Original Article

Neonatal Maternal Separation Alters Gelatinase Activity in Mouse Ovarian Preantral Follicles

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Abstract

Objective: The early life environment is critical for normal growth and development for future reproductive function. This study aims to investigate the effect of neonatal maternal separation (MS) on gelatinase activity of mouse ovarian follicles.

Materials and Methods: In this experimental study, infants from female NMRI mice were randomly allocated into two groups immediately after birth: i. MS isolated from their mothers for 6 hours per day, from postpartum days 2 to 16) and ii. Control (undisturbed during the 16 days). Ovarian tissues were dissected to perform differential counts of the ovarian follicle type by haematoxylin and eosin staining. The isolated follicles were cultured for 12 days. Gelatinase activity and the gene expressions of matrix metalloproteinases, *MMP2* and *MMP9*, and their tissue inhibitors, *TIMP1* and *TIMP2*, were evaluated by zymography and real-time polymerase chain reaction (PCR), respectively.

Results: Follicle counts at the different developmental stages were significantly different between the control and MS groups. There was a significant decrease in gelatinase activity in the MS group compared to the control group. The MS group showed significantly decreased gene expression levels of *MMP2* and *MMP9* compared to the control group. In contrast, the gene expression levels of *TIMP1* and *TIMP2* significantly increased in the MS group compared to the control group.

Conclusion: MS is a stressor agent that compromises ovarian follicle development, at least via disruption of gelatinase activity and its related gene expressions.

Keywords: Gelatinase, Ovarian Follicle, Stress

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Introduction

Frequent exposure to different stressors is an unavoidable part of modern life that compromises health (1, 2). A deficiency in ovarian follicular development, as an important reason for female subfertility or infertility, is one of the consequences of stressors (3). Stress affects the reproductive system through increasing the level of cortisol hormone and impairs the function of the pituitary-gonadal (HPG) axis, which leads to inhibition of ovarian synthesis of steroidal hormones (1, 4). Although several changes in physiological and pathological conditions are suggested in response to stressors, the precise mechanisms of stress-induced failure of ovarian follicular development are not well-known (2, 3).

Under normal conditions, remodelling of extracellular matrix (ECM) components is regulated by the activity of a complex of zinc-dependent proteins, known as matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), during growth and development (5). The MMP system plays pivotal roles in the reorganization of the ECM in the ovarian tissue during follicular development. The MMP system resides in the basement membrane of the ECM and it degrades the components of this membrane, such as different types of collagen, laminin, elastin, and gelatin. MMP2 (gelatinase A) and MMP9 (gelatinase B) play key roles in impairment of the basement membrane, cell growth, activity of growth factors and cellular reorganization during follicular development. MMP2, as a gelatinase in the ECM, degrades fibronectin, collagens type V and VI, laminin, elastin and gelatin. Furthermore, MMP2 expedites MMP9 activity and regulates follicular development, while tissue inhibitors of MMP2 and MMP9 (TIMP1 and TIMP2, respectively) control their proteolytic activities (6). Dysfunctions of the MMP system induce destructive effects on ECM remodelling during follicular growth and ovulation (5).

Maternal separation (MS) is a typical model for postnatal stress (7, 8). MS induces adverse effects on the reproductive system (7). MS has been shown to injure follicular reserves under in vivo conditions by changing HPG function (9, 10). Stress increases the production of reactive oxygen species (ROSs) and oxidative stress

(OS), and has dangerous effects on female fertility (7, 11). The role of ROS in regulation of ECM remodelling has been previously demonstrated (6). OS, at least through impairment of the ECM, appears to compromise female fertility.

Although, the implications of stress on some aspects of the adult female reproductive system are well-studied (1-3, 12), the effect of early life stress on ovarian follicular development remains to be completely elucidated. The effects of stress on adult ovarian tissues may be reversible and short-lived; however, the effects of early life stress may be more long-lasting. In the present study, the preantral follicles (PF) of MS mice were used to investigate the effects of adverse early life events on the activities and expression patterns of MMP2 and MMP9, and TIMP1 and TIMP2, respectively, during *in vitro* culture conditions.

Materials and Methods

All reagents and materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Animals

In this experimental study, 6-8-week-old male (n=2) and female (n=6) NMRI *Mus musculus* animals (Razi Vaccine and Serum Research Institute, Tehran, Iran) were used. The mice were allowed to acclimatize to the animal facility for one week. In order to ensure pregnancy, the female and male mice were kept in pairs throughout the one-week breeding period in polypropylene cages equipped with nesting materials of soft sawdust. Food and water were available ad libitum. The animals were on a 12/12 hour light/dark cycle with lights off at 8 pm at 22 ± 2°C and 52 ± 2% humidity. All procedures were approved by the Damghan University Ethical Committee of Animal Care and Use (IR.BSDU.REC.1396.11) according to the Declaration of Helsinki (2004 Tokyo revision).

Maternal separation

The cages of the pregnant females were checked daily for delivered litters and the day of birth was defined as postnatal day 0 (P0) for each litter. Figure 1 outlines the experimental timeline. Sex determination was not done at birth to minimize the stress of the offspring; however, the animals were sexed at P16 when the female litters were classified into two groups, MS and control. MS was performed from P1 to P16, starting at 10 am for 180 minutes. Briefly, the dam was removed daily from the cage and placed in a different cage that had similar conditions in terms of lighting cycle, temperature and humidity, and food and water. The litters were kept in the home cage. During MS, the litter cage was placed on a heating pad at a temperature of 32°C. No food or water was available for litters during the MS. After 180 minutes, the dams were reunited with their offspring in the home cage.

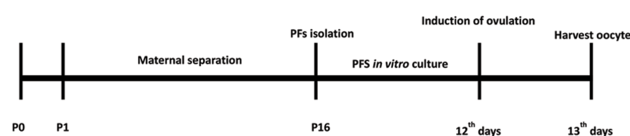

Fig.1: Timeline of maternal separation (MS), *in vitro* culture of isolated PFs and induction of ovulation. PF; Preantral follicles, P0; Postnatal day 0, P1; Postnatal day 1, and P16; Postnatal day 16.

Isolation of follicles and *in vitro* culture

All of the female offspring (n=20) were sacrificed by cervical dislocation on P16. Their ovaries were aseptically dissected and immersed in alpha minimum essential medium (α-MEM) with 10% foetal calf serum (FCS). The PF were mechanically dissected from the ovaries by using 29-gauge syringe needles. The PF (n=810) that had diameters of 140-150 µm and intact round follicular structures that contained at least two to three layers of granulosa cells, one or two layers of theca cells and a centrally located oocyte were chosen for the study. We identified the granulosa and theca layers according to the characteristics described earlier (13, 14). The granulosa cells had shaped dense layers around the oocyte. The theca layer was typically distributed through a single layer, or rarely two continuous layers, around the healthy granuloma cells and were characterized by a long or flattened shape and large nucleus. All of the selected follicles were randomly cultured in microdroplets of culture medium (20 uL, 1 PF/droplet) in a culture dish (25 droplets/dish) under mineral oil and incubated at 37°C in 5% CO_2 in air for 12 days. The culture medium was α-MEM supplemented with 100 mIU/ml follicle-stimulating hormone (FSH, Fostimon, Switzerland), foetal bovine serum (FBS, 5%), insulin-transferrin-selenium (ITS, 1%), and recombinant epidermal growth factor (rEGF, 20 ng/mL). The medium were replaced by fresh medium (10 µl) every other day as previously described (7). On the twelfth day, ovulation was induced by the addition of human chorionic gonadotropin (1.5 IU/ml) to the culture medium. After 16-18 hours, the follicles ruptured and oocyte extrusion occurred.

Histology

The ovaries were harvested from each group and fixed in Bowen's solution for three hours. Next, the ovaries were embedded in paraffin and serially sectioned at 5 µm sections. Thereafter, they were mounted on slides and stained with haematoxylin and eosin. The numbers of follicles at the different developmental stages were counted in every fifth section of each ovary visualized on the largest cross-section. The classification defined by Peters (15) was used with some modifications, as follows: type A follicles were composed of a layer with less than 20 granulosa cells; type B follicles were composed of a layer of 20-60 granulosa cells; type C follicles were composed of two layers of 60-

100 granulosa cells; and type D follicles consisted of three or more layers of 100-200 granulosa cells. Since follicles that are more advanced than type D do not appear in the ovaries of 14-day-old mice, we classified the cells only until type D.

Zymography for MMP2 and MMP9

The activities of gelatinases (MMP2 and MMP9) from 15 PFs in an equal amount of culture medium were evaluated on days 2, 4, 6, 8, 10 and 12 by zymography, as described elsewhere (7). The experiments were repeated three times. Both the mixture of follicles and culture medium were subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% SDS that contained 0.05% (w/v) gelatin under non-reducing conditions. The gels were washed for 20 minutes at 22-24°C in 2% Triton X-100 and subsequently digested for eight hours at 37°C using tris-HCl (50 mM), $CaCl_2$ (2.5 mM), NaCl (200 mM), and $ZnCl_2$ (1 mM); pH=7.4 for the entire solution. After that, the gels were stained with 0.5% Coomassie Brilliant Blue (Bio-Rad, Canada) in 50% (v/v) methanol and 20% (v/v) acetic acid, and finally decolorized for five hours at 22-24°C in 30% v/v methanol and 10% v/v glacial acetic acid. Gelatinolytic activity was detected as cleared bands. Intensity and surface of the cleared bands were analysed by ImageJ software (University of Wisconsin, USA) and considered as gelatinolytic activity.

Gene expression analysis

RNA was isolated from 15 PF on days 1, 6 and 12 of the culture period using an RNX-Plus kit (CinnaGen, Tehran, Iran) according to the manufacturer's instructions. A total of 500 ng of the extracted RNA with a ratio above 1.8 for 260/280 optical density was used for cDNA synthesis with a cDNA Synthesis Kit (Fermentas, MD, USA) according to the manufacturer's protocol.

The mRNA expression levels of *MMP2, MMP9, TIMP1* and *TIMP2* were determined by an ABI StepOne real-time PCR machine (Applied Biosystems, ABI, USA) using SYBR Green (Amplicon, Denmark). The gene-specific primer sets were designed to span introns or cross exon/exon junctions using Oligo software version 7 (DBA Oligo, Inc., USA, Table 1). All real-time polymerase chain reaction (PCR) protocols were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (16). *GAPDH* was used as the reference gene. The thermal profile was adjusted to one cycle of denaturation at 95°C for 15 minutes, followed by 40 cycles at 95°C for 30 seconds, and one cycle at 60°C for 45 seconds. Finally, the relative expression fold was calculated using the $2^{-\Delta\Delta CT}$ method. The specificity of each amplification reaction was confirmed by melting-curve analysis. A no-template control (NTC) tube for each gene was included in all of the experiments.

Statistical analysis

SPSS software (SPSS Inc., Chicago, IL, USA, version 24) was applied for analysing the statistically significant differences between the MS and control groups via the independent sample's t test. A $P<0.05$ was considered as a statistical of significance. All experiments were repeated at least four times, and data are presented as mean ± SD.

Table 1: Primers for real-time polymerase chain reaction (PCR) analysis of the matrix metalloproteinase (MMP) and tissue inhibitor (TIMP) genes

Gene	Primer sequence (5´-3´)	Product size (bp)	Melting temperature (°C)	Accession number
MMP9	R: CATAGTGGGAGGTGCTGTCG	247	62.5	NM_013599
	F: CTGTCCAGACCAAGGGTACAG		63.2	
MMP2	R: CTGTTGTAGGAGGTGCCCTG	265	62.5	NM_008610
	F: GAGAAGGACAAGTGGTCCGC		62.5	
TIMP1	R: TTCAGTTTTTCCTGGGGGAAGG	202	62.1	NM_011593
	F: GGGTGTGCACAGTGTTTCCC		62.5	
TIMP2	R: TCCCAGGGCACAATGAAGTC	281	60.5	NM_011594
	F: GCAGACGTAGTGATCAGAGCC		63.2	
GAPDH	R: CCCTGTTGCTGTAGCCGTATTC	203	62.1	NM_008084
	F: TGACATCAAGAAGGTGGTGAAGC		61.3	

Results

Histology

The morphology of the ovaries changed considerably between the MS and control groups. Large follicles in the MS group underwent various degrees of degeneration and extensive stroma formed from the degenerated follicles. The numbers of the different types of follicles were determined in the ovaries of both groups and we calculated their mean numbers for each group (Table 2). The ovaries of the MS group looked similar to the control group. The ovaries were compact and contained cells and follicles. The developed follicles were located in the proximal part of the cortex, although the medulla had more stroma in the MS group (Fig.2). Nevertheless, there were significantly less types A-D follicles in the MS group compared to the control group (P<0.05). There were significantly more degenerated follicles in the MS group compared to the control group (P<0.05).

Table 2: Differential counts of follicles in one ovary

Follicle type	Control	MS
Type A	486.0 ± 23.6	349.3 ± 21.1*
Type B	169.3 ± 11.3	138.3 ± 9.9*
Type C	141.0 ± 13.0	81.5 ± 7.4*
Type D	39.0 ± 6.1	14.8 ± 2.5*
Degenerated	5.75 ± 1.71	11.75 ± 2.7*

Data are presented as mean ± SD. *; Indicate significant (P<0.05) difference compared to the control group and MS; Maternal separation.

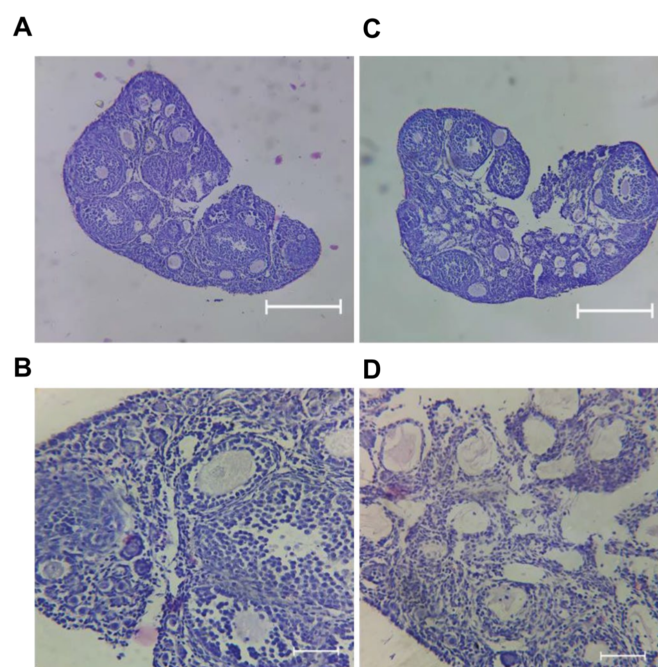

Fig.2: Histological sections of hematoxylin-eosin (HE) stained postnatal mouse ovaries. **A, B.** Control group and **C, D.** The maternal separation (MS) group (scale bar: A, C: 200 µm, B, D: 100 µm).

Changes in gelatinase activity during *in vitro* culture

The gelatinase activities (MMP2 and MMP9) in the culture medium of PF during the *in vitro* culture were detected by zymography. Figure 3 provides a summary of the intensities of their bands. The detectable gelatinase activity at 95 kDa was considered to be MMP2, whereas the activity at 72 kDa was considered to be MMP9. We observed a significant decrease in the activities of MMP2 (P=0.011) and MMP9 (P<0.000) in the MS group and in the control group (both, P<0.000) until day 10 of the culture period. Their activities in both the MS and control groups increased significantly at ovulation post- human chorionic gonadotropin (hCG) administration compared to other times during the cultivation period. Furthermore, there was a significant decrease in MMP2 activity in the culture medium of MS follicles compared to the control group during the culture period. There was less gelatinase activity of MMP9 in the culture medium collected from the MS groups compared to the control groups (P<0.000, Fig.3).

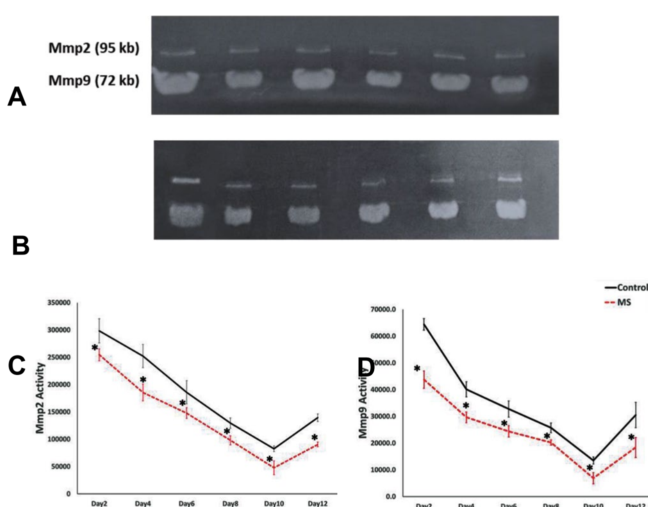

Fig.3: Zymogeraphy of matrix metalloproteinases 2 (MMP2) and MMP9 of preantral follicles (PFs) during *in vitro* cultur. **A.** Representative gelatin zymography gels of PFs from the maternal separation (MS) group. **B.** Gelatin zymography gels of the control group during the *in vitro* culture. **C** and **D.** Densitometry analysis of MMP2 and MMP9 activity. *; Significant (P<0.05) difference compared to the control group.

Relative mRNA expressions of *MMP2*, *MMP9*, and *TIMP1* and *TIMP2*

The expressions of *MMP* and *TIMP* mRNA were analysed by real-time qPCR. Both *MMP* and *TIMP* mRNA were detectable in both groups (Fig.4). The relative gene expressions of *MMP2* and *MMP9* decreased significantly during the culture period in the MS (P<0.000 and P<0.000, respectively) and control (P=0.000 and P=0.001, respectively) groups. However, the relative gene expressions of *TIMP1* and *TIMP2* increased significantly during the culture period in both the MS (P<0.000 and P=0.005, respectively) and control (P=0.007 and P=0.001,

respectively) groups. Relative mRNA expressions of *MMP2* and *MMP9* elevated significantly on day 12 (post-luteinizing hormone administration) in the both MS (P<0.000) and control (P<0.000) groups. Also, the relative gene expression levels of *MMP2* (P<0.000) and *MMP9* (P<0.000) in the PF of the MS group were significantly lower compared to the control group on days 1, 6 and 12 of the culture period. The relative gene expression levels of *TIMP1* and *TIMP2* in follicles of the MS group were significantly higher than the control group on days 1, 6 and 12 of the culture period (P<0.000).

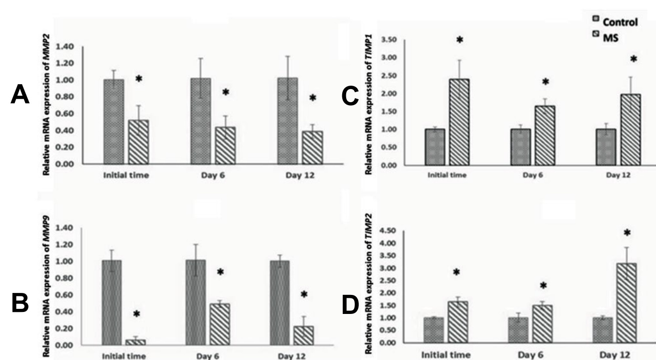

Fig.4: Relative mRNA expressions of matrix metalloproteinase 2 (*MMP2*) and *MMP9*, and tissue inhibitors (*TIMP1*) and *TIMP2* in the preantral follicles (PF) of the maternal separation (MS) and control groups. The data are presented as relative fold changes (mean ± SD) of three independent experiments. *; Significant difference compared with the control group (P<0.05).

Discussion

The data in the present study show the effects of MS as a social model of stress in mice on the development of ovarian follicles, its gelatinolytic activity, and mRNA expressions of *MMP2* and *MMP9,* and their tissue inhibitors, *TIMP1* and *TIMP2*. The results indicate that MS decreases follicle growth and development in addition to changes in gelatinolytic activity and related gene expressions. Both social and physical stress act as key factors in disrupting reproductive physiology homeostasis, at least by targeting ovarian tissues (17). In this regard, our previous study has shown that MS significantly decreased the developmental competence of in vitro cultured PFs (7). Several studies have been performed on the effects of different types of stress on female fertility and the results showed the destructive effects of stress on reproductive physiology (18) at least through induction of ovarian failure (19). The results of a study conducted in 2001 showed a significant increase in glucocorticoid secretion in postnatal mice exposed to MS for six hours/day (8). It was established that high concentrations of glucocorticoid hormone compromise both follicle and oocyte maturation (20). Therefore, postnatal stress could directly disrupt ovarian function and folliculogenesis (7). Wu et al. (21) have reported that maintenance of 6-week-old mice in a tight cage for 23 days was associated with decreased oocyte chromatin disconfiguration and reduction in oocyte development. Researchers in 2012 used a hunter model to induce psychological stress and reported reductions in oocyte development and blastocyste formation (22). Bhat and Yajurvedi (23) reported that neonatal stress caused apoptosis in granulosa cells, which increased the rate of follicle atresia and decreased the number of follicles and oocytes in a rat model.

Despite numerous studies, the mechanisms of the effect of stress on reproductive function have not been exactly described. The present study also assessed gelatinase activity and relative gene expressions. The results showed significant decreases in expressions of the *MMP2* and *MMP9* genes and significant increases in expressions of the *TIMP1* and *TIMP2* genes in the MS group compared to the control group. Homeostasis of the ECM of ovarian tissue is essential for normal folliculogenesis and that, in turn, is controlled by the activity of various proteolytic enzymes, in particular MMP enzymes and TIMPs (6). Therefore, any changes in expression and activity of the MMPs disrupt follicular development (7, 24). Under normal conditions, MMP2 and MMP9 are active during folliculogenesis. Their activities are precisely controlled and any alterations in their activities could cause impaired follicular development (24). TIMP1 and TIMP2 are specific inhibitors of MMP9 and MMP2, respectively. Low activity levels and expression of gelatinases in the MS group were associated with increased levels of their inhibitor, which was probably due to the devastating effects of MS. In contrast to our results, another study showed that stress increased expressions of MMP2 and MMP9 in a mouse model of cancer (25). Similar results were also observed in a mouse model of ovarian cancer (26). The difference between recent studies and our results may be due to differences in cell types. On the other hand, it has been demonstrated that psychological stress produces high levels of ROS, which can induce OS (2). Furthermore, OS was observed in cultured PF of mice that experienced early life stress (7). OS might be a causative factor for MMPs system dysregulation (27). Thus, it seems that psychological stress changes the regulation of the MMPs system through induction of OS.

Conclusion

The decrease in gelatinolytic activity and changes in mRNA expressions of *MMP2* and *MMP9,* and TIMPs in stressed neonatal mice suggest that stress might reduce the developmental competence of ovarian follicles and reproductive potential, at least through changing the MMPs system of ovarian follicles.

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Authors' Contributions

Z.A.; Performed experiments and collected the data. S.Z.; Participated in development of the study concept and design, performed data analysis, and wrote the manuscript. T.L.; Conducted the biochemical and molecular experiments, and real-time PCR analysis. M.E.; Contributed extensively to data interpretation and the conclusion. B.M.; Performed the histopathology experiments and interperations. Also, aided in interpreting the results and worked on the manuscript. All authors edited and approved the final version of this manuscript for submission.

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