect children not only in their early years of life but also in their adult life. It seems that the repeated activation of the hypothalamic-pituitary-adrenal (HPA) axis and disruptions in pathways of the immune response and the neurotrophin production could play a critical role in the long-lasting effects of early-life stress.1,2

Stress can trigger the activity of the adrenergic system that change the function of the endocrine and immune systems.3 Cooperation between the immune and endocrine systems is necessary for normal development and function of the female reproductive system.4

According to psychoneuroimmunology studies, the physiological response to stressors affects function of the innate immune system even in the absence of pathogens that is known as sterile inflammatory response.5 Although it remains unknown how innate immune function is stimulated by stress, the role of different factors including glucocorticoids, danger signals and catecholamines are evidenced by several studies.2,6–10 The transcription of inflammatory mediators such as cytokines and chemokines are activated during the inflammatory process.11 Inflammasomes are intracellular multi-protein complexes which include a NOD-like receptor/an AIM-like receptor, the adapter molecule apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1.12–14 NLRP3 along with the adaptor protein ASC, active caspase-1 through assembly of the inflammasome, although the exact mechanism for this process is unclear. The NLRP3, the most described inflammasome, can be triggered by a wide range of exogenous and endogenous stress signals. Caspase-1 activation leads to the cleavage of pro-interleukin (IL)-1beta, pro-IL-18 into active forms of IL-1β, IL-18, which play potential role in modulating innate immune function.15–17 Moreover, caspase-1 plays a role in cell apoptosis via stimulation of caspase-3 and -7.18

On the other hand, psychosocial stressors via stimulation of inflammatory cytokines such as TNF-α, IL1-β and IL-6 can lead to the suppression of gonadotropin-releasing hormone (GnRH) secretion.19 Since the main target of GnRH is the gonadotropes of anterior pituitary gland, stress-induced GnRH suppression can affect the secretion of gonadotropic hormones and consequently reproductive functions.

Mitochondria are responsible for the reactive oxygen species (ROS) production, ATP generation and apoptotic control.20 Owing to the high levels of antioxidant enzymes, the mitochondria can regulate the balance between ROS production and degradation.21 The findings of studies have confirmed that chronic stress can lead to disruption of mitochondrial function and free radicals production.22 It is apparent that mitochondria play a significant role in female reproductive processes including oocyte maturation, fertilization and early embryogenesis.23 The evidence for the involvement of oxidative injury in the
pathogenesis of infertility in females has been accumulating since long.\textsuperscript{24}

Since the adverse effects of early-life stress on the neuroendocrine and innate immune system, also adverse effects of oxidative stress on fertility status have been well described in previous studies, stress-induced maternal separation during early life may have an adverse effect on the female reproductive system. Therefore, this study was designed to investigate the effect of maternal separation stress on ovarian function in adult female mice.

Materials and Methods

Experimental Animals

Pregnant Naval Medical Research Institute mice were obtained from the Pasteur Institute of Iran. The animals were housed under a cycle of 12 h:12 h light/dark at controlled temperature (22–25°C) and humidity (55–65%). All animal procedures were carried out according to guidelines approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1395.2507). To produce maternal separation model, pups were separated from their mothers and placed in a clean cage for 3 h every day (9 am to 12 pm each day) from post-natal days (PND) 2 to 14. The birthday of pups was considered as PND 0.\textsuperscript{25–27} In this study, female pups were randomly divided into two groups: control and maternal separation group. The pups of the control group were left untouched. Mice at PND 70 were sacrificed by deep anesthesia and the ovaries were removed. One ovary from each mouse was used for histological assessment and the second ovary was used for molecular assessments.

Histological Assessment

The ovaries for histological assessment were immediately transferred to Bouin’s fixative solution. The fixed ovaries were dehydrated in ascending graded series of ethanol (Merck, Darmstadt, Germany) and then emerged in paraffin wax. The serial sections (5 μm thick) were prepared with a rotary microtome (Microm, Walldorf, Germany) and rehydrated in descending graded series of ethanol. The sections were cleared in xylene, stained with hematoxylin and eosin (H&E) and mounted with DPX. For histological assessment, transverse sections from nine different regions of the ovaries were examined. The number of primordial, primary, secondary and Graafian follicles were counted by light microscopy and the ovaries were removed. Mice at PND 70 were sacrificed by deep anesthesia and the ovaries were removed. One ovary from each mouse was used for histological assessment and the second ovary was used for molecular assessments.

ROS Assay

The level concentration of ROS production in ovarian tissues was measured in the FL-1 channel using a BD FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).\textsuperscript{29} The expression level of NLRP3, ASC, caspase-1, TLR4, BAX, BCL2 and TNFα genes was analyzed by real-time reverse transcription polymerase chain reaction (RT-qPCR). The total RNA extraction from ovarian tissues was performed with TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The complementary DNA was produced via reverse transcription reaction using a PrimeScript RT reagent kit ( Takara, South Korea) according to the manufacturer’s protocol. The RT-qPCR was carried out with gene specific primers and the HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) by an ABI7500 (Applied Biosystems, Foster City, CA, USA). The reference gene glyceraldehyde-3-phosphate dehydrogenase messenger RNA (mRNA) expression are used as the internal control to normalize mRNA expression levels. The level of target genes expression was calculated using 2−ΔΔCT. List of primer sequences used for RT-qPCR analysis are presented in Table 1.

Enzyme-linked Immunosorbent Assay

The ovarian tissues were homogenized in PBS on ice, then centrifuged at 800 × g for 5 min. The collected supernatants were used in the enzyme-linked immunosorbent (ELISA) assay. The level concentrations of ATP and GPx were measured with specific ELISA kits (R&D Systems, Minneapolis, MN, USA) and the level concentrations of IL-1β and IL-18 in ovarian tissue were measured using ELISA kits (Koma Biotech, Seoul, Korea) according to the manufacturer’s protocols.

Immunocytochemical Analysis

To determine caspase-3 and NLRP3 immunoreactivity, the fixed ovarian samples were degraded in graded ethanol and embedded in paraffin. After removing paraffin, samples were rehydrated through a graded series of ethanol and permeabilized with 10 mM sodium citrate and 0.05% Tween 20. The samples were blocked in a blocking solution including 1% (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and then incubated with primary antibodies against caspase-3 (Abcam, Cambridge, MA, USA) and NLRP3 (R&D Systems, Minneapolis, MN, USA) overnight at 4°C with primary antibodies against caspase-3 (1:1000 dilution, Abcam, Cambridge, MA, USA) and NLRP3 (1:1000 dilution, Abcam, Cambridge, MA, USA). The tissue sections were then incubated with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA). After washing with PBS, the sections were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) and mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA) and then observed under a fluorescent microscope (Nikon Eclipse 80i, Tokyo, Japan). The images were captured using a Nikon DS-Qi1 camera and analyzed using ImageJ software (ImageJ U. S. National Institutes of Health, Bethesda, MD, USA).

Real-time Reverse Transcription Polymerase Chain Reaction Analysis

The expression level of NLRP3, ASC, caspase-1, TLR4, BAX, BCL2 and TNFα genes was analyzed by real-time reverse transcription polymerase chain reaction (RT-qPCR). The total RNA extraction from ovarian tissues was performed with TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The complementary DNA was produced via reverse transcription reaction using a PrimeScript RT reagent kit ( Takara, South Korea) according to the manufacturer’s protocol. The RT-qPCR was carried out with gene specific primers and the HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) by an ABI7500 (Applied Biosystems, Foster City, CA, USA). The reference gene glyceraldehyde-3-phosphate dehydrogenase messenger RNA (mRNA) expression are used as the internal control to normalize mRNA expression levels. The level of target genes expression was calculated using 2−ΔΔCT. List of primer sequences used for RT-qPCR analysis are presented in Table 1.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
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<tr>
<td>NLRP3</td>
<td>5′-GGACCCACAGTG-TAACCCTGAGCAG-3′</td>
<td>5′-AGGGCTG-CAGTGTTGTAATCCAGG-3′</td>
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<td>ASC</td>
<td>5′-CACAATACGTCTC-CAACCAC-3′</td>
<td>5′-AACTTAATTCACTT-TCACA-3′</td>
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<td>Caspase-1</td>
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<td>GADPH</td>
<td>5′-TGAACATCAAGAAGT-GTGAGG-3′</td>
<td>5′-CAGTTGGAAGT-GGGAG-3′</td>
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Maternal separation affects reproductive system in female mice
K. Khodamoradi et al.

(1:1000 dilution, Abcam, Cambridge, MA, USA). The samples were incubated with secondary antibody (1:500 dilution, Abcam, Cambridge, MA, USA) for 2 h at 37°C and then the cells’ nuclei were stained with PI (1:1000, Sigma-Aldrich, St. Louis, MO, USA). The cell counting and merging of the pictures were performed by Image J software (ImageJ U. S. National Institutes of Health, Bethesda, MA, USA).

**Statistical Analysis**

The collected data were analyzed using SPSS version 20.0 software. The values were tested for normality using the Kolmogorov–Smirnov test. The statistical significance of the results was determined using the independent samples t-test and Mann–Whitney U test. The results were presented as the mean ± standard deviation (SD) and P ≤ 0.05 was considered as statistically significant.

**Results**

**Histological Assessment**

Histological analysis of ovarian follicles was assessed with H&E staining (Fig. 1). The results showed that percentage of primordial follicles in the maternal separation group (17.226 ± 9.096) was significantly lower compared with the control group (53.188 ± 4.485) (P < 0.001). There was no significant difference between percentage of primary follicles in the maternal separation group (5.584 ± 3.229) and percentage of primary follicles in the control group (5.839 ± 2.387) (P > 0.05). Percentage of secondary follicles in the maternal separation group (28.247 ± 5.621) was significantly higher compared with the control group (16.899 ± 5.639, P < 0.01). In addition, percentage of Graafian follicles in the maternal separation group (48.941 ± 7.826) was significantly higher compared with the control group (24.072 ± 1.908, P < 0.001, Fig. 2).

**ROS Evaluation**

Reactive oxygen species production in ovarian tissue was detected using the DCFH-DA assay. The level of ROS production in ovarian tissue of the maternal separation group (614.4183 ± 28.6545) was significantly higher compared with the control group (363.9886 ± 48.3404, P < 0.01, Fig. 3).

**RT-qPCR Analysis**

The results of RT-qPCR analysis showed that expression of the following significantly increased in the maternal separation group compared with the control group: TNF-α (P < 0.001), NLRP3, ASC, caspase-1, TLR4 and BAX (P < 0.01). In contrast, the expression of BCL2 gene significantly decreased in the maternal separation group compared with the control group (P < 0.01) (Fig. 4).

**Enzyme-linked Immunosorbent Assa**

Enzyme-linked immunosorbent assay results showed that the level concentrations of ATP and GPx were significantly lower in the maternal separation group compared with the control group (2.4992 ± 0.03 vs. 3.03497 ± 0.02, P < 0.001 and 147.46 ± 11.58 vs. 399.3414 ± 23.39, P < 0.001 respectively). The IL-1β and IL-18 concentrations in the maternal separation group were higher than the control group (2.4773 ± 0.01 vs. 1.8753 ± 0.02 and 3.3494 ± 0.04 vs. 1.9481 ± 0.08 respectively, P < 0.001 for both) (Fig. 5).

**Immunocytochemical Analysis**

Caspase-3 and NLRP3 markers were used to label the ovarian cells in the control and maternal separation groups. The nuclei were stained with PI (Fig. 6). Immunocytochemical analysis showed that the mean percentage of caspase-3 positive cells in the maternal separation group was significantly higher compared with the control group (50.666 ± 1.76% vs. 24.00 ± 0.58%, P < 0.001). Also, the mean percentage of NLRP3 positive cells was significantly higher in the maternal separation group...
Maternal separation affects reproductive system in female mice

K. Khodamoradi et al.

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Maternal separation affects reproductive system in female mice

1. Inflammation and apoptosis including NLRP3, ASC, caspase-1, TLR4, BAX, BCL2 and TNF-α were significantly affected in the maternal separation group. Our results also showed that maternal separation stress significantly increased percentage of caspase-3 and NLRP3 positive cells.

2. According to a large body of evidence, maternal separation has long-lasting effects on neurodevelopmental and behavioral health that can increase susceptibility to psychopathology in adulthood. It is known that the function of HPA axis, an essential hormonal response system to manage stress, adversely affected by maternal separation stress. Considering the regulatory role of HPA axis in many homeostatic systems, these adverse modifications in HPA axis activity can result in functional alterations in the biological systems such as neuroendocrine, immune and reproductive systems.

3. Furthermore, stress can affect the function of the adrenergic system that change the activities of the endocrine and immune systems. Cross-talk between the endocrine and immune systems is necessary for normal development and function of the female reproductive system. Although it remains unknown how innate immune function is stimulated by stress, the role of different factors including glucocorticoids, danger signals and catecholamines are evidenced by several studies.

4. Previous studies have indicated that NLRs play a regulatory role in the reproductive and innate immune systems in mammals. The NLRP3 inflammasome, as a well-known NLR, is a cytoplasmic complex which is activated by danger signals derived from pathogens and metabolic dysregulation. Activation of NLRP3 inflammasome lead to regulating the secretion of the pro-inflammatory cytokines IL-1β and IL-18.

5. Our findings showed that stress induced by maternal separation significantly affect the NLRP3 inflammasome components and inflammatory molecules including NLRP3, ASC, caspase-1, TLR4, TNF-α, IL-1β, IL-18, BAX and BCL2 were significantly affected in the maternal separation group. Our results also showed that maternal separation stress significantly increased percentage of caspase-3 and NLRP3 positive cells.

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9. Our findings showed that stress induced by maternal separation significantly affect the NLRP3 inflammasome components and inflammatory molecules including NLRP3, ASC, caspase-1, TLR4, TNF-α, IL-1β, IL-18 and caspase-3. Furthermore, maternal separation stress can significantly affect mitochondrial activation and resulted in increase of ROS production and decrease of ATP level. In addition, this chronic stress can affect the mRNA expression of BCL2 and BAX genes which are involved in apoptosis pathways.

10. It is well known that increased ROS production is associated with mitochondrial dysfunction and apoptosis. Recently, it has been identified that mitochondria play a key role in activation of NLRP3 inflammasome. Mitochondrial damage can trigger signals of apoptosis and induce NLRP3 inflammasome

Discussion

The findings of this study showed that maternal separation stress led to significant histological alterations in the ovarian tissue, including decreased percentage of primordial follicles and increased percentage of secondary and Graafian follicles. In addition, maternal separation stress during early life increased ROS production and decreased ATP and GPx concentrations. Furthermore, expression of cytokines and genes involved in inflammation and apoptosis including NLRP3, ASC, caspase-1, TLR4, TNF-α, IL-1β, IL-18, BAX and BCL2 were significantly affected in the maternal separation group. Our results also showed that maternal separation stress significantly increased percentage of caspase-3 and NLRP3 positive cells.

According to a large body of evidence, maternal separation has long-lasting effects on neurodevelopmental and behavioral health that can increase susceptibility to psychopathology in adulthood. It is known that the function of HPA axis, an essential hormonal response system to manage stress, adversely affected by maternal separation stress. Considering the regulatory role of HPA axis in many homeostatic systems, these adverse modifications in HPA axis activity can result in functional alterations in the biological systems such as neuroendocrine, immune and reproductive systems.

Furthermore, stress can affect the function of the adrenergic system that change the activities of the endocrine and immune systems. Cross-talk between the endocrine and immune systems is necessary for normal development and function of the female reproductive system. Although it remains unknown how innate immune function is stimulated by stress, the role of different factors including glucocorticoids, danger signals and catecholamines are evidenced by several studies.

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Our findings showed that stress induced by maternal separation significantly affect the NLRP3 inflammasome components and inflammatory molecules including NLRP3, ASC, caspase-1, TLR4, TNF-α, IL-1β, IL-18 and caspase-3. Furthermore, maternal separation stress can significantly affect mitochondrial activation and resulted in increase of ROS production and decrease of ATP level. In addition, this chronic stress can affect the mRNA expression of BCL2 and BAX genes which are involved in apoptosis pathways.

It is well known that increased ROS production is associated with mitochondrial dysfunction and apoptosis. Recently, it has been identified that mitochondria play a key role in activation of NLRP3 inflammasome. Mitochondrial damage can trigger signals of apoptosis and induce NLRP3 inflammasome
activation through release of oxidized mitochondrial DNA into cytosol. Furthermore, the findings of studies indicated that mitochondrial ROS and DNA play an important role in NLRP3 inflammasome activation. In addition to its role in apoptosis, it has been suggested that BCL2 might play a role in the inhibition of NLRP3 inflammasome activation. Shimada et al. have described that increased expression of BCL2 lead to decrease in IL-1β levels. From this, it can be concluded that apoptosis plays a role in NLRP3 inflammasome activation. In addition, recently some studies have described that TNF, as a proinflammatory cytokine, can regulate the NLRP3 inflammasome activation. Given these data and our findings, plus the known effects of stress on the neuroendocrine system, it seems that maternal separation stress can affect the NLRP3 inflammasome components and inflammatory molecules with alteration in level of ROS, BCL2 and TNF-α and subsequently lead to increase of IL-1β and IL18- production.

Our findings showed that maternal separation resulted in histological alterations in the ovarian tissue, including decreased percentage of primordial follicles and increased percentage of secondary and Graafian follicles. In addition to reducing the number of primary follicles, stress likely accelerates the process of evolution of primary follicles to secondary and Graafian follicles. It seems that maternal separation stress lead to fast down the folliculogenesis process that may reduce the ovarian follicular reserve and induce a shorter reproductive lifespan.

In our study, maternal separation stress cause to increase of ROS production and decrease of GPx concentration. Cells have defensive strategies to prevent oxidative injury caused by excessive production of ROS, such as anti-oxidant enzymes like catalase (CAT), superoxide dismutase and GPx. Although ROS play physiological roles during folliculogenesis process, maturation of oocyte, and fertilization, increased ROS production and oxidative stress can suppress the antioxidant defense and influences the fertilization capacity. The findings of studies indicated that oxidative stress lead to follicular atresia and ovarian follicle aging. Furthermore, excessive production of ROS and oxidative stress can lead to pathologic events in the ovary including inflammation and apoptosis.

Findings of studies described that IL-1 is expressed in the mammalian ovary and play a physiological role in ovary. Although inflammation is very important in reproductive processes such as ovulation, menstruation and implantation, uncontrolled inflammation has adverse effects on normal function of ovary. Uri-Belopolsky et al. have reported that IL-1 may enhance the inflammatory genes expression and promote apoptotic signaling pathways that leads to exhaust the ovarian reserve. On the other hand, it is well known that follicular atresia can occur via mitochondrial pathway or binding of death receptors to their ligands such as TNF-α and Fas. According to the above-mentioned data and the results of this study, we can conclude that maternal separation stress via activation of inflammatory and apoptosis pathways may have adverse effects on folliculogenesis process and the number of ovarian follicles.

Conclusion
This study provides a glimpse of the effects of maternal separation stress on ovarian tissue in female mice as adults. Based on this study, maternal separation stress had detrimental effects on ovarian tissue, in addition to the known effects of stress on the HPA axis and neuroendocrine system, probably through increase of ROS production and impact on mitochondrial function, inflammatory process and apoptosis pathways. An improved insight into the mechanisms by which maternal separation stress affects the reproductive system may provide necessary information to keep the reproductive system healthy.

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Conflict of Interest
The authors declare that there is no known conflict of interest regarding this publication.

References