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Research article

The effects of maternal separation stress experienced by parents on male reproductive potential in the next generation

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ABSTRACT

There is little information available about the effects of early-life parental stress on the reproductive potential of the next generation. The aim of this study is to examine the reproductive potential of male mice whose parents experienced maternal separation stress. In the present study, male first-generation offspring from parents were undergone of maternal separation (MS) were examined. Sperm characteristics, histological changes in testis, reactive oxygen species (ROS) production, expression of apoptotic and inflammatory genes and proteins were assessed. Findings showed that MS experienced by parents significantly decreased the morphology and viability of spermatozoa. Furthermore, significant changes in testicular tissue histology were observed. Increased production of ROS, decreased glutathione peroxidase (GPX) and adenosine triphosphate (ATP) concentrations, and affected the expression of genes and cytokines involved in inflammation. Finally, the mean percentage of caspase-1 and NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) positive cells was significantly higher in first-generation group. MS experienced by parents may negatively affect the reproduction of first generation offspring.

1. Introduction

It is well proven that early-life stress has long-lasting impacts on an individual's behavior in adulthood (Kikusui et al., 2005; Oomen et al., 2010; Nishi et al., 2013; Korosi et al., 2012). Early-life events that increase reactions to stress lead to increased stress hormone exposure and consequently increased susceptibility to stress-induced disease throughout the life span (Francis and Meaney, 1999).

Furthermore, there is a large amount of evidence that stress can interfere with the functions of the reproductive system (Rai et al., 2003; McGrady, 1984; Rabin et al., 1988; Khodamoradi et al., 2019a). Studies have examined the effects of different types of stress on sex hormones, and investigators are still trying to understand the mechanisms through which stress affects reproductive functions in both sexes. Sexual functions can be affected by stress-related hormones at all three levels of the hypothalamic–pituitary–gonadal axis (HPG axis) (Rivier and Rivest, 1991). Early research demonstrated that stress is attended by an increase in hypothalamic-pituitary-adrenal (HPA) axis activity and a decline in reproductive functions that can be a way to preserve the activity of the adrenal cortex at the expense of gonadal activity (Selye, 1939). It is well known that adrenal corticosteroids, corticotropin releasing factor (CRF), and adrenocorticotropic hormone (ACTH) have key roles in modulating the impact of stress on reproductive functions (Rivier and Rivest, 1991). Previous studies have determined that experienced stressful conditions by parents could negatively affected male reproductive function and reduced the quality of sperm in offspring (McNamara et al., 2014; Rodgers et al., 2013; Lavoie et al., 2019). It has further been shown that maternal separation stress has long-term effects on male sexual development (Rhees et al., 2001). Moreover, our previous study demonstrated that early-life stress induced by maternal separation has damaging impacts on testicular tissue and sperm parameters that may be mediated through influence on mitochondrial function, activation of reactive oxygen species (ROS) production, apoptosis pathways, and inflammatory processes (Khodamoradi et al., 2019b). In this regards it has been

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determined that inflammatory response and overproduction of inflammatory markers including TLR4, NLRP3 and TNFα could activated the apoptotic reactions which consequently impose negative effect on sperm traits (Khodamoradi et al., 2019b; Ahmed, 2015; Taylor et al., 2004). There is concern about whether the effects of early-life stress can be transmitted to subsequent generations. Although some studies have evaluated the harmful effect of early-life stress on behavioral responses across generations (Schmauss et al., 2014), there is little information available about the effects of early-life parental stress on the reproductive potential of their next generation offspring. Maternal separation stress, as a well-studied model of early-life stress, can result in long-lasting alterations in the central nervous system development, such as the stress-response regulatory systems. According to previous studies, higher maternal behavior has been observed in adult females who received intensive maternal care in the early stage of life. Thus, it has been suggested that maternal behavior may be transmitted to the next generation through non-genetic inheritance means (Kikusui et al., 2005; Champagne et al., 2003a; Francis et al., 1999a, 1999b). In addition, a mother's behavior with her offspring can play a significant role in the programming of behavioral and neuroendocrine responses to stress in adulthood. These effects are linked with constant alterations in gene expression in regions of the brain that are mediators of stress response (Meaney, 2001).

Previous investigations showed that stress-induced ovarian tissue changes can be transmitted to first-generation offspring. However, there has been little investigation into whether maternal separation stress experienced by parents can affect the male reproductive potential of their offspring. In the other word, there is little evidence about the underlying mechanisms mediating the negative and harmful effect of experience stress by parents on sperm quality in next generation offspring. Hence, this study was designed to examine the reproductive potential of male mice whose parents experienced maternal separation stress focusing role of inflammatory responses, oxidative stress and apoptosis.

2. Materials and methods

2.1. Experimental animals

Pregnant NMRI (Naval Medical Research Institute) mice were purchased from the Pasteur Institute of Iran. The animals were kept under controlled humidity (55–65%) and temperature (22–25 °C) conditions, with a light-dark (12 h–12 h) cycle. All procedures were performed according to guidelines approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1395.2507). In this study, pups were randomly distributed into two groups: maternal separation (MS) parents and control parents groups. In the MS parents group, pups were isolated from their mothers and housed in a separate cage for 3 h between 9 am and 12 pm each day from postnatal day (PND) 2–14 (Lorigooini et al., 2019, 2020). The birth day was considered as PND 0. The pups of the control parents group did not separate and were with their mother. Pups of both groups were used to generate the first-generation litters (Figure 1). Then, the male pups of the first-generation were used in this study: MS offspring and control offspring groups. Each group was contained 16 mice. Mice at PND 70 were sacrificed under deep anesthesia and had their testes were removed. One testis from each mouse was used for molecular assessments and the second testis was used for histological assessment. All experiments were performed in triplicate (three measurements repeated on the same set of individuals).

2.2. Semen analysis

The epididymis was separated from the testis and placed in a Petri dish containing 1 ml of Ham's F-10 medium (Life Technology, Carlsbad, CA, USA) supplemented with 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA). Sperm fluid was recovered from cauda epididymis by dissection of the epididymis using needled-tuberculin syringes and incubated for 30 min at 37 °C and 5% CO2. Then, the obtained suspension was centrifuged at 300 x g for 5 min and the sperm pellet was re-suspended in 1 ml phosphate-buffered saline (PBS, Sigma-Aldrich, Madrid, Spain). Epididymal spermatozoa were evaluated for count, total motility, progressive motility, morphology, and viability according to the world health organization (WHO) manual guidelines (Organisation, 1999). About 200 spermatozoa per replicate were assessed at 400X magnification.

2.3. Sperm count

The sperm suspension was diluted (1:20) with PBS, and then 10 μl of this diluted suspension was transferred onto a Neubauer slide and spermatozoa were counted under a light microscope.

2.4. Sperm motility

For assessing sperm motility, 10 μl of the sperm suspension was placed on a Neubauer slide and the percent of motile sperm was assessed under a light microscope according to the following criteria (Organisation, 1999); Grade A: percent of sperm with rapid progressive motility; Grade B: percent of sperm with slow or sluggish progressive motility; and Grade C: percent of sperm with vibrating motility. Total motility was calculated by adding Grades A, B, and C.

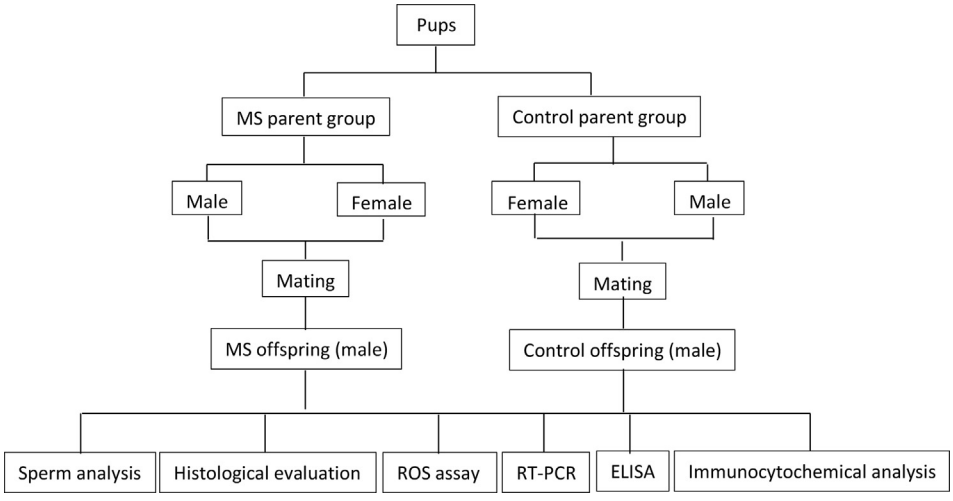

Figure 1. Schematic of study design.

2.5. Sperm morphology

Diff-Quik stain was used for sperm morphology assessment according to WHO guidelines (Organisation, 1999). The stained slides were observed at 1000X magnification using a light microscope. At least 100 spermatozoa per slide were counted to calculate the percentage of normal spermatozoa.

2.6. Sperm viability

The one-step eosin-nigrosin staining technique was used for assessment of sperm viability (Björndahl et al., 2003). The semen sample and eosin-nigrosin solution (1:1) were transferred into a microtube and mixed by pipetting. This suspension was incubated at room temperature for 30 s and then a smear was prepared. Air-dried slides were observed under a light microscope to calculate the percentage of alive sperm (colorless) and dead sperm (red color in head) (Klimowicz-Bodys et al., 2012).

2.7. Histological evaluation

The testicular samples for histological analysis were immediately placed in Bouin's fixative solution. The fixed testes were dehydrated through graded ethanol (50%, 70%, 90% and absolute; Merck, Darmstadt, Germany) and then embedded in paraffin. The serial 5-μm thick sections were obtained using a rotary microtome (Microm, Walldorf, Germany) and rehydrated in a graded series of ethanol (absolute, 90%, 70%, and 50%). After clearing in xylene, the sections were stained with haematoxylin and eosin and mounted with dibutyl phthalate in xylene (DPX). For histological evaluation, transverse sections from nine different regions of the testis were examined. The inner diameter, outer diameter, and thickness of seminiferous tubules were measured by light microscopy (Mazaud-Guittot et al., 2011) and ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA).

2.8. ROS assay

The concentration of ROS production in testicular tissues was measured by flow cytometry using 2′, 7′ -dichlorofluorescin diacetate (DCFH-DA; Sigma, USA) after enzymatic digestion of mechanical minced tissue (Dym et al., 1995). The testicular tissues were mechanically homogenized in Ham's F-10 medium (Life Technology, Carlsbad, CA, USA) and then centrifuged at 10,000 x g for 5 min. The testicular homogenates were incubated with 20 μM DCFH-DA at 37 °C in the dark for 45 min. After washing with PBS, Dichlorofluorescein (DCF) fluorescence (green) was measured in the FL-1 channel by a BD FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) (Fatemi et al., 2014).

2.9. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

The expression levels of NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3), TLR4 (toll like receptor 4), ASC (apoptosis-associated speck-like protein containing a CARD), caspase-1, TNFα (Tumor Necrosis Factorα), BAX (BCL2-associated X protein), and BCL2 (B-cell lymphoma 2) genes were analyzed using RT-PCR. The isolation of total Ribonucleic acid (RNA) from testes tissue was performed with TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The complementary DNA (cDNA) was produced in a reverse transcription reaction using a PrimeScript RT reagent kit (Takara, South Korea) according to the manufacturer's protocol. The RT-PCR was carried out with gene specific primers and HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) using an ABI7500 (Applied Biosystems, Foster City, California, USA). The mRNA expression levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. The target genes' expression levels were calculated by $2^{-\Delta CT}$ (Nouri et al., 2020). The primer sequences used for RT-PCR are shown in Table 1.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The testicular tissue samples were mechanically homogenized in PBS on ice. After centrifuging, the collected supernatant was used in the ELISA assay. The concentrations of interleukin 1β (IL-1β) and interleukin -18 (IL-18) in testicular tissue were detected using general ELISA kits (Koma Biotech–Korea) and the concentrations of glutathione peroxidase (GPX) and adenosine triphosphate (ATP) were detected by specific ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocols.

2.11. Immunocytochemical analysis

The fixed testicular tissues were dehydrated in a graded series of ethanol and then the 5-μm thick sections were prepared from paraffin-embedded testicular tissues. After deparaffinization, the tissue sections were rehydrated in graded ethanol to determine caspase-1 and NLRP3 immunoreactivity. The tissue sections were permeabilized with 10 mM sodium citrate and 0.05% Tween 20, and blocked in a solution of 1% (w/v) BSA (Sigma-Aldrich, St. Louis, MO, USA) in PBS. The tissue sections were incubated overnight at 4 °C with primary antibodies against caspase-1 (1:1000 dilution, Abcam, Cambridge, MA) and NLRP3 (1:500 dilution, Abcam, Cambridge, MA). After the secondary antibody (1:500 dilution, Abcam, Cambridge, MA) incubation for 2 h at 37 °C, the cellular nuclei were stained with propidium iodide (PI; 1:1000, Sigma-Aldrich, St. Louis, MO, USA). The cell counting and merging pictures were done using “ImageJ” software (Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA).

2.12. Statistical analysis

Statistical data analysis was performed using SPSS version 20.0 software. The normality was checked using the Kolmogorov–Smirnov test and the statistical significance of the results was determined using the independent samples t-test and the Mann-Whitney U. Data were reported as the mean ± SD (standard deviation), and $p \leq 0.05$ was considered significant.

3. Results

3.1. Semen analysis

Our results (Table 2) showed that there was no significant difference in sperm count between the MS offspring and control offspring groups ($p > 0.05$, $t = 0.2401$, $df = 4$). Moreover, there was no difference in any grade of motility (Grade A: rapid progressive motility) ($p > 0.05$, $t = 2.424$, $df = 4$); Grade B: slow or sluggish progressive motility ($p > 0.05$, $t = 0.1450$, $df = 4$); Grade C: vibrating motility ($p > 0.05$, $t = 1.239$, $df = 4$) between the groups. However, our findings showed that normal morphology ($p < 0.05$, $t = 3.063$, $df = 4$) and the viability ($p < 0.05$, $t = 4.027$, $df = 4$) of spermatozoa significantly decreased in the MS offspring group compared with the control offspring group. Figure 2 shows representative images of sperm morphology and viability.

3.2. Histological evaluation

Histological analysis of seminiferous tubules was evaluated with haematoxylin and eosin (H & E) staining (Figure 3). The results showed that the mean outer and inner diameters of the seminiferous tubules were

Table 1. Primer Sequences used for RT-qPCR analysis.

Genes	Forward Primers	Reverse Primers
NLRP3	5′GGACCCACAGTGTAACTTGCAGA 3′	5′ AGGCTGCAGTTGTCTAATTCCAG 3′
TLR4	5′ TGAGTGGTCAGTGTGATTGTGGT 3′	5′ TGTAGTGAAGGCAGAGGTGAAAG 3′
ASC	5′ CACAAATCAGTCTCCAACACC 3′	5′ TAACCATTACCTTGTTCCCA 3′
Caspase-1	5′ CACTCGTACACCTCTTGCCCTC 3′	5′ CTTTCACCTCTTTCACCATCTCCA 3′
TNFα	5′ TGTCTCAGCCTCTTCTCATTCCTG 3′	5′ AGGCCATTTGGGAACTTCTCATCC 3′
BAX	5′ GCAAACTGGTGCTCAAGG 3′	5′ CAGCCACAAAGATGGTCA 3′
BCL2	5′ ACTTTTAGGCGTGGCTGATG 3′	5′ GTGCTGCTCACTGTATTTTATTTT 3′
GADPH	5′ TGACATCAAGAAGGTGGTGAAG 3′	5′ CGAAGGTGGAAGAGTGGGAG 3′

Table 2. Sperm parameters in the control offspring and the MS offspring groups. Data are reported as mean ± standard deviation (SD). *$p < 0.05$ compared with the control offspring group.

Groups	Count (x10^6)	Total Motility (%)	Progressive Motility Grade A (%)	Progressive Motility Grade B (%)	Progressive Motility Grade C (%)	Morphology (%)	Viability (%)
Control offspring	1.67 ± 0.5	82 ± 16.32	11 ± 4.8	66 ± 15.5	5 ± 2.7	96.3 ± 1.7	91.1 ± 1.9
MS offspring	1.6 ± 0.07	77.4 ± 13.56	4.00 ± 1.41	64 ± 18.17	9.4 ± 4.76	92.428 ± 1.38*	83.00 ± 2.92*

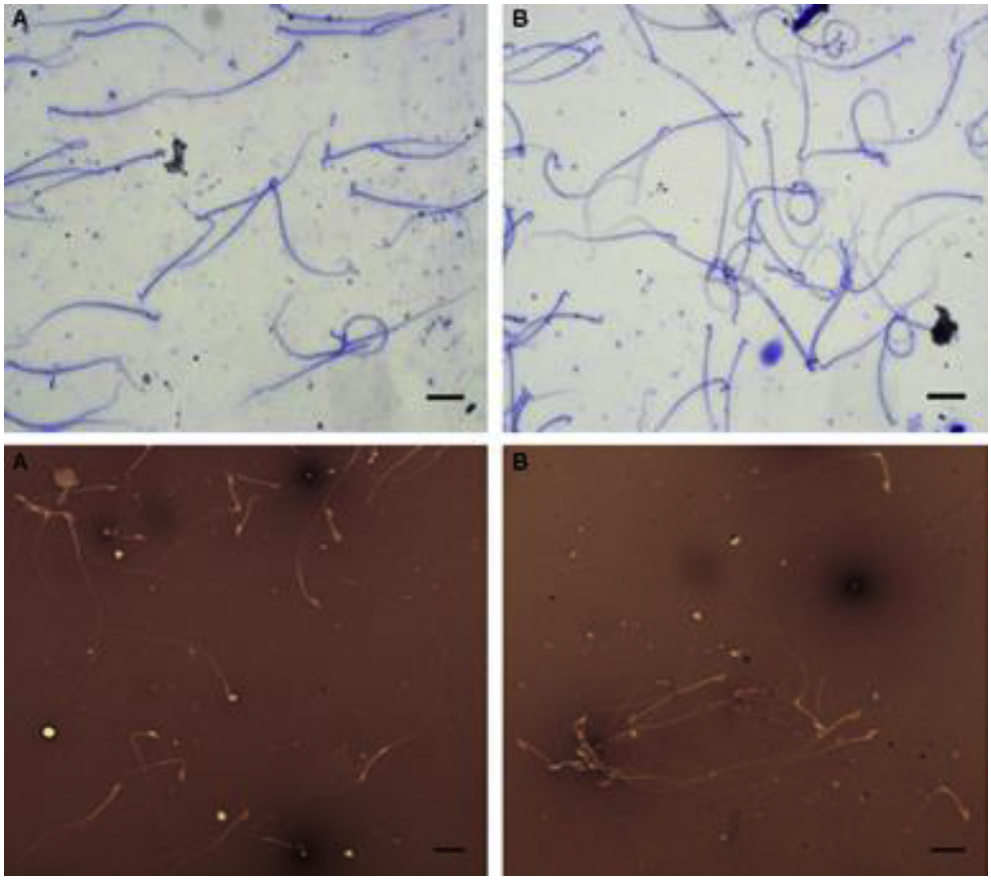

Figure 2. Representative images of sperm morphology and viability. (A) Control offspring group; (B) MS offspring group; upper panel: Diff-Quik staining to evaluate sperm morphology, scale bars are 10 μm; lower panel: Eosin and Nigrosin staining to evaluate sperm viability, scale bars are 30 μm. Samples were analyzed in triplicate.

significantly lower in the MS offspring group compared with the control offspring group ($p < 0.001$, $t = 9.515$, $df = 6$ for inner diameter, $p < 0.001$, $t = 6.060$, $df = 6$ for outer diameter, Table 3). There was no significant difference in the mean thickness of the seminiferous epithelium between the control offspring and MS offspring groups ($p > 0.05$, $t = 2.284$, $df = 6$).

3.3. ROS evaluation

ROS production in testicular tissue was evaluated using the DCFH-DA assay. The concentration of ROS production significantly increased in the MS offspring group compared with the control offspring group ($p < 0.05$, $t = 3.634$, $df = 6$, Figure 4).

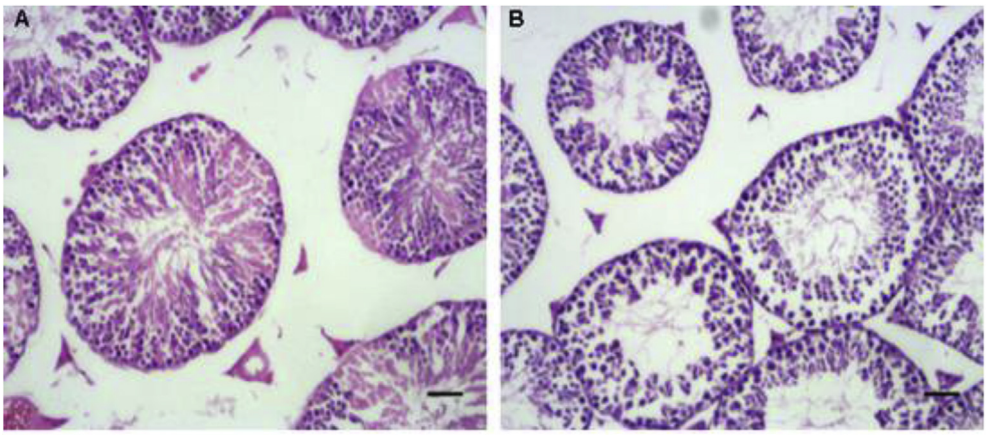

Figure 3. The histopathological features provided from H&E (Haemotoxylin and Eosin)-stained seminiferous tubules sections in the control offspring (A) and the MS offspring (B) groups. Scale bars are 50 μm. Samples were analyzed in triplicate.

Table 3. Diameters of seminiferous tubules and seminiferous epithelial thicknesses in the control offspring and MS offspring groups. Data are reported as mean ± standard deviation (SD). ***p < 0.001 compared with the control offspring group.

Parameter	Control offspring	MS offspring
Outer diameter of the seminiferous tubules (μm)	274.696 ± 5.223	240.636 ± 8.671***
Inner diameter of the seminiferous tubules (μm)	121.873 ± 1.94	98.782 ± 3.831***
Seminiferous epithelial thickness (μm)	152.823 ± 6.562	141.854 ± 6.584

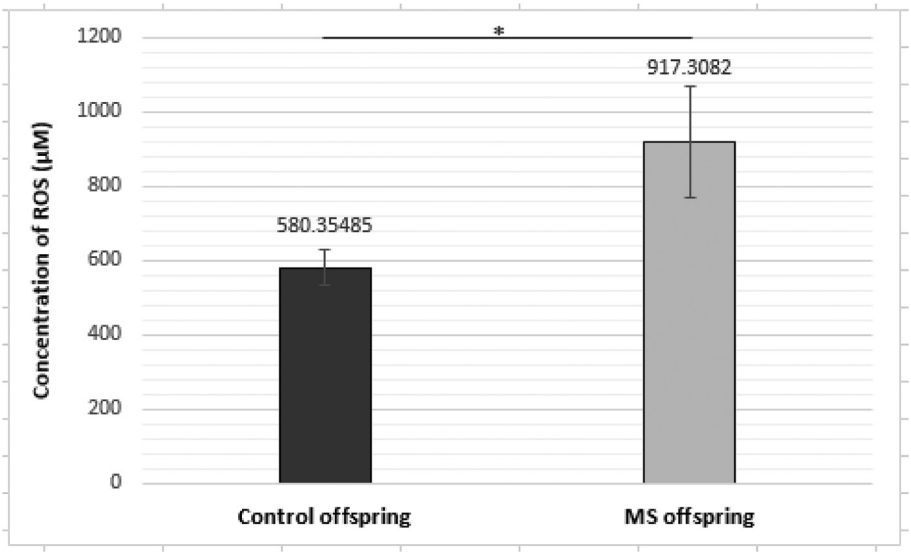

Figure 4. The concentration of ROS production in the testicular tissue by flow cytometry using 2′, 7′ -dichlorofluorescin diacetate (DCFH-DA). Values are reported as mean ± SD (standard deviation). *p < 0.05 compared with the control offspring group. Samples were analyzed in triplicate.

3.4. RT-PCR analysis

The results of RT-PCR analysis showed that expression of TLR4 significantly increased in the testicular tissue of the MS offspring group ($p < 0.001$, $t = 9.086$, $df = 6$) compared with the control offspring group. Expression of the NLRP3 ($p < 0.05$, $t = 3.486$, $df = 4$), TNFα ($p < 0.05$, $t = 2.538$, $df = 6$), and caspase-1 ($p < 0.001$, $t = 6.213$, $df = 6$) in the MS offspring group was also higher compared with the control offspring group. Furthermore, expression of ASC ($p > 0.05$, $t = 1.312$, $df = 6$) and BAX ($p > 0.05$, $t = 1.590$, $df = 6$) in the MS offspring group did not change in compared to the control group. In contrast, the expression of BCL2 ($p < 0.01$, $t = 3.991$, $df = 6$) gene in the MS offspring group was lower compared with the control offspring group (Figure 5).

3.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA results showed that IL-1β ($p < 0.01$, $t = 5.286$, $df = 6$) and IL-18 ($p < 0.001$, $t = 11.27$, $df = 6$) concentrations significantly increased in the MS offspring group compared with the control offspring group. The GPx ($p < 0.001$, $t = 11.11$, $df = 6$) and ATP ($p < 0.001$, $t = 12.22$, $df = 6$) concentrations significantly decreased in the MS offspring group compared with the control offspring group (Figure 6).

3.6. Immunocytochemical analysis

Testicular cells in the control offspring and MS offspring groups were labeled with caspase-1 and NLRP3 markers. The cells' nuclei were stained

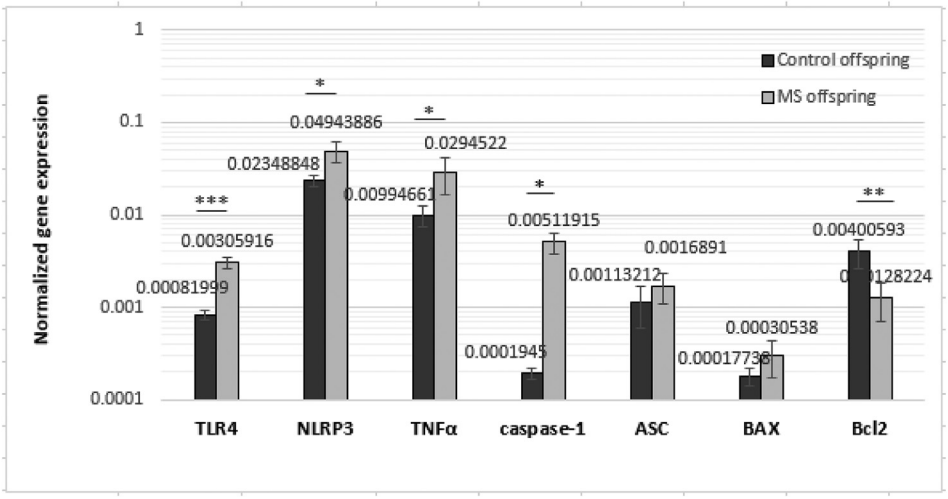

Figure 5. The gene expression of TLR4, NLRP3, TNFα, caspase-1, ASC, BAX, and BCL2 using RT-PCR. Values are reported as mean ± SD (Standard Deviation). *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the control offspring group. Samples were analyzed in triplicate.

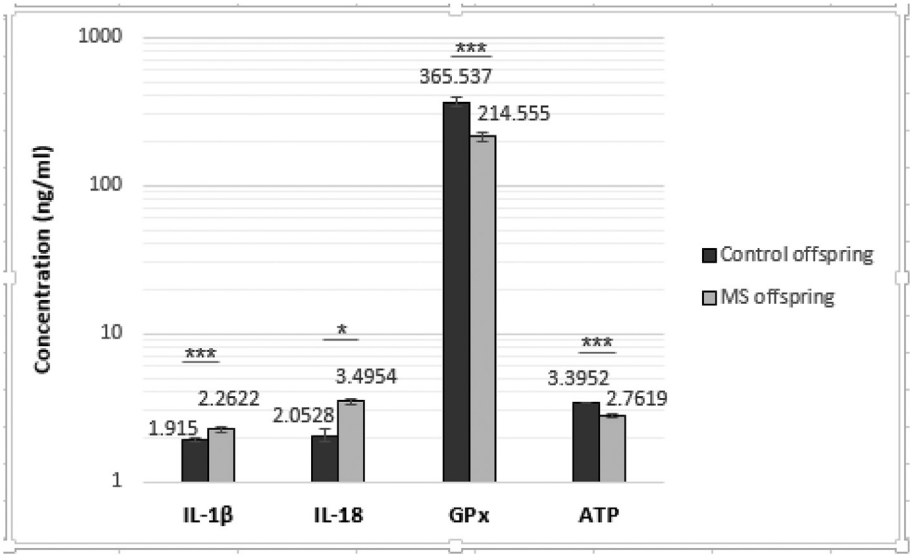

Figure 6. The concentrations of IL-1β, IL-18, GPx and ATP measured using ELISA. Values are reported as mean ± SD (standard deviation). *p < 0.05 and ***p < 0.001 compared with the control offspring group. Samples were analyzed in triplicate.

with PI (Figure 7). Immunocytochemistry staining showed that the mean percentage of caspase-1 ($p < 0.001$, $t = 25.35$, $df = 4$) positive cells significantly increased in the MS offspring group (49.00 ± 1.00) compared with the control offspring group. Also, the mean percentage of NLRP3 positive cells significantly increased in the MS offspring group (50.5 ± 0.5) compared with the control offspring group ($p < 0.001$, $t = 50.61$, $df = 4$) (Figure 8).

4. Discussion

Our findings showed that MS stress experienced by parents significantly decreased the morphology and viability of spermatozoa in the MS offspring group. Furthermore, significant changes in testicular tissue histology, including decreased diameters of the seminiferous tubules, were observed in this group. MS stress experienced by parents also significantly increased production of ROS, and decreased GPx and ATP concentrations, and significantly affected expression of genes and cytokines involved in inflammation, including NLRP3, TLR4, TNFα, caspase-1, IL-1β, IL-18, and BCL2. Finally, the mean percentage of caspase-1 and NLRP3 positive cells was significantly higher in the MS offspring group compared with the control offspring group.

Early-life stressful experiences can influence the development and shaping of the brain and cause lifelong impacts affecting behavioral and physiological responses (Heim et al., 2002; Apter-Levy et al., 2013). Early-life events can also influence health during adulthood by affecting the development of neural systems which play a role in modifying the behavioral and endocrine responses to stress (Francis and Meaney, 1999; Seckl and Meaney, 1993; Nemeroff, 1996; Sroufe, 1997; Heim et al., 2000). It has been demonstrated that intensive maternal attention during PND 2 to 14 can significantly influence several aspects of behavior, including memory, learning, maternal behavior, and fearfulness (Francis et al., 1999b; Meaney, 2001; Caldji et al., 2000).

The results of other investigations have shown that stress is attended by an increase in HPA axis activity and a decline in reproductive functions (Selye, 1939). Sexual functions can also be affected by stress-related hormones at all three levels of the HPG axis (Rivier and Rivest, 1991). In addition, repeated activation of the HPA axis disturbs the coordination between the neurotrophic and immune pathways that can play a role in

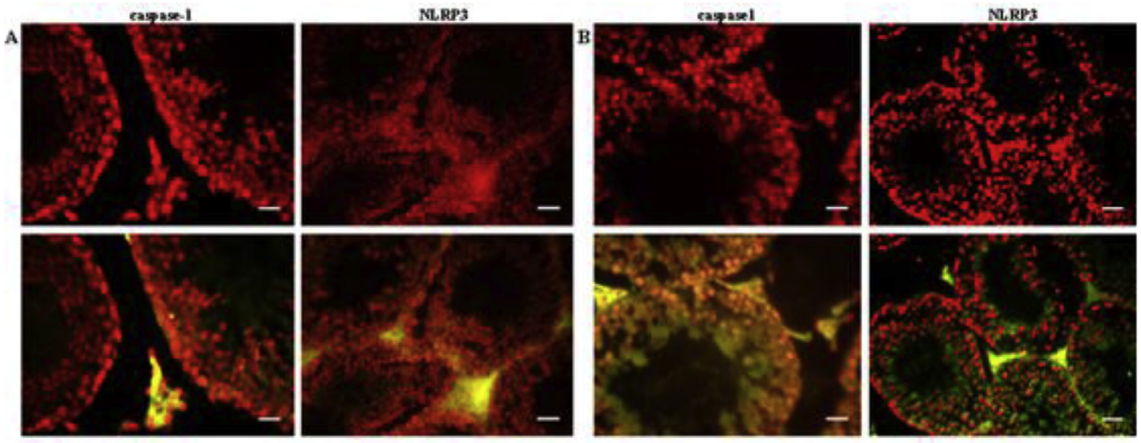

Figure 7. Immunocytochemical analysis of testicular cells for caspase-1 and NLRP3 markers. (A) Control offspring group; (B) MS offspring group; upper panel: PI stained pictures; lower panel: merged pictures of PI and secondary antibody stained cells. Scale bars are 10 μm. Samples were analyzed in triplicate.

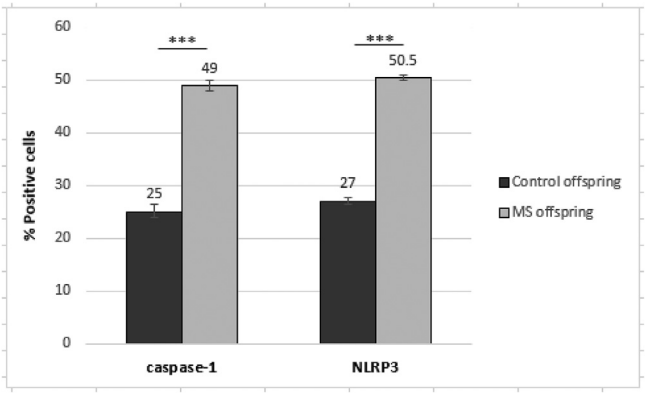

Figure 8. Comparison of the mean percentage of positive cells for caspase-1 and NLRP3 markers byimmunocytochemical assessment. Values are reported as mean ± SD (Standard Deviation). ***$p < 0.001$ compared with the control offspring group. Samples were analyzed in triplicate.

the long-lasting consequences of early-life stress (Tractenberg et al., 2016). Stress-induced activities in the adrenergic system can affect the function of the endocrine and immune systems that are necessary for normal fertility (Jóźków and Mędraś, 2012; Ostanin et al., 2007). Previous study has shown that MS stress significantly affects expression of genes and cytokines involved in inflammatory responses. MS led to increased expression of TLR4, NlRP3, TNFα, caspase-1, ASC, IL-1β and IL-18 (Khodamoradi et al., 2019b).

Although there is a vast amount of evidence showing the negative effects of prenatal stress on male sexual differentiation including abnormal levels of testosterone and elevated androstenedione level during the prenatal period (Ward, 1972, 1984; Wilke et al., 1982; Rhees and Fleming, 1981), the long-term consequences of postnatal MS on sexual behavior in males have not been well examined (Rhees et al., 2001). However, it does appear that the adverse effects of prenatal stress on sexual behavioral and morphological alterations are the result of changes in the testosterone levels of male fetuses whose mothers were stressed during pregnancy (Ward, 1984; Ward and Weisz, 1980).

The results of other studies have shown that early life physical and sexual abuse can result in an increase in endocrine and autonomic responses to stress in adulthood (Heim et al., 2000; De Bellis et al., 1994). Rhees et al. reported that, similar to stress during the prenatal period, maternal separation stress in the postnatal period in male mice can influence sexual differentiation (Rhees et al., 2001). Our previous study showed that chronic stress induced by maternal separation had harmful effects on sperm characteristics and testicular tissue, probably through the activation of ROS production and its influence on mitochondrial function, inflammatory processes and apoptosis pathways.

The vulnerability of spermatozoa to oxidative stress has been well-known as a factor affecting fertility status. Increased ROS production disrupts the mitochondrial function and activates the caspases and apoptosis (Makker et al., 2009; Abbaszadeh et al., 2018). The roles of caspases 8, 9, 1, and 3 in spermatozoa have been investigated to study the main apoptotic pathways (Paasch et al., 2004). Activated caspase-1 mediates inflammatory signaling pathways and links inflammation processes with cell apoptosis (Gupta et al., 2001; Solary et al., 1998). Conversely, mitochondrial injury can lead to the activation of apoptotic signals and also NLRP3 inflammasome, which triggers the secretion of the pro-inflammatory cytokines IL-1β and IL-18 (Tschopp and Schroder, 2010; Shimada et al., 2011, 2012). It has been demonstrated that bcl-2 plays a key role in protecting cells, most likely through mechanisms which decrease production of ROS (Kane et al., 1993). In addition to its role as an inhibitor of apoptosis, BCL2 may inhibit NLRP3 inflammasome activation. It has also been reported that an increase in BCL2 expression can lead to a decline in levels of IL-1β (Shimada et al., 2012). Moreover, recent studies have shown that the activities of NLRP3- inflammasome can be regulated by the proinflammatory cytokine TNF (Wree et al., 2018; McGeough et al., 2017). According to this data, it seems that stress-induced maternal separation can disturb the activities of NLRP3 inflammasome through changes in the levels of ROS, BCL2 and TNFα, and consequently causes an increase in the production of IL-1β and IL-18.

Little is known about how the effects of maternal separation stress experienced by parents can be transmitted to the next generation. It has been demonstrated that early-life stress can induce alterations in DNA methylation of the brain that may play a role in the physiological adaptation to stress in adulthood. Furthermore, next-generation sequencing has shown changes in DNA methylation of the genes which encode the insulin receptor and its downstream target genes (McCoy et al., 2016). Franklin et al. investigated the transgenerational effect of chronic stress induced by maternal separation on behavioral responses. They reported that chronic stress in early life changed the behavioral response of the animals in their study to aversive environments during adulthood. This stress changes DNA methylation of germline in in males exposed to stress. Thus, most behavioral changes in response to maternal separation stress are transmitted to male offspring (Franklin et al., 2010). Although the mechanisms of sex-dependent alterations in behavior are not known, sex steroids may be playing a role (Franklin et al., 2010; Mani and Thakur, 2006; Singh and Prasad, 2008). On the other hand, since the transition of stress-induced behavioral changes across generations involves environmental factors, it seems to be caused by epigenetic changes (Cameron et al., 2008; Weaver et al., 2004). Weaver et al. showed that an

increase in the licking and grooming behavior of rat mothers with pups can alter the epigenome of their offspring at the promoter of the hippocampal glucocorticoid receptor gene (Weaver et al., 2004). The actions of estrogen to regulate gonadotrophin-releasing hormone (GnRH) and luteinising hormone (LH) secretion are mediated by oestrogen receptors in the anteroventral periventricular nucleus (AVPvN) (Petersen et al., 1993, 2003; Herbison, 1998). Several studies have investigated the maternal effects on the promoter methylation status of steroid receptor genes in brain (Weaver et al., 2007; Szyf et al., 2005).

It has been reported that maternal separation stress experienced by parents can induce the apoptotic process and subsequently interfere with ovarian folliculogenesis in the first generation, but its mechanism has not been studied. Cameron et al. reported that maternal effects can influence neuroendocrine functions linked with sexual behavior in female rats. It seems that epigenetic modifications at the estrogen receptor alpha (ERα) promoter are the cause of these maternal effects (Cameron et al., 2008). Furthermore, Champagne et al. reported that variations in maternal care are linked with alterations in ERα expression in the hypothalamic medial preoptic area (MPOA) that are transmitted to female offspring by their mothers (Champagne et al., 2003b).

Our studies show that maternal separation stress can affect male reproductive potential not only in the stressed mice but also in their offspring, but the mechanisms of this are not well understood. Epigenetic alterations may play a role in transmitting the deleterious effects of the MS stress experienced by parents on the reproductive potential of their next generation. Therefore, further studies are needed to clarify the mechanisms involved in the transgenerational effects of maternal separation stress such as possible epigenetic effects.

5. Conclusion

Our results provide evidence that maternal separation stress experienced by parents significantly influenced the morphology and viability of spermatozoa as well as testicular tissue in first-generation offspring. Chronic stress induced by maternal separation had harmful effects on sperm characteristics and testicular tissue, probably through the activation of ROS production and its influence on mitochondrial function, inflammatory processes, and apoptosis pathways. However, there is little information about how the effects of maternal separation stress experienced by parents can be transmitted to their offspring. Therefore, future studies are necessary to investigate the involved mechanisms of the negative effects of maternal separation stress experienced by parents on the male reproductive potential of their next generation.

Declarations

Author contribution statement

K. Khodamoradi: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Z. Khosravizadeh: Performed the experiments.

H. Amini-Khoei: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

S. Hosseini: Contributed reagents, materials, analysis tools or data.

A. Dehpour and G. Hassanzadeh: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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