

## Research Paper



# Prenatal Stress-induced Spatial Memory Deficit in a Sex-specific Manner in Mice: A Possible Involvement of Hippocampal Insulin Resistance

Masoomeh Mohammadi<sup>1</sup> , Ali Haeri Rohani<sup>1</sup> , Parichehr Yaghmaei<sup>1</sup> , Hedayat Sahraei<sup>2\*</sup>

1. Department of Biology, Faculty of Basic Sciences, Science and Research Branch, Azad Islamic University, Tehran, Iran.

2. Neuroscience Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.



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## ABSTRACT

**Introduction:** In the present study, the effects of prenatal stress on spatial learning and memory deficit and its relationship with hippocampal insulin resistance were examined in male and female offspring.

**Methods:** Female NMRI mice were mated with males overnight, and the 0-day of pregnancy was detected (Gestational day 0-GD0). The pregnant mice were then randomly divided into stress and control groups. The stress group received stress from the GD0 to GD10. On post natal day 30 (PND30), the offspring were divided into 4 subgroups, namely: male-control, female-control, male-stress, and female-stress. Barnes maze method was used for spatial learning evaluation. Plasma cortisol and insulin levels were measured at the beginning of the experiments. At the end of the experiments, the animals' brains were removed, and their hippocampus was extracted. The hippocampus was homogenized, and its insulin and insulin-receptor contents were evaluated.

**Results:** The stressed animals needed more time for reaching to target hole. In addition, they spend more distance to find the target hole, which was more pronounced in the male offspring. Both plasma and hippocampal insulin content were reduced in the stressed groups. Moreover, the hippocampal insulin receptors protein was reduced in the stressed animals. There was a positive relationship between plasma and hippocampal content and memory deficit in the stressed groups.

**Conclusion:** These results indicated that prenatal stress could induce spatial learning and memory deficit in offspring, which is associated with plasma and hippocampal insulin and receptor content reduction (hippocampal insulin resistance) in these animals.

**\* Corresponding Author:****Hedayat Sahraei, PhD.****Address:** Neuroscience Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.**Tel:** +98 (21) 88620811**E-mail:** h.sahraei@bmsu.ac.ir

## Highlights

- Maternal stress is very harmful for fetus.
- The effect of stress is significant during the early days of gestation.
- This effect is due to several hormonal and neuronal disturbances including Insulin resistance.
- The effects of stress on the fetus is gender dependent.

## Plain Language Summary

The possible effectiveness of prenatal stress on learning and memory in neonates and also the changes in hippocampus as of essential part of the brain involved in learning and memory. We found that prenatal stress can reduce the insulin effects in hippocampus and it may be the main cause of stress on neonatal memory deficits.

### 1. Introduction

**I**ntrauterine life is thought to be a very important developing period that is sensitive to both external and internal environmental changes. Maternal hormonal and nutritional status effects, which are the consequences of both internal and external (environmental) factors on a pregnant mother's brain stress system function, are among the main factors which can affect the growth and programming of the fetus. In this regard, studies have shown that stress can induce adverse effects on normal fetus brain development, especially the (hypothalamic-pituitary-adrenal) HPA axis, both in the human and animal models (Glover, O'Connor, & O'Donnell, 2010). Among the most important parts of the brain which is affected by prenatal stress is the hippocampus (Weinstock, 2001). The resulting alteration in offspring brain morphology induced by prenatal stress is the change in the offspring's behavior (Weinstock, 2017), including learning deficit. It is well known that glucocorticoid hormones which are released during stressful events from the mothers' adrenal glands, and also different inflammatory mediators are produced during and or after stress (Hantsoo, Kornfield, Anguera, & Epperson, 2019; McEwen, Nasca, & Gray, 2016) can easily reach the fetus via blood placenta barrier and cause abnormal growth and development in the fetus (Bronson & Bale, 2016; McEwen, 2019).

Moreover, it is established that maternal malnutrition due to the experience of stressful events can also cause retardation in the fetus (Maghami et al., 2018; Nätt et al., 2017). Data also indicate that prenatal stress adversely affects the offspring's glutamate N-methyl-D-aspartate

(NMDA) receptor dysfunction in the hippocampus (Fang et al., 2018). In their experiments, Fang et al. indicated that offspring from dams that experienced chronic restraint stress from gestational day 8 (GD8) to GD20 show lower expression of glutamate NMDA receptor NR1 subunit in the hippocampal dentate gyrus, CA1, and CA3 regions (Fang et al., 2018) which was abundant in the male offspring. In another study, it is indicated that prenatal mild chronic stress from GD7 to GD20 in the pregnant female Wistar rats reduced the expression of glutamate NMDA NR2B subunit in the offspring hippocampus in a sex-specific manner (Wang et al., 2016). Interestingly, the offspring in these studies show spatial memory deficiency as they could not find the target page in Morris Water Maze (MWM) task (Fang et al., 2018; Wang et al., 2016), which also was sex-specific. In addition to alteration in glutamate NMDA receptor expression in the hippocampus, studies revealed that the expression of the metabotropic glutamate receptors type 2/3 was also reduced in males but not in female offspring hippocampus with prenatal stress history (Wang et al., 2015).

In addition to the rodents, experiments on primates also revealed that prenatal stress can affect the offspring's hippocampus development. In this regard, Coe et al. indicated that babies of pregnant Rhesus monkeys which experienced dark room staying stress combined with noise shocks showed an increased basal serum cortisol levels and reduced hippocampal dentate gyrus neurogenesis, and decreased hippocampal volume (Coe et al., 2003). Considering the role of hippocampus neurogenesis in spatial learning and memory and also the pivotal role of the NMDA glutamate receptors within the hippocampus in this regard, it is not surprising that prenatal stress, which can affect both of these two factors, can

impair the spatial learning and memory in the offspring (Kim & Diamond, 2002).

On the other hand, increasing research has indicated a role of hippocampal insulin resistance in hippocampal malfunction (Biessels & Reagan, 2015a). Hippocampal insulin resistance is indicated by the lower insulin level and insulin receptor in the hippocampus (Biessels & Reagan, 2015a). Interestingly, prenatal stress is associated with glucose intolerance and insulin resistance (Entringer et al., 2008; Karbaschi, Sadeghimahalli, & Zardooz, 2016; Karbaschi et al., 2017; Roostamkhani, Zardooz, Parivar, & Roodbari, 2013), and deficit in working memory performance. (Entringer et al., 2009).

However, less attention is paid to the effects of prenatal stress on hippocampal insulin resistance and spatial learning and memory. In the present study, attempts were made for further evaluation of the effects of prenatal stress on spatial learning and memory in offspring and its relation to hippocampal insulin resistance.

## 2. Materials and Methods

### Study animals

Female NMRI mice ( $n=20$ ; average weight: 25 g), purchased from Pasture Institute, Tehran, Iran, were mated overnight with male (F/M ratio: 2/1), and GD0 was determined by sperm observation in their vaginal smear. After mating, the male mice were removed. The pregnant females were divided into stress and control groups ( $n=10$ /group). Animals were kept in cages (2/cage) until delivery at a controlled temperature ( $22^{\circ}\text{C}\pm 2^{\circ}\text{C}$ ). Standard mouse chow (Pars Animal Food Co, Tehran, Iran) and tap water were available ad lib except during the experiments. Experiments were carried out according to the animal care guidelines, Baqiyatallah University of Medical Sciences Animal Ethics Committee (BMSU/AEC#256).

### Experimental procedure

Figure 1 shows the experimental timeline. Briefly, animals in the stress group experienced electric foot shock stress during GD1 to GD10, remaining undisturbed until delivery. The control group did not experience any stress during pregnancy until delivery. Offspring of all animals were taken care of by their mothers until postnatal day 30 (PND30). On this day, the offspring were separated and randomly assigned to male control, female control, male stress, and female stress groups ( $n=8$ /group). On PND31-PND35, Barnes Maze (BM) test was performed. On PND35, the animals were deeply anesthetized, and

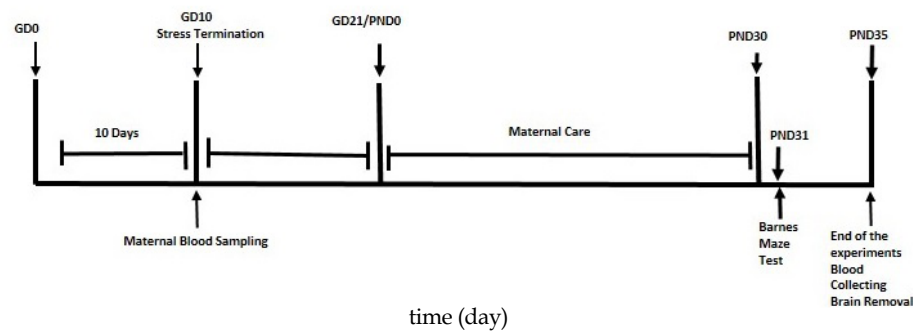
their brains were removed for hippocampus extraction. At the same time, animals' blood was collected from their trunk.

### Electric foot shock stress procedure

Induction of electric foot shock was applied to the pregnant mice between 9 AM and 12 noon from GD1 to GD10. For this purpose, each pregnant female mouse was placed in a communication box (Borj-e-Sanat Co., Tehran, Iran) chamber ( $15\times 10\times 50$  cm;  $L\times A\times H$ ) 30 min before the shock. Then a brief electric shock (0.04 mA) was applied to the animal's foot (10 Hz) for 6 seconds. The animals were kept in the chambers for an additional 30 min, and after this time, the animals were removed to their home cages. This procedure was repeated in the coming days until GD10.

### Spatial learning and memory testing

The spatial learning and memory tests were performed using a Barnes maze (BM) according to Maghami et al. (2018) with minor modifications. The maze platform was made of opaque blue circular Plexiglas (D: 100 cm) with 18 holes (D: 10 cm) placed at the platform's edge with equal spacing. The platform was on a base (H: 120 cm) from the ground. An escape box made of black Plexiglas ( $20\times 20\times 20$  cm) was attached under one of the holes, which considered as target hole. The target hole had the same position for each animal throughout the test. For spatial cues, black strips with different shapes were attached to the walls of the experimental room, and the experimenter was hidden behind a curtain during the experiments. Animals' activity in the maze was monitored and recorded by a CCTV camera located 90 cm above the maze platform. This device recorded all animal activity and software manufactured by Borj-e-Sanat Co., Tehran, Iran, and could analyze the animals' movement in the maze. The software offered all factors mentioned in this study, including time and distance traveled by the animal. Each animal experienced four trials per day. For this purpose, each animal was brought to the test room 60 min before the learning trial. The animal was put in the maze's center under a black bucket while the lights were off. Then the lights were on, the bucket was removed, and the animal was allowed for 90 s (cut-off time) to find the target hole. If the animal did not find the target hole after this time, it was guided by the experimenter to the target hole. To familiarize the animals with the maze environment, one day before starting the learning trials, the animals were put in the escape box for 2 min, then placed directly in the target hole and allowed to enter and stay in the escape box, beneath the hole, for another 2 min.



**Figure 1.** Experiments Time-Line

GD: Gestational Day; PND: Post Natal Day.

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When the animal entered the target hole, it was allowed to stay there for 2 min and then removed to its cage for 15 min. After each trial session, the maze and the target hole were cleaned using 70% ethanol. This procedure was repeated for four consecutive days. For spatial memory testing, on the fifth day, each animal was placed in the maze while the target hole was covered by a dark plate, and each animal was allowed to move in the maze for 90 s freely. The time the animals spent on the plate was recorded as an indicator of the spatial memory index.

### Hippocampus extraction

The animals were deeply anesthetized, and their brain was fixed by cold saline transcardiac infusion. The animals' brains (8 mice/group) were removed surgically and placed on ice for hippocampal removal. After hippocampal removing, it placed in a tube containing the lysis buffer solution (sodium deoxycholate 0.25%=0.025 g, NaCl=0.08 g, SDS=0.01 g, EDTA=0.003 g, protease inhibitor cocktail=1 tablet, Triton X-100 (0.01%)=10  $\lambda$ ) at a rate of 4 times of hippocampus volumes. After homogenization, the suspension was removed and centrifuged at 3500 g for 10 min at 4°C. Then the supernatant was separated for insulin and insulin receptor assessment.

### Blood and hippocampal insulin and hippocampal insulin receptor content assessment

An ultra-sensitive mouse insulin ELISA kit (minimum detection: 0.02  $\mu$ g/L; Mercodia, Sweden) and a mouse (Murine) Insulin Receptor ELISA Kit (Cloud-Clone Corp., TX, USA) were used to measure the blood and hippocampus insulin, and hippocampal insulin receptor content, respectively. The measurements were performed in one run, and the intra-assay coefficients of variation were 9.2% and 7.1%.

### Statistical analysis

Data are presented as Mean $\pm$ SEM (standard error of the mean). To better understand differences, the Area Under The Curve (AUC) was calculated for 'time and distance spent for reaching target hole' variables. One-way ANOVA followed by Tukey post hoc test was used. Moreover, The Pearson correlation test was performed to assess the relationship between the variables. In all cases,  $P < 0.05$  was considered statistically significant.

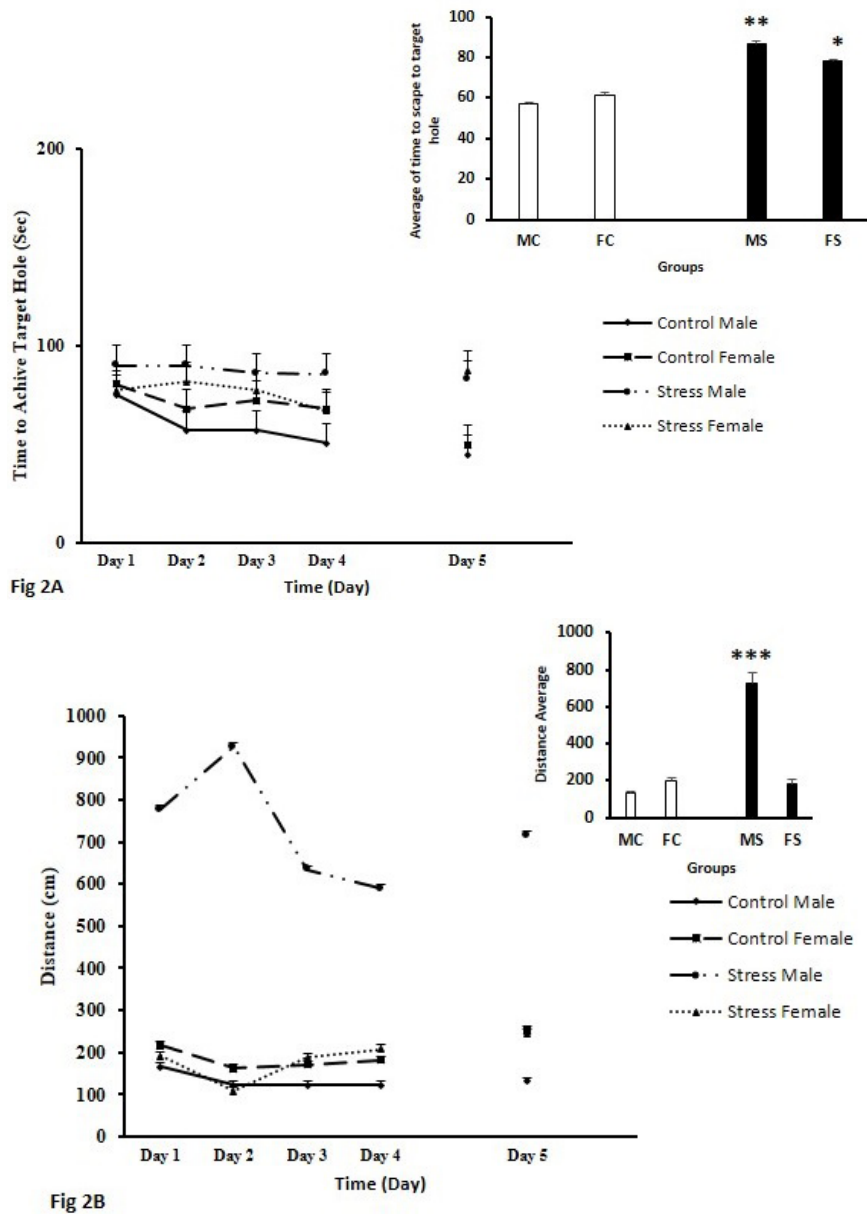
## 3. Results

### Effects of prenatal stress on time elapsing to reach the target hole by offspring

The time elapsed by the offspring from the stress and control groups is presented in Figure 2A. The data analysis indicated that the male stress and female stress groups needed more time to reach the target hole than the male control and female control groups. (one-way ANOVA;  $F_{3,24} = 2.415$ ,  $P < 0.01$ ; Figure 2A). Further post hoc analysis indicated that the male stress group needed more time than the female stress group.

### Effects of prenatal stress on distance traveling to reach the target hole by offspring

Simultaneous distance recording of the animals' activity also revealed that the male stress and female stress groups traveled longer to reach the target hole than the control groups (one-way ANOVA:  $F_{3,24} = 12.83$ ,  $P < 0.0001$ ; Figure 2B). Again, post hoc analysis indicated that the male stress group traveled more distances than the female stress group.



**Figure 2.** (A-B): The effects of prenatal stress on offspring spatial learning and memory

The time elapsed to reach the target hole (A) and the distance traveling to reach the target hole (B) were determined (n=8/group). \*P<0.05, \*\*P<0.01, and \*\*\* P<0.001 different from control groups.

MC: Male Control; FC: Female Control; MS: Male Stress; FS: Female Stress.

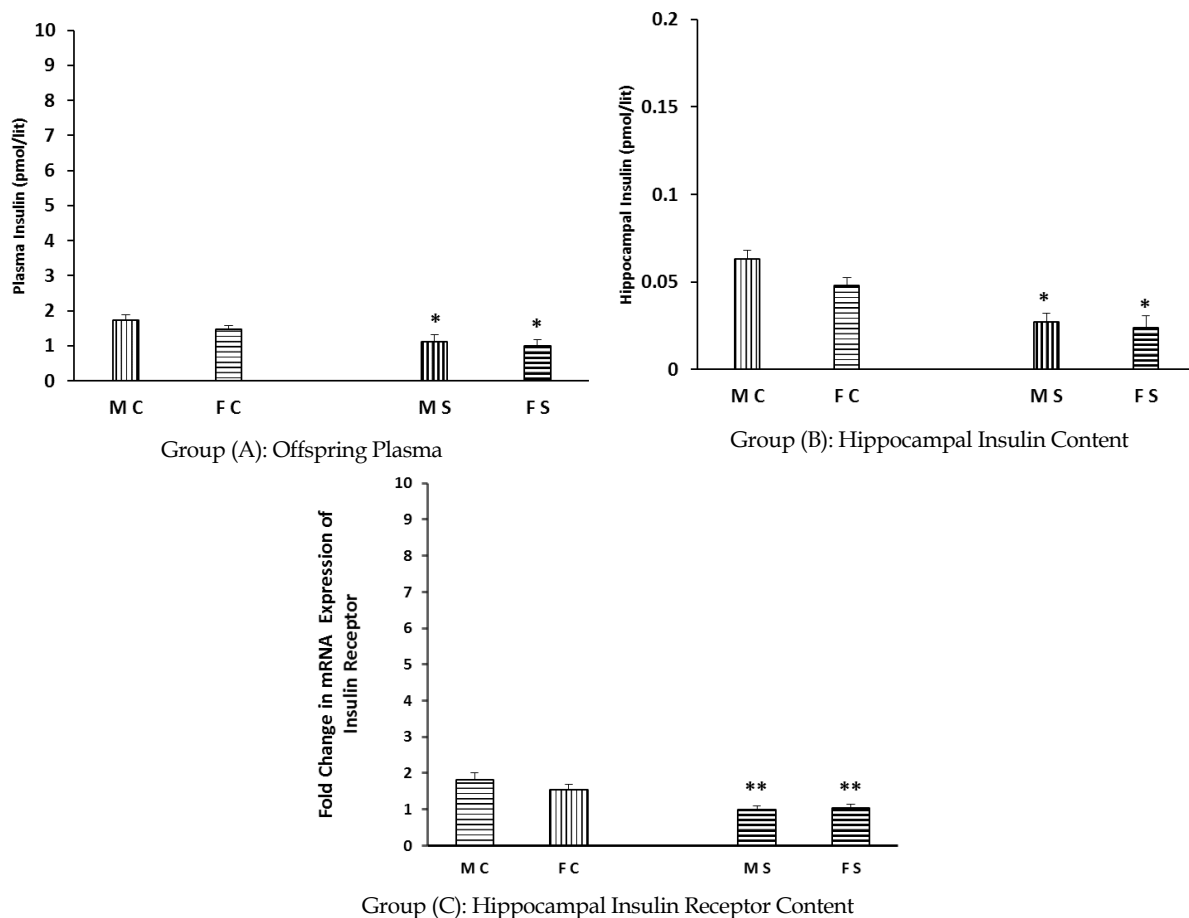
### Effects of prenatal stress on offspring blood and hippocampal insulin levels

The offspring's plasma insulin level is shown in Figure 3A. Clearly, the male stress and female stress groups had a lower plasma insulin concentration than the control groups (one-way ANOVA;  $F_{3, 24}=3.68$ ,  $P<0.01$ ; Figure 3A). The hippocampal insulin level of the male stress and female stress groups also was lower

than the control groups (one-way ANOVA;  $F_{3, 24}=4.9$ ,  $P<0.01$ ; Figure 3B).

### Effects of prenatal stress on offspring hippocampal insulin receptor level

The results of the offspring hippocampal insulin receptor levels are shown in Figure 3C. One-way ANOVA indicates that the offspring belonging to the stressed mothers have fewer insulin receptors in their hippocampus than the



**Figure 3.** (A-C): Offspring Plasma (A), Hippocampal Insulin Content (B), and Hippocampal Insulin Receptor Content (C) in offspring of the stressed and non-stressed mothers

Each point indicated the Mean $\pm$ SEM for 8 animals. \*P<0.05 and \*\*P<0.01 statistical difference with the corresponding control group.

MC: male control; FC: female control; MS: male stress; FS: female stress.

offspring belonging to the control (non-stressed) mothers (one-way ANOVA;  $F_{3,24}=2.24$ ,  $P<0.1$ ; Figure 3C).

### Relationship between offspring blood and hippocampal insulin level and hippocampal insulin receptor content and their spatial learning

The Pearson correlation analysis indicates a positive correlation between the stress offspring's spatial learning deficit and their blood and hippocampal insulin level and their hippocampal insulin receptor content as well (Table 1). However, this relationship is not seen in the control groups.

## 4. Discussion

Prenatal stress-induced spatial learning and memory deficit in offspring and hippocampal insulin resistance were more abundant in the male offspring than in female ones. These findings suggest the importance of prenatal stress on the hippocampus development and function in

the offspring. In addition, these findings may indicate the sex difference in response to prenatal stress as the male offspring were more sensitive than the female offspring.

Results of the present study indicate that the male and female offspring from the stressed dams spend more time and travel more distance to reach the target hole than their counterparts in the control groups. These findings are in agreement with other findings that indicate that prenatal stress can induce spatial learning and memory in rats and mice, which is accompanied by atrophy in the hippocampal neurons and a decrease in neurogenesis in the hippocampus as well (Benoit, Rakic, & Frick, 2015; Bock, Wainstock, Braun, & Segal, 2015; Hosseini-Sharifabad & Hadedoushan, 2007; Lemaire, Koehl, Le Moal, & Abrous, 2000). For example, Benito et al. (2015) have shown that prenatally stressed C57BL/6 mice (dams received chronic unpredictable stress during gestational days) perform less spatial learn-

**Table 1.** Correlation of the hippocampal insulin content, hippocampal insulin receptor content, and plasma insulin level with the spatial learning and memory

Variables		Hippocampus Insulin Content		Plasma Insulin Content		Hippocampus Insulin Receptor Content		Spatial Memory	
		The Pearson Coefficient	P	The Pearson Coefficient	P	The Pearson Coefficient	P	The Pearson Coefficient	P
Control	Male	0.241	0.51	0.31	0.21	0.154	0.16	0.343	0.38
	Female	0.173	0.23	0.18	0.27	0.213	0.27	0.321	0.321
Stress	Male	0.589	0.015*	0.57	0.023*	0.645	0.018*	0.734	0.0114*
	Female	0.3514	0.0911	0.43	0.054*	0.35	0.057*	0.651	0.034*

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Values are expressed as the Mean±SEM for 8 male or female mice. \*P<0.05 for the stress vs the control groups. Weinstock, M. (2017). Prenatal stressors in rodents: effects on behavior. *Neurobiology of Stress*.

ing and memory than non-stressed ones in the Morris water maze task. The prenatally stressed animals swam slower and needed a longer time to reach the platform in the task. These researchers also showed several epigenetic changes in the hippocampal neurons, which they proposed to be involved in the spatial learning and memory deficit. In addition, it is shown that prenatal restraint stress from day 15 until delivery (3 times, 45 min) also induced spatial learning deficit in Sprague-Dawley rats. This deficit was accompanied by inhibiting neurogenesis in the hippocampus (Lemaire et al., 2000). It is also shown that prenatal restraint stress (1 h/day from day 15 of pregnancy until delivery) can induce spatial learning deficit and decrease CA3 cell dendritic tree size in Wistar rats' offspring (Hosseini-Sharifabad & Hadinedoushan, 2007). Interestingly, sex differences are also shown in the previous studies in relation to prenatal stress. In this regard, it is shown that prenatal restraint stress on the female pregnant Sprague-Dawley rats from day 17 of pregnancy until delivery (1 h/day) can inhibit the spatial learning and memory in T and Y maze and also affect passive avoidance test in the offspring (Gué et al., 2004). The male offspring were more vulnerable to deficit than females in this experiment. These findings which are in agreement with our findings, indicated that prenatal stress could affect the hippocampus function in spatial learning and memory. Even though we did not investigate the morphological and epigenetic changes in the hippocampus, similar changes may happen in the animals in our study as well.

A significant part of our findings was prenatal stress-induced insulin resistance in the hippocampus, which was in relation to spatial learning and memory deficit in the male and female offspring. Our data indicated that prenatal stress decreased plasma insulin levels in male

and female offspring. This finding agrees with previous studies in this regard (Lesage et al., 2004). Previous studies indicate that prenatal stress can induce glucose intolerance and reduced basal plasma insulin levels in the offspring (D'mello & Liu, 2006; Lesage et al., 2004; Tamashiro, Terrillion, Hyun, Koenig, & Moran, 2009). Our data also indicated hippocampal insulin content was reduced in male and female stressed offspring. Insulin can cross the blood-brain barrier via an active process, and any decrease in plasma insulin level could be reflected in the brain as well (Banks, 2004; Banks, Owen, & Erickson, 2012; Baura et al., 1993). Prenatal stress-induced plasma insulin level decrement in the offspring might be responsible for our observation.

In addition to hippocampal insulin content decline, a decrease in the hippocampal insulin receptors was also observed in our study. According to previous studies, hippocampal insulin resistance is characterized by hippocampal insulin content and hippocampal insulin receptor content decrement (Biessels & Reagan, 2015b). Our data indicate that prenatal stress can induce hippocampal insulin resistance in male and female offspring. Since hippocampal insulin resistance is considered the main cause of spatial learning and memory deficit (Craft, 2007), it is not surprising that the male and female offspring belonging to the stressed dams showed a decrease in learning and memory in the Barnes Maze task. Interestingly, the spatial learning and memory deficit and hippocampal insulin receptor and insulin content show a significant correlation. This correlation indicates that hippocampal insulin resistance must be considered one of the possible mechanisms involved in the effect of prenatal stress on spatial learning and memory deficit in the offspring. However, the relationship between hippocampal insulin resistance and spatial learning and

memory deficit also was sex-specific and the male offspring were more affected than the females. This finding has a potential interest to be the object of future studies in this regard.

## Ethical Considerations

### Compliance with ethical guidelines

All the experiments were conducted according to the animal care guidelines of Animal Ethics Committee of Baqiyatallah University of Medical Sciences.

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The current work was granted from the Neuroscience Research Center, Baqiyatallah University of Medical Sciences.

### Authors' contributions

Investigation: Masoomeh Mohammadi; Conceptualization: Ali Haeri-Rohani; Data curation and methodology: Parichehr Yaghmaei; Writing, review and editing, visualization, supervision, project administration, and funding acquisition: Hedayat Saharei.

### Conflict of interest

The authors declared no conflict of interest

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