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Original Article

Nanoparticles of Magnesium Oxide Improve Autistic-Like Behaviors Induced by the Maternal Separation Model without Affecting Gonads Structure

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Abstract

Maternal separation in the early days after birth can induce autistic-like behaviors in animals. Nanoparticles of Magnesium oxide (nano-MgO) can decrease anxiety, pain perception and improve animal memory. Also, sex hormones are involved in the formation of many behaviors. In this study, the effects of nano-MgO on behavioral responses in immature rats and gonadal histological structures of adult rats in the autistic-like model of maternal separation were investigated. Juvenile male and female offspring (60±5g) were divided into control and maternal-separated groups. The maternal separation was done by separation of the pup from the mother for 1 hour/daily/1-10 postnatal days. Nano-MgO 2.5 and 5 mg/kg were injected intraperitoneally during the 31±2-35±2 postnatal days. Pain perception and memory were evaluated after the first, third, and last injections and on 45±2 postnatal days. Social interaction, anxiety level, and motor activity were evaluated on 36±2 postnatal days. Tissue samples were removed from the testis and ovary for histological studies on 73±2 postnatal days. Maternal separation increased pain perception, anxiety, and motor activity, and also decreased social interaction index, and impaired memory in animals. Nano-MgO improved anxiety, and social interaction, induced analgesia, and modulated hyperactivity. Also, memory impairment was reversed by the nano-MgO2.5 mg/kg while it was not significant. There were no alterations in the histological and histometrical structure of the testis and ovary of adult rats between the studied groups. The behavioral complications caused by the autistic-like model can be corrected by nano-MgO; however, the gonads were not affected by the autism condition and nano-MgO application.

Keywords: Autism, Gonadal tissues, Magnesium oxide, Maternal Separation, Nanoparticles.

1. Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder that has been reported in recent years [1]. This disease was characterized by persistent deficits in communication, social interaction, limited and repetitive patterns of behavior, interests, or activities that manifest clinically during early development [1, 2]. Multiple factors such as genetic mutations, activation of the maternal immune system and environmental stimuli can cause ASD [3, 4]. Maternal separation (MS)

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during the first weeks after birth is stressful behaviors that can cause symptoms of ASD which include changes in the brain and behavior and can affect neurogenesis and cause a decrease in hippocampal neurons [5]. Also, this kind of stress can lead to hyperactivity, inactivity, fear, anxiety and, defensive behaviors [6].

On the other hand, an imbalance in the trace ions levels such as copper, zinc, magnesium and selenium has been observed in the hair and nail of children with ASD [7]. A significant magnesium deficiency was observed in ASD patients [8]. Magnesium is involved in essential cellular processes such as nucleic acid formation and energy metabolism and neurodevelopment; magnesium regulates glutamate-activated channels in neuronal membranes, a process that highly correlated with ASD pathogenesis [9]. Magnesium deficiency can increase memory impairment and induce behaviors such as anxiety and depression [10, 11]. Also, magnesium increases the pain threshold [12].

In recent years, nanotechnology has been expanded with a wide range of important applications at the cellular and molecular level [13-15]. Nanoparticles of MgO (nano-MgO) can affect anxiety, learning and memory, and pain perception [13, 14, 16-18]. However, there has been no specific report on the use of nano-MgO in the treatment of ASD yet.

On the other hand, it seems that early life stress caused by MS has destructive effects on sperm parameters and testicular tissue [19]. Maternal separation in male rats in the first days after birth has a decreasing effect on the number, morphology, and lifespan of sperms at maturity, increases the level of reactive oxygen species, and decreases the concentration of glutathione peroxidase and adenosine triphosphate [19]. In the female rat, MS causes a decrease in primary follicles and an increase in secondary follicles and graft follicles [20].

Some nanoparticles may cause reproductive toxicity and damage in the male reproductive system too [21-23]. Bioaccumulation of nanoparticles in the testicles affects the number of sperm, their motility and morphology [24, 25]. In addition, causes disruption in Leydig cells, which causes a decrease in testosterone level [26, 27]. It has been shown that nano-MgO have a dose-dependent adverse effects or on the testis and ovary of rats [21].

Since there were no specific studies about the effects of nano-MgO on behavioral responses and sexual tissue histology in an autistic-like model of MS, in this study, the effects of nano-MgO on the behavioral parameters, including anxiety, social interaction, motor activity, memory, and pain perception in immature rat have been evaluated. Also, histological structures of the testis and ovary after puberty have been evaluated in autistic-like model of MS in male and female rats.

2. Materials and Methods

2.1. Animals

In this experimental study, the parent male and female Wistar rats were obtained from the breeding center of laboratory animals in the Shahid Chamran University of Ahvaz.

Rat's offspring were randomly divided into four groups. All rats were kept under suitable

conditions (25C˚ and 12/12 light-dark) with sufficient access to water and food at all times except during the tests. Training and tests were conducted during daylight between 8:00 AM; and 2:00 PM.

2.2. Autistic-like model induction and animal grouping

Male and female Wistar rats were allowed to mate. Pups were divided into control and MS groups. In the control group, pups were kept with their mothers for all 21 days after birth and did not undergo any treatment (2 male and 6 female pups). In the MS group, pups were separated from the mother, daily for 1 hour (1-10 postnatal days) and kept in a plastic box (9×8 cm, under normal temperature) without bedding [28].

The process of the maternal separation was done between 9-11 Am. At the end of the lactation period, the maternal separated pups were divided into three groups, including sham (recipient of saline 0.9%) (4 male and 3 female pups) and recipients of nano-MgO 2.5 mg/kg (2 males and 5 female pups), and 5 mg/kg (2 male and 6 female pups) (US Nano, Co USA) [17]. All components were injected intraperitoneally once before puberty for five constant days at the age of 31±2 - 35±2 days. Behavioral assessment according to **Figure 1** was done in two stages. All procedure following, the institutional guidelines for animal care followed by Shahid Chamran University of Ahvaz (EE/1401.2.24.118713/scu.ac.ir).

2.3. Behavioral tests

All behavioral tests were done on juvenile rats (60±5-75±5 gr) between days of 30±2-45±2 and the interval time of thirty minutes was used between tests (**Fig. 1**).

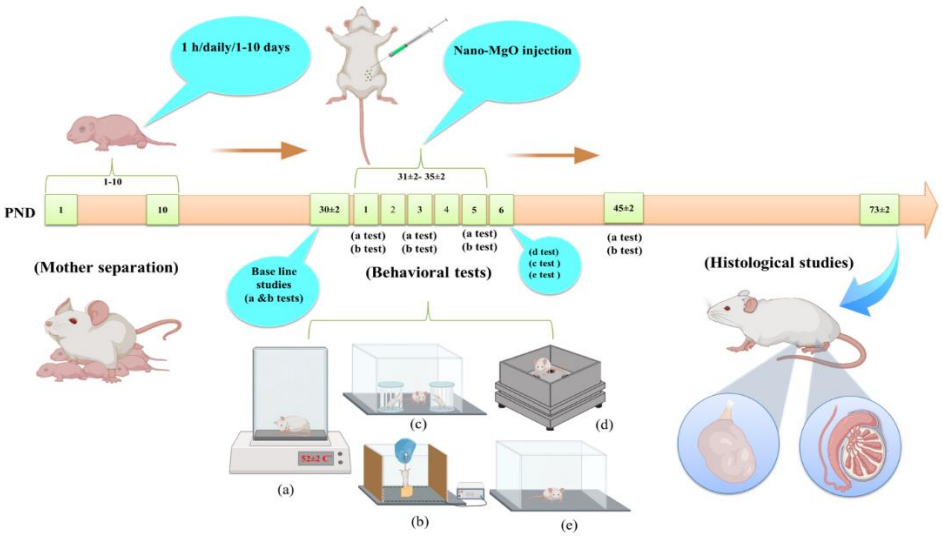

Figure 1. Schematic design of the study. Maternal separation was done between 1-10 PNDs. Behavioral tests, including hot plate (a), step-down (b), social interaction (c), hole board (d), and open field (e) were done between 30±2-45±2 PNDs. Baseline of the pain perception and step -down training were evaluated on 30±2 PND. Hot plate and step-down tests were done on 31±2, 33±2, 35±2 and 45±2 PNDs. Hole board, social interaction, and open field tests were done on 36±2 PND. Gonadal histological studies were done on 73±2 PND. PND= postnatal day.

2.3.1. Hot plate test

Pain perception was evaluated by the hot plate test. Baseline of the pain level was measured 24 hours before injections (on 30±2 postnatal day), and pain test was done 30 minutes after the first, third and the last injection. For evaluating the stability of the nano-MgO effects, pain perception was evaluated on 45±2 postnatal days. Animals were placed on a hot plate apparatus (52 ± 2 °C) and the latency to lick the hind paw was recorded on 31±2 and 35±2, and 45±2 postnatal days [14].

Analgesia was expressed as a percentage of maximum possible effect (MPE %) as follows:

$$\text{MPE\%} = (\text{post\_treatment time} - \text{pre\_treatment time}) / (\text{Cutoff time} - \text{pre\_treatment time}) \times 100$$

Where pre-treatment time is =latency time on 30±2 postnatal days; and post-treatment time is =latency time on 31±2, 33±2, 35±2, and 45±2 postnatal days [29].

2.3.2. Step-down apparatus

Memory was assessed by the step-down apparatus. This device was a Plexiglas box with stainless steel grid floor and a safe plastic platform in the center of the box. Animals were trained on 30±2 postnatal days. The memory test was done 30 minutes after the first, third and the last injection on 31±2, 33±2 and, 35±2 postnatal days, and for evaluating the stability of the nano-MgO effects, memory test was evaluated on 45±2 postnatal days. On training day, rat was placed on safe platform and received an electrical shock (15 volt/ 15 second) when all its paws placed on the grid floor. On test day, rat was placed on safe platform and step-down latency (SDL) recorded during 300 seconds [16].

Social interaction, anxiety, and locomotor activity were evaluated 24 hours after the last injection (on 36±2 postnatal day).

2.3.3. Social interaction test

Social interaction test was conducted for 5 min in a square space (40x40x40 cm), which had a short wall (15×40×2 cm). Two plastic cylindrical enclosures with removable lids were located on opposite sides of the small wall in the middle of the box, in which the strange rats were placed. At habituation time in order to adapt to the environment each rat was placed in a square space with empty chambers for five minutes and then a strange rat was placed in one of the chambers for ten minutes. On the test time, second strange rat was placed in another chamber and the time that the test rat spent near the cage of this strange rat, and the time spent away from the cage of rats was recorded during five minutes [28]. The index of social interactions was calculated with the following formula:

$$\text{Sociability} = \frac{\text{The amount of time spent near the cage of a strange rat}}{\text{The amount of time spent away from the strange rat cage}}$$

2.3.4. Hole board test

The anxiety test was conducted with hole board test on the day of 36±2. The anxiety test device consisted of a 40 x 40 cm board with 16 circular holes and glass walls with a height of 40 cm. The animals were placed on this device for five minutes and the time of the first time the rat stuck its head in one of the holes and then the number of times this action was recorded [30]

2.3.5. Open field test

Open field apparatus was a Plexiglas box (40×40×40 cm diameters). Each rat was placed on the center of the box and the number of crossed lines during a 5 min was considered animal locomotor activity [31].

2.4. Histological analysis

Animals were sacrificed, and the testes and ovaries were removed on 73 ± 2 postnatal days and fixed in buffered formalin solution for 48 hours. Then, tissue samples were excised, and processed for paraffin embedding sections. Sections with 5μm thickness were stained with hematoxylin and eosin and used for histological and histometrical studies at a light microscopic level (Olympus BH, Tokyo, Japan). In each rat, 90 round cross-sections of seminiferous tubules were chosen randomly. The diameters and germinal epithelium height of each seminiferous tubule cross-section were measured at a magnification of ×40 in male rats. Also, the diameters and numbers of ovarian follicles were evaluated in different experimental groups.

2.5. Statistical analysis

Statistical analysis was done by the Instat 3 software one-way analysis of variance (ANOVA) with Tukey post hock testwas used. The significance level was considered as $p<0.05$.

3. Results and Discussion

3.1. Behavioral analysis

3.1.1. Hot plate test

Figure 2 shows, MPE % was significantly decreased in the MS group on 31±2 postnatal days ($p<0.05$), and it was not improved by the acute injection of nano-MgO on this day ($p>0.05$). Significant difference was no observed between MPE % of maternal separated group and control group on 33±2, 35±2, and 45±2 postnatal days ($p>0.05$). MPE% was improved by the nano-MgO 2.5 mg/kg and it was significant in compared to saline or nano-MgO 5 mg/kg on 33±2, and 35±2 postnatal days ($p<0.01$). Also, there was not significant differences between MPE% of nano-MgO recipient groups and saline group on 45±2 postnatal days ($p>0.05$).

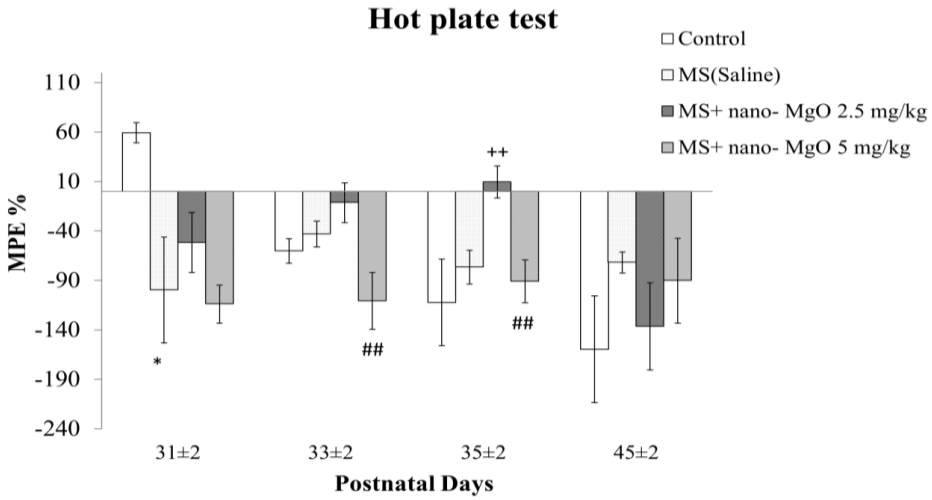

Figure 2. Hot plate test in juvenile rats. *P < 0.05 is in comparison to the control (no maternal separation) group, ++P < 0.01 is in comparison to the MS (saline) group, and##P<0.01 is in comparison to MS+ nano-MgO 2.5 mg/kg. Each bar shows mean ± S.E.M. (N=7-8). MS= maternal separation, nano=nanoparticles.

3.1.2. Step-down test

As **figure 3** shows, in the step-down test, latency time was decreased in the MS rats, 24 hours after training of 31±2 postnatal days ($p<0.05$). Significant differences were not observed between control (no maternal separation) and the MS rats on 33±2, 35±2, and 45±2 postnatal days ($p>0.05$). Latency time was improved by the nano-MgO 2.5 mg/kg, on 31±2, and 33±2postnatal days ($p>0.05$), but it was not significant. Also, significant difference was not observed between saline and nano-MgO recipient groups on 35±2 postnatal days ($p>0.05$) even though nano-MgO 2.5 mg/kg could improve latency on compared to nano-MgO 5 mg/kg on 45±2 postnatal days ($p<0.05$).

3.1.3. Social interaction test

The social interaction index was decreased in the MS rats ($p<0.001$), and it was significantly increased by the nano-MgO 2.5 mg/kg ($p<0.001$) (**Fig. 4**). Even though the social interaction index was increased by the nano-MgO 5 mg/kg, it was not significant.

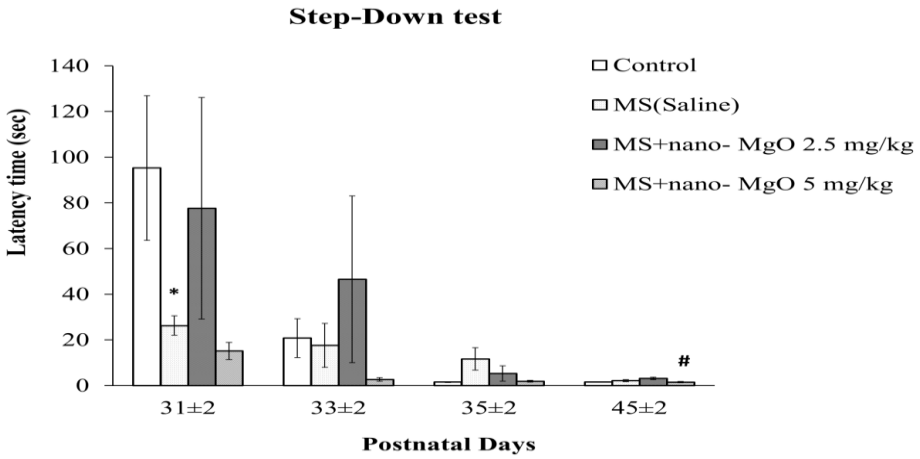

Figure 3. Step-down test in juvenile rats. *P<0.05 is in comparison to the control (no maternal separation) group on the same day, and #P<0.05 is in comparison to nano-MgO 2.5 mg/kg group on the same day. (N=8).MS= maternal separation, nano=nanoparticles.

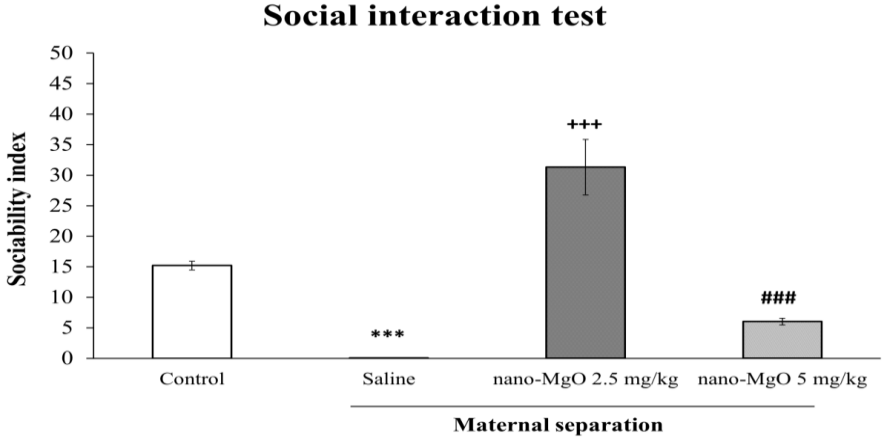

Figure 4. Social interaction test in juvenile rats. ***P < 0.001 is in comparison to the control (no maternal separation) group, +++P < 0.001 is in comparison to the MS (saline) group, and ###P<0.001 is in comparison to nano-MgO 2.5 mg/kg. Each bar shows mean ± S.E.M. (N = 6-8).

3.1.4. Hole board test

Anxiety-like behavior was measured by the hole board test one day after the last injection (Day of 36±2). Latency time was decreased in the MS rats ($p<0.001$) while it was increased by the nano-MgO 2.5 mg/kg ($p<0.001$) (**Fig. 5A**).

The number of head dipping was increased in the maternal separated rats too ($p<0.001$) and treatment by the nano-MgO significantly decreased this parameter ($p<0.001$) (**Fig. 5 B**). It was notable that the efficacy of nano-MgO 2.5 mg/kg was much more than nano-MgO 5 mg/kg.

3.1.5. Open field test

Locomotor activity was increased in the MS group ($p<0.001$) (**Fig. 5**), and was decreased significantly by the nano- MgO ($p<0.001$) to the level of the control group. Also, locomotor activity was improved by nano-MgO 2.5 mg/kg better than nano-MgO 5 mg/kg ($p<0.05$) (**Fig. 6**).

The results of this study have shown that MS during 1-10 days after birth could induce autistic-like behaviors, including social interaction disorder, anxiety, hyperalgesia, hyperactivity, and memory impairment in juvenile male and female rats (**Figs. 2-6**).

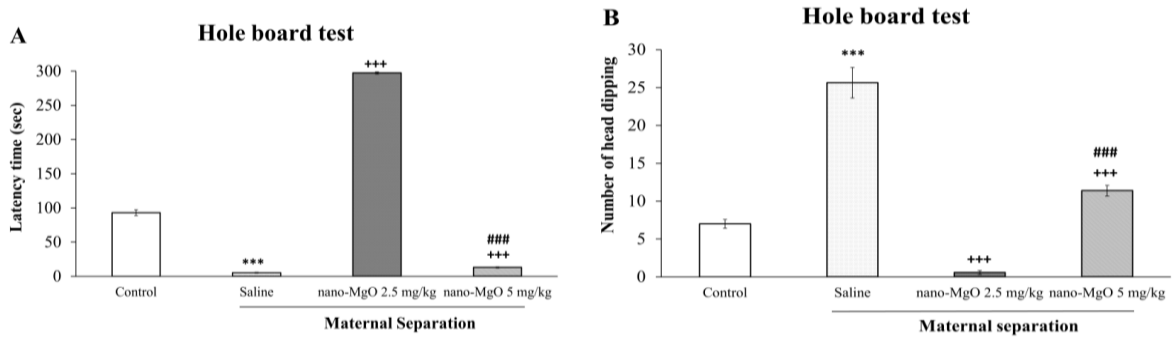

Figure 5. Hole board test in juvenile rats. ***P < 0.001 is in comparison to the control (no maternal separation) group, +++P < 0.001 is in comparison to the MS (saline) group, and ###P<0.001 is in comparison to nano-MgO 2.5 mg/kg. Each bar shows mean ± S.E.M. (N = 6-8).

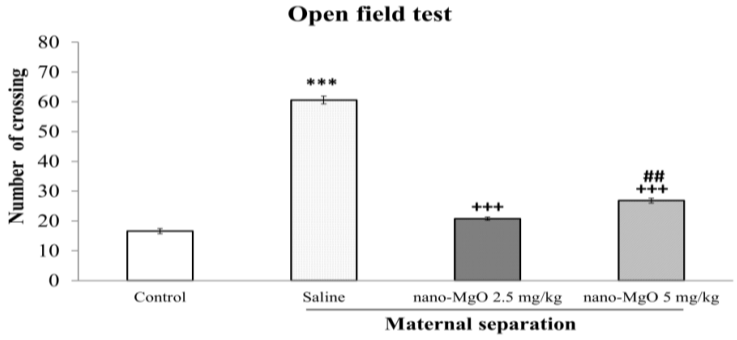

Figure 6. Open field test in juvenile rats. ***P < 0.001 is in comparison to the control (no maternal separation) group, +++P < 0.001 is in comparison to the MS (saline) group, and ##P<0.01 is in comparison to MS+ Nano-MgO 2.5 mg/kg. Each bar shows mean ± S.E.M (N=6).

The MS model is a widely accepted animal model for studying autistic-like behaviors [4, 32, 33].

Maternal separation can cause depression, anxiety, and social interaction disorders, which are accompanied by neurogenesis disorders in adult mice [34]. Also, it leads to long-term changes in memory loss, lowering the pain perception threshold and increasing motor activity [3, 33, 35]. In this study, following previous experiments, it was confirmed that maternal separation is an acceptable model for inducing autistic-like behaviors.

On the other hand, use of the nano-MgO in juvenile rats significantly improved autistic-like behaviors (Figures 2-6). Nano-MgO can increase analgesia, memory improvement, anxiolytic effect, and decrease motor activity in rats [13, 14, 36]. Torabi and et al (2020 and 2018) have shown that nano-MgO induced analgesia and improved anxiety, and in high dose decreased motor activity in rats under acute restraint stress [14, 36, 37]. They also have shown that nano-MgO could decrease glutamate levels and increase the expression of the NR2A subunit of the N-Methyl-D-Aspartate (NMDA) receptor in the hippocampus of rats under normal and stress conditions and significantly change the magnesium, iron, and calcium ions level in these rats [14, 36].

Kesmati and et al (2021) have shown that usage of nano-MgO in the Alzheimer-like model of rat improved short and long-term memory, increased the magnesium ion level and total antioxidant capacity in the blood serum, and improved cell damage induced by streptozocin in different areas of the hippocampus of male rats [13].

Nabaee and et al (2022) have shown that the nano-MgO significantly improved memory impairment induced by sleep deprivation by increasing the level of magnesium ions in the hippocampus of rats without changing the level of oxidant/ antioxidant factors as well as brain-derived neurotrophic factor in the hippocampus area [38]. They also showed that the nano-MgO in high dose caused hypoactivity in rats [38].

Since, maternal separation in the early days after birth can affect the number of neurons in the rat hippocampus by decreasing neurogenesis or increasing neuronal apoptosis [4]. As well magnesium ion level decrease in the brain of autistic children [39]. So that it seems the use of nano-MgO in the present study has been able to improve autistic-like behaviors by increasing the magnesium content of the brain.

3.2. Histological analysis

3.2.1. Testis

The histological study of the testis in control rats showed that the testicular tissue consists of several seminiferous tubules with normal structure such that each seminiferous tubule is composed of different types of spermatogenic cells along with Sertoli cells (**Fig. 7A** and **B**). Microscopic structure of the testis in maternally separated rats alone, and the MS rats receiving nano-MgO indicated that the normal histologic structure of seminiferous tubules, and no structural differences were observed between studied groups (**Fig.7 C-E**).

The histometric analysis of the testis structure showed no statistically significant difference between the mean germinal epithelium height and the seminiferous tubule diameters in all studied groups (**Table 1**).

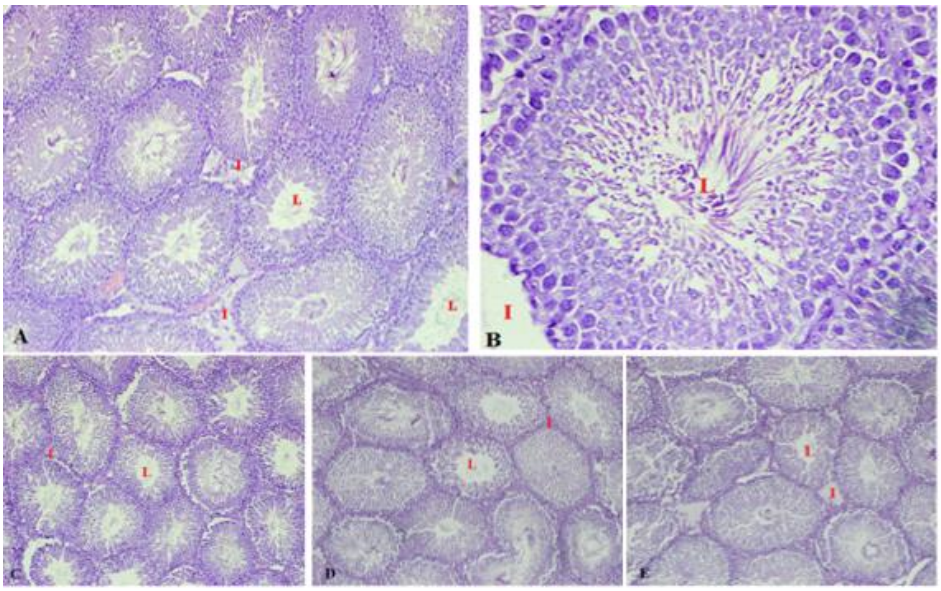

Figure 7. Testis structure in control (A and B), MS (C), and MS+ nano-MgO 2.5 or 5 mg/kg (DandE) groups. Transverse sections of seminiferous tubules with normal germinal epithelium and spermatozoa cells in the lumen of some of them are observed (10x magnification, Hematoxylin-Eosin staining, A, C-E), (400x magnification, Hematoxylin-Eosin staining, B). L: Lumen, I: Interstitial tissue.

Table 1. Mean (±S.E.M) germinal epithelium height and seminiferous tubule diameters in the testis of all studied groups.

Groups	Seminiferous tubules diameter (µ)	Germinal epithelial height (µ)
Control	149.8±0.71	45.9±0.63
MS +saline	150.5±0.7	44.8±0.59
MS+ nano-MgO (2.5mg/kg)	151±0.67	45.5±0.71
MS+ nano-MgO (5 mg/kg)	149.4±0.69	44.9±0.59

3.2.2. Ovary

The histological study of the ovary showed the normal ovarian structure, consisting of different ovarian follicles, including primordial, primary, secondary and antral follicles along with corpus luteal, the in cortex of ovary and loose connective tissue containing blood vessels in the medulla of the ovary, in all studied groups (**Fig. 8**).

Table 2 shows the histometric analysis of the ovary in adult female rats. There is no statistically significant difference between the mean numbers of ovarian follicles in all studied groups.

Table 2. Mean (±SEM) numbers of ovarian follicles in all studied groups.

Groups	Primordial	Primary	Secondary	Antral
Control	6.20±0.15	3.76±0.11	4.70±0.13	5.83±0.13
MS +saline	6.06±0.16	4.20±0.17	4.26±0.16	5.56±0.17
MS+ nano-MgO (2.5mg/kg)	6.06±0.16	0.18±4.10	0.15±4.36	0.13±5.70
MS+ nano-MgO (5 mg/kg)	6±0.19	3.90±0.11	4.46±0.14	0.17±5.86

Histological results have shown that the maternal separation and treatment with nano-MgO could not affect histological parameters of the testicular tissues, such as the average height of the germinal epithelium, the diameter of the seminiferous tubules and ovarian tissues, such as the average number of ovarian follicles and also corpora lutea did not exist (**Figs. 7** and **8**).

Probably in this study, maternal separation could not affect sexual tissues due to the shortness of the daily separation time (one hour) or the period of separation (ten days), the long interval between the end of the separation and the collection of the tissues, the lack of connection between the separation from the mother and the changes in the tissue.

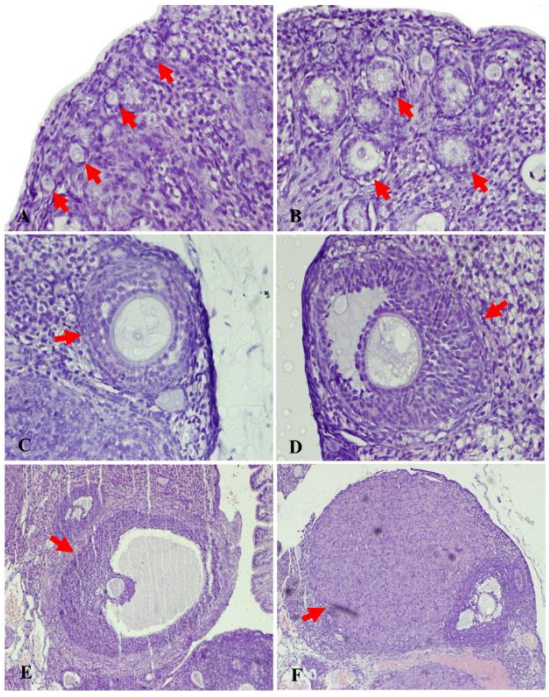

Figure 8. The normal structure of primordial(A), primary (B), secondary (C), and antral (D and E) follicles and the corpus luteum (F) were observed in the control group (40x magnification, hematoxylin-eosin staining).

Also, Naguib and et al (2023) have shown that nano-MgO in high dose produced considerable changes in sex hormones, stress parameters, and histopathological alterations in the testicular and ovarian tissues of male and female rats [21].

According to this study, no harmful effect of nano-MgO was observed on sexual tissues, which can show its positive role in long-term use.

4. Conclusion

According to the results of this study, it seems that the autism model of separation from the mother during a critical period can affect many behaviors, such as pain perception, memory, motor activity, and social interaction, by affecting the brain, and this disorder improves remarkably with nano -MgO, while the structure of the gonads tissue are less affected by separation from the mother, so it can be concluded that the brain tissue is more sensitive than the gonads and nano-MgO is a good candidate for improving the complications of autism. In this way, it is suggested that the neurochemical changes of different parts of the brain after the induction of autism in the presence of nano -MgO should be studied and to prevent some clinical complications of the toxicity of the above substance, it should be investigated more precisely.

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Conflict of interest

The authors declare to have no conflict of interest.

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