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Chronic Stress Diminishes the Oocyte Quality and In Vitro Embryonic Development in Maternally Separated Mice

Leila Dehdehi[1], Marefat Ghaffari Novin[1,2], Yousef Sadeghi[1], Mohammad Amin Abdollahifar[1], Seyed Ali Ziai[3], Hamid Nazarian[2,1*]

Abstract

Objectives: This study aimed to use a valid mouse model of chronic stress like a maternal separation (MS) to determine the effect of early life chronic stress on oocyte quality and subsequent in vitro embryo development.

Materials and Methods: This study was based on case-control, interventional, and quantitative applied research. Mice were subjected to 180 minutes of MS stress paradigm at postnatal day (PND) 2–14. Then, corticosterone and serotonin levels were measured in the serum and ovary samples, respectively. In addition, relevant behavioral tests including an elevated plus maze (EPM) and open field test (OFT) were performed for evaluating anxiety-like behaviors at PND 48. Finally, oocyte number, nuclear maturation, reactive oxygen species (ROS) and intracellular glutathione (GSH) levels, as well as in vitro embryo development were evaluated as well.

Results: Our findings showed that MS provokes anxiety-like behavior and increases serum corticosterone concentration ($P<0.05$). On the other hand, the number of oocytes ($P<0.001$), nuclear maturation ($P<0.05$), and the concentration of ovarian serotonin ($P<0.01$) decreased following MS. Further, the fertilization ($P<0.001$) and blastocyst rate ($P<0.05$) significantly decreased in MS mice. Eventually, chronic stress led to a reduction in the level of GSH ($P<0.01$) while it increased the level of ROS production ($P<0.001$).

Conclusions: Chronic stress through, at least in part, oxidative stress in the oocytes of mice undergoing MS paradigm negatively affected the oocyte competency and embryo development.

Keywords: Maternal separation, Oocyte maturation, Embryonic development, Oxidative stress

Introduction

According to some researchers (1,2), the fertility problem has increased the demand for assisted reproductive techniques (ARTs) and evidence demonstrates that psychological stress has detrimental influences on the reproduction system and may affect the ART outcomes (3-5). In this regard, some studies have reported the effects of stressful experiences in the life span that reduces the success of in vitro fertilization (6,7).

Experiencing stressful conditions in early life negatively influences the behaviors in adulthood that can be considered as a reason for vulnerability to psychiatric disorders like anxiety and depression in the future (8-11). Although previous research has focused on the effects of anxiety and depression on the outcomes of ARTs, very little is found about the influence of chronic stress on the oocyte quality and in vitro embryonic development.

To examine the effects of psychological stress on ART outcomes, considerable heterogeneity and variability are observed in human being studies in terms of mean age, infertility duration, etiology, as well as the timing of stress evaluation and final reports (3). In addition, employing animal models of chronic stress provide conditions for studying the neuroendocrine and behavioral effects of early adversity (12,13) and yielded experimental procedures for examining the involved mechanisms by which chronic stress exerts its influences on the reproductive function. In this concept, maternal separation (MS) as an early life chronic stress is confirmed as an approved paradigm leading to anxiety-like behaviors in later life (14-16). In the MS, model rodent offspring' is detached from its mother before weaning which is accompanied by an alteration in behavioral and hypothalamic–pituitary–adrenal (HPA) axis responsiveness (17,18).

Serotonin or 5-hydroxytryptamine (5-HT) is a known neurotransmitter implicated in behavioral and mood dysfunction such as depression and anxiety (19) and is involved in brain development and embryogenesis (20). In addition to the brain, there is a local serotonergic network in the mammalian reproductive system where it may affect oocyte, granulosa, and cumulus cells, as well as early embryos (21,22).

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[1]Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [2]Infertility and Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [3]Departmentof Pharmacology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
***Corresponding Author:** Hamid Nazarian, Tel: +98-2122712234, Fax: +98-2122716383, Email: H.Nazarian@sbmu.ac.ir

In addition, chronic stress is known to affect homeostasis (23), HPA axis (24), and serotonin regulation (25). Studies with acute stress models exerted stresses on the short period in the time of final follicular growth and maturation that did not mimic the natural condition (26,27) and the evidence of the exact influence of chronic psychological stress on the follicular development and oocyte competency after the postnatal period is bounded. Considering the above-mentioned discussion, this study aimed to examine the effects of chronic stress on oocyte quality and later in vitro embryo development using a novel MS model and to observe if early life stress may affect the reproductive system in adult female mice and if these changes could be related to altered ovarian serotonin content.

Materials and Methods

All reagents and chemicals of this research, unless otherwise specified, were purchased from Sigma Chemical Company (Sigma, USA).

Maternal Separation Model

Pregnant Naval Medical Research Institute (NMRI) mice (Pasteur Institute, Tehran, Iran) were kept in 12 hours light/dark photoperiod at controlled temperature (22 ± 1°C) with adequate food and water in control and maternally separated groups. The first 24 hours after birth was considered as postnatal day 0 (PND 0). Then, mother-pup separation was performed for 180 min/d during PND 2-14 (starting between 09:00 AM and 12:00 AM) in the MS paradigm (28). Further, the pups were brought back to the nesting cage after the separation period. At PND 21, female offspring were housed together until evaluation day PND 48 (10,28,29). There were 8 to 10 animals in each experimental group.

Behavioral Tests

Elevated Plus Maze

The elevated plus maze (EPM) is a plus-shaped Plexiglas apparatus with two opposite open (30 × 5 cm) and closed (30 × 5 × 15 cm) arms with a center of a platform (5 × 5 cm) that is approved for evaluating the anxiety-like behaviors in rodents (30). Animals were placed individually in the center platform of the apparatus in the dimly illuminated testing room and 5 minutes period of the behavioral session was recorded for each animal. Furthermore, the number of entries into open arms, as well as the total spent time was recorded over 5 minutes. Then, the recordings were analyzed by EthoVision XT software (Noldus, Netherland) and 70% ethanol was used to clean the apparatus after testing each mouse.

Open-Field Test

The open-field test (OFT) was applied to examine the depression and anxiety-like behaviors of mice in different conditions (31). This behavioral apparatus consisted of a large square box measuring 50 cm× 50 cm × 30 cm with the surrounding walls. Each mouse was placed in the 30 cm × 30 cm central zone arena of the OFT box. The number of entrances and the time spent to the central area were recorded by a camera for a 5-minute test period. Then, video analysis was performed by EthoVision XT software (Noldus, Netherland) and the open-field apparatus was cleaned between each mouse using 70% ethanol.

Sample Collection and Biochemical Analysis

Cardiac blood samples were taken and centrifuged at 4200 rpm for 12 minutes at 4°C. Then, the serum was aliquoted in micro-tubes and frozen at -20°C until the analysis day (32), followed by performing the serum corticosterone measurement using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's manuals (MyBioSource, San Diego, USA, MBS494312). For ovarian 5-HT and 5-hydroxyindolacetic acid (5-HIAA) determination, tissues were placed in ice-cold phosphate buffer saline (PBS) and weighed before homogenization (33). After centrifuging the homogenates for 10 minutes at 5000 rpm, the supernatant was removed and stored for ELISA analysis and then assayed based on manufacturer's instructions (MyBioSource, San Diego, USA, MBS723181, MBS700811 for 5-HT and 5-HIAA, respectively). Eventually, each treatment was tested three times and the concentrations of corticosterone and serotonin were determined according to their respective standard curves.

Cumulus-Oocyte Complexes Isolation

To obtain cumulus-oocyte complexes (COCs), female mice of 7-8 weeks were primed with 10 IU pregnant mare serum gonadotropin (PMSG, Gestyl, Organon), followed (46-48 hours later) by the second injection of 10 IU human chorionic gonadotrophin (hCG, Pregnyl, Organon). After 14-16 hours, mice were killed by cervical dislocation and the COCs were immediately isolated from the oviducts, and finally, oocyte denudation was performed with human tubal fluid (HTF) medium containing 80 IU/mL hyaluronidase (34).

Measurement of Intracellular Reactive Oxygen Species and Reduced Glutathione Levels

Denuded mature oocytes at the metaphase II (MII) stage were selected and GSH and intracellular reactive oxygen species (ROS) levels were measured by using 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (Cell Tracker Blue CMF2HC Molecular Probes; Invitrogen) and 2-7-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen), respectively, according to previously described criteria (35, 36). In brief, 25-30 oocytes were incubated in PBS containing polyvinyl alcohol (PBS/PVA, 1 mg/mL), 10 μM Cell Tracker Blue and 10 mM H2DCFDA for 30 minutes. Following incubation, the oocytes were washed with PBS/PVA and observed by excitation at 370 nm

and 460 nm for GSH and ROS under the fluorescence microscope, respectively (Labomed Lx 400). Fluorescence intensity quantification on images was analyzed by ImageJ software (version 1.41, National Institute of Health) and this experiment was replicated at least three times.

In Vitro Fertilization

According to previous studies (26,37), the sperm was obtained from fertile NMRI male mice and dispersed in the HTF medium with 10 mg/mL BSA at 37 °C for 10 minutes. The collected COCs from the oviducts were placed in fertilization drops (HTF containing 15 mg/mL BSA) and then the capacitated sperm was added and concentration was adjusted to 1 million/mL. After incubation for six hours, fertilized oocytes displaying extruded second polar body and two pronuclei (2-PN) were cultured for further embryonic progress. The numbers of embryos up to the blastocyst stage were evaluated daily under an inverted microscope (Nikon, Tokyo, Japan).

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism software, version 7. The results are summarized as the means ± standard error of mean (SEM). Differences between the two groups were analyzed using the unpaired two-tailed Student's *t* test. A P<5% was considered statistically significant. Finally, the correlation between ovarian serotonin level and ROS production was analyzed using the Pearson correlation.

Results

Effect of Maternal Separation on Anxiety-Like Behaviors in Mice

In the EPM, it was observed that MS mice have a lower number of open arm entries and percentage time spent in open arms in comparison with the control mice ($P<0.01$ for both), the details of which are illustrated in Figure 1A-B. In addition, the number of entries and the spent time to the central arena in the OFT significantly decreased in the MS mice compared to the control mice ($P<0.05$ and $P<0.001$, respectively), the related data are displayed in Figure 1C-D.

Analysis of Serum Corticosterone

Figure 2 depicts the serum corticosterone levels after MS procedure. T-test analysis revealed that corticosterone levels increased significantly in the maternally separated mice when compared to the control group ($P<0.05$).

Effect of MS on Intracellular ROS and Glutathione Levels

Oxidative stress was analyzed by measuring intracellular ROS production in oocytes. Based on the results, the intracellular ROS level was significantly greater in the maternally separated oocytes in comparison to the control oocytes ($P<0.001$, Figure 3A-B). Along with these results, the intracellular levels of GSH, as a parameter of

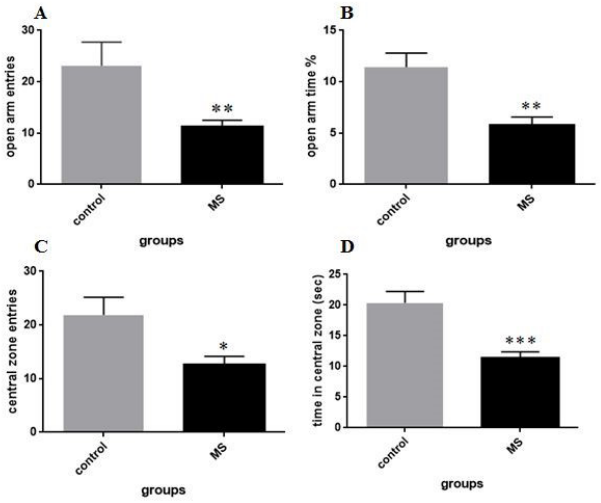

Figure 1. Effect of MS on the Numbers of Open arm Entries (A) and Percentages of Open arm Time (B) in the EPM.
Note. The number of central zone entries (C) and time spent in the central zone in the OFT (D). All values are expressed as the mean± S.E.M from 8 to 10 animals and analyzed using the t-test; $^{*}P$<0.05, $^{**}P$<0.01, and $^{***}P$<0.001 in comparison to the control group.

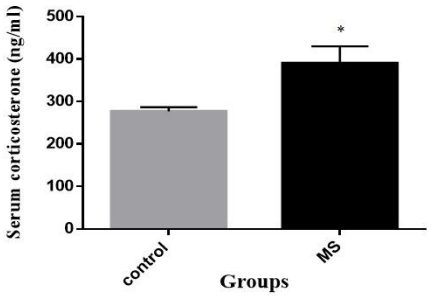

Figure 2. Corticosterone Levels in the Serum of Control and MS Groups.
Note. All values are expressed as the mean± SEM and analyzed using the *t* test; $^{*}P<0.05$ compared with the control group.

antioxidant capacity, were significantly lower in the MS oocytes when compared to the control oocytes ($P<0.01$, Figure 3C-D).

Effect of MS on Oocyte Number, Nuclear Maturation, and In Vitro Embryonic Development

After ovarian induction, the number of retrieved oocytes in the control mice was statistically higher than the MS group ($P<0.001$, Figure 4). The results of the MS effect on the nuclear maturation of oocytes are represented in Table 1. This chronic stress elevated the number of the oocyte at metaphase I (MI) stage ($P<0.05$) while it reduced the number of the oocyte at the metaphase II stage ($P<0.05$) when compared with the control mice. Figure 5 illustrates the representative images of 2-cell and 4-cell embryos, as well as blastocyst stage embryos. The rates of fertilization, cleavage, and blastocyst demonstrated statistically significant differences. As shown in Table 1 and Figure 5, the 2-PN formation rate was lower in the stressed mice ($P<0.001$) and 2-cell ($P<0.01$), 4-cell ($P<0.001$),

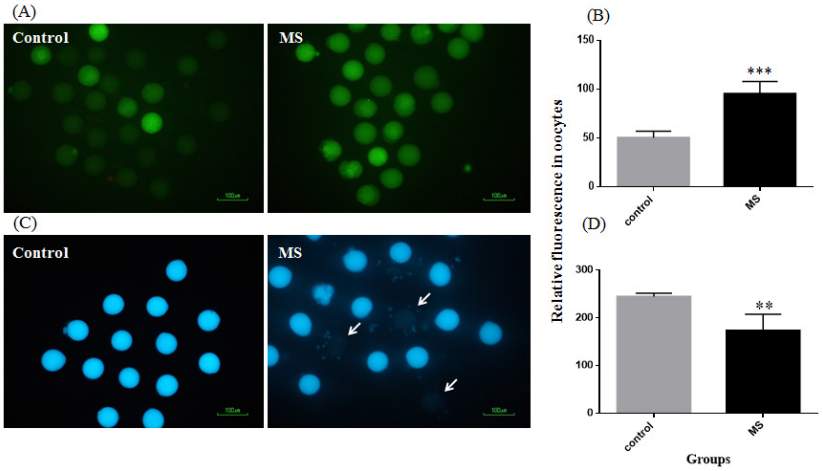

Figure 3. Intracellular Reactive Oxygen Species (ROS) (A, B) and Reduced Glutathione (GSH) Levels (C, D) in MII Oocytes From Control and MS Mice. *Note*. Fluorescence intensity for the ROS significantly increased in MS oocytes. On the other hand, the intensity of the cell tracker blue for the GSH reduced in MS oocytes in comparison with the control group. The data indicates the lowest amount of cell tracker blue in the oocyte quantification of ROS (B) and GSH (D) levels in oocytes from control and MS groups. In addition, the arrows show the oocytes with the lowest intensity; $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control group (Bar=100 µm).

and blastocyst rates ($P<0.05$) decreased in maternally separated oocytes in comparison to the control group.

Analysis of Ovarian 5-HT and 5-HIAA Concentration

5-HT concentration in the ovaries of separated mice decreased significantly compared to the control group ($P<0.01$) while its metabolite 5-HIAA represented no change (Figure 6A-B).

The Correlation Between Oxidative Stress and Serotonin Level

In maternally separated mice, a significant correlation was observed among reduced ovarian serotonin concentration and increased production of ROS (Figure 6C).

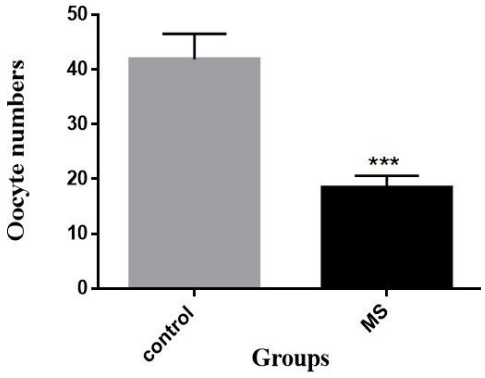

Figure 4. Oocyte Number Retrieved After Gonadotropin Injection. *Note*. All values are expressed as the mean± SEM and analyzed using the *t* test; $^{***}P<0.001$ compared with the control group.

Discussion

The present study examined the possible influence of early chronic stress on the oocyte quality and embryonic development of MS oocytes. According to the results of this study, early MS, as an animal model of chronic stress, deteriorated the oocyte quality and embryonic development in adult female mice. The results indicated that MS stress decreased the nuclear and cytoplasmic maturation and impaired in vitro embryonic development. In addition, these effects were accompanied by a decrease in the ovarian serotonin level.

In the current study, the evaluation of mice responses in behavioral tests showed that the MS stress increased anxiety-like behaviors significantly and elevated the serum corticosterone concentration. Exposure to this arena in the OFT causes an anxiety reaction because rodents do not like large open spaces, which is defined by fewer entries and less time spent in the central part of this test. Further, less spent time in the open arms and entries in the EPM are mostly related to anxiety levels (12). Our findings are in agreement with the results of the other studies, showing that MS procedure increases the behaviors related to anxiety behaviors, as well as increased corticosterone levels (18,38).

Furthermore, the corticosterone level was higher in

Table 1. Nuclear Maturation and Fertilization Rates

Groups	MI (%)	MII (%)	2-PN Rate (%)	2-Cell Rate (%)	4-Cell Rate (%)	Blastocyst Rate (%)
Control	22.64±3.75	71.15±2.76	79.00±1.45	70.38±2.71	66.30±3.469	38.23±3.496
MS	46.18±6.14 [a]	49.58±5.80 [a]	64.48±2.59 [c]	46.89±4.145 [b]	42.03±2.148 [c]	19.89±5.04 [a]

Note. Values are shown as the mean ± SEM.

[a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. MI: Metaphase I; MII: Metaphase II.

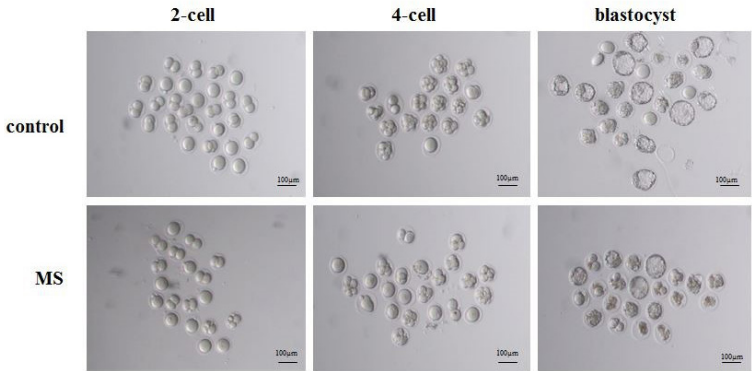

Figure 5. 2-Cell, 4-Cell and Blastocyst Rate of Control and Maternally Separated Group.
Note. Oocytes in MS group showed lower 2-cell (*P*<0.01), 4-cell (*P*<0.001), and Blastocyst rate (P<0.05) compared to control group (Bar = 100 µm).

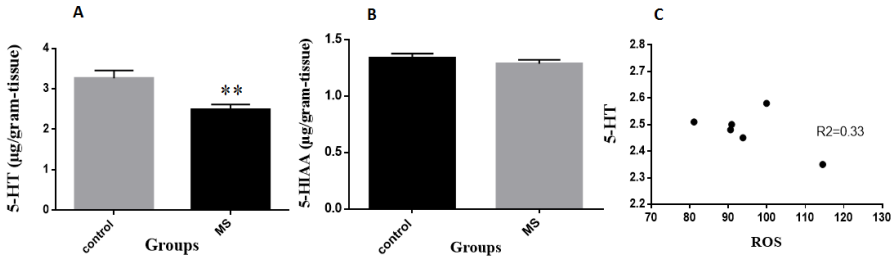

Figure 6. (A) 5-HT Levels in the Ovaries of Control and MS Groups, (B) 5-HIAA Levels in the Ovaries of Control and MS Groups, (C) Correlations Between Ovarian Serotonin Content and Production of Reactive Oxygen Species in Maternally Separated Mice Analyzed Using Pearson's Correlation.
Note. All values are expressed as the mean ± SEM and analyzed using *t* test; **P<0.01 in comparison to the control group.

maternally separated than normal mice that could be a sign of HPA axis activity. The corticosterone is considered as the major glucocorticoid in stress responses (26). In similar studies, an alteration in the HPA axis was related to anxiety-like behaviors in the EPM and OFT (39,40). Moreover, a significant correlation was found between cortisol as a stress hormone and anxiety in human studies (41).

Our results demonstrated that MS stress causes deterioration in ovarian 5-HT content. Serotonin contributes to brain development and is involved in embryogenesis (42). Additionally, there is a local serotonergic network between mammalian follicles and oocytes that have a distinct role in embryo formation (20,43-45). It is worth to mention that 95% of serotonin is produced in peripheral organs and regulates major functions (46). Likewise, serotonin not only is implicated in the regulation of the hypothalamus-hypophyseal-gonadal axis but also its effects could be observed in the ovary. One possible explanation for decreasing the concentration of the serotonin in maternally separated mice is serotonin transporter presence in ovaries that deteriorate the extracellular level of serotonin (47). Evidence has indicated that MS stress disrupts the completeness of the serotonergic system that controls anxiety-like behaviors (16,48). Furthermore, some studies have revealed that elevated glucocorticoid concentration impairs the serotonergic neurotransmission system and inhibits serotonin synthesis (46,49). In this study, there was no difference in the 5-HIAA level, as was previously reported it does not always alter in the same direction as 5-HT (50).

In this study, the number of oocytes that retrieved after the pregnant mare serum gonadotropin priming and hCG triggering, were extremely lower in stressed mice. To support our finding, some studies showed that the administration of glucocorticoids reduced the number of ovarian follicles in neonatal animals which, finally, affected the number of retrieved oocytes (51,52).

Oocyte maturation involved both cytoplasmic and nuclear maturation that warranted the proper fertilization and embryo development (53). Meiosis resumes and first polar body extrusion, as well as germinal vesicle breakdown occur and M II competent oocyte is formed after growing and triggering the follicle with gonadotropins. Impairment in this transition and the absence of first polar body (M I oocyte) make incompetency (26,54). In this research, the nuclear maturation of oocytes in MS oocytes was affected and the percentage of MI oocytes was increased as well. In this regard, although Liu et al reported that the nuclear maturation was not affected in predatory stress but they reported cytoplasmic maturation is more vulnerable to stress (26).

The present study also explored the influence of MS on ROS and intracellular GSH levels. In addition, stress was associated with increased ROS levels in oocytes. These findings are in accordance with recent observations, indicating that mouse serum, ovaries, and oocytes

affected by oxidative stress in restraint stress. (55). Other studies showed that an increased level of ROS resulted in the peroxidation of lipids (56) that recently have been reported this can be modified by serotonin (57). In supporting the effects of chronic stress on ROS generation, our data demonstrated that ovarian serotonin is inversely correlated with the ROS level in oocytes.

Our results further indicated that the fertilization rate, as well as the percentage of cleavage and blastocyst rate was lower in stressed mice. Previous studies reported that acute stress such as predatory stress and restraint models during 24 hours can impair the oocyte developmental potential (26,55). Zhang et al. concluded that restraint stress as acute stress can decrease the rate of blastocyst formation in the stressed mice but the fertilization was unaffected by this model (58). In another study, Gao et al. found that restraint stress more than four days impairs oocyte competency but in a few days, it did not differ with the control group. They also used several physical stressors (i.e., hot, cold, and shaking) synchronously that reflected no natural condition (27). Our separation model was more similar to the conditions that arose from early life difficulties. In ovarian folliculogenesis, primordial to primary follicle transition occurs in postnatal time (59) that can be affected by this early life stress model.

The decrease in the embryonic development observed in the MS mice possibly results from the deterioration of intra-oocyte GSH and an increase in the ROS level. Evidence reported that oxidative stress can impair oocyte activation and male pronucleus (PN), and consequently blastocyst formation by decreasing the Ca^{2+} reserves (37, 60). Another study found that an increase in intracellular ROS induces cell cycle arrest and apoptosis (7). The impairment in the serotonergic system is another explanation for these significant differences in embryonic development. Serotonin may be a local modifier in mouse cumulus cells, and oocytes by its action on Ca^{2+} and cAMP signaling that is essential for fertilization and proper embryo development (22).

Employing animal models of psychiatric disorders provides opportunities to study the underlying mechanism of disorders. Anxiety and mood disorders are the most frequent psychiatric disorders in children and adolescents (61,62). It was revealed that separation anxiety in children leads to social and emotional disturbances in the future (63). Taken together, it is possible that anxiety in children can be a risk factor for vulnerability to reproductive disorder in adulthood. On the other hand, some In vitro fertilization undergoing patients suffer from anxiety and depression that can be due to psychiatric disorders in the past and thus recognizing this matter can lead to therapeutic strategies.

Conclusions

In general, our results indicated that experiencing MS as chronic stress impairs the oocyte quality and in vitro embryo development in adult female mice. In addition, these changes were partly associated with a considerable increase in the intracellular ROS level of MS oocytes and a decreased level of ovarian serotonin. In spite of limitations related to blood volume and sampling, our ELISA tests ran correctly. More investigation is needed to obtain further knowledge about the other neurotransmission and underlying mechanisms involved in the influences of MS in embryonic development in later life.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

All experimental protocols were approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1395.370) and institutional guidelines for animal care and use (Department of Biology and Anatomical Sciences, School of Medicine, SBMU).

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