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PERINATAL EFFECTS OF LITHIUM CARBONATE NANOPARTICLES ON PITUITARY-GONADAL AND PITUITARY-THYROID AXES OF RATS' FEMALE PROGENIES

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ABSTRACT

Fertility and reproduction are from of the most important issues of communities. Considering the interaction of reproduction system and thyroid gland plus various behaviors of nanoparticles and their main materials, this study was carried out to investigate the perinatal effects of lithium carbonate nanoparticles on ovarian and thyroid textures and the amount of sexual and thyroid hormones in rats' female progenies. Forty female pregnant rats were divided into control, placebo (0.5 ml of distilled water), 1.26 and 1.90$_{mg/kg/bw}$ doses of lithium carbonate nanoparticles. All injections were done intraperitoneal for twenty days. At the end of lactation period, female progenies were separated and ten rats were selected randomly from each group. Blood samples were taken and the amount of sexual and thyroid hormones were measured. Also, by separating thyroid and ovary glands, cell count was performed in these tissues. Results showed that nanoparticles reduced estrogen, progesterone, LH and FSH hormones in both doses and also reduced pre-antral and antral follicles, the number of corpus luteum and increased corrupted follicles significantly ($P<0.01$). On the other hand, these particles reduced T3, T4, thyroid follicles, epithelial height of follicles and nuclear TSH and colloidal space of follicles significantly ($P<0.01$). According to results, nanoparticles of lithium carbonate damaged performance and structure of pituitary-gonadal axis and pituitary-thyroid axis of rats.

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Introduction

Lithium (derived from the Greek word of lithos meaning stone) with Li symbol is an alkaline silver-white metal which is soft with atomic number of three [1]This element is the lightest metal with the lowest density under the standard conditions of temperature and pressure. Lithium, similar to other alkaline metals, is very reactive and flammable so it can never be found as

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a free element in nature [2]. Lithium was discovered at 1817 and was used in medicine at 1817 for mania treatment. At 1886, a non-organic lithium (carbonate), which is used today, was introduced for depression treatment (Granados et al. 2002)
Lithium carbonate is the most frequently used form of lithium. Salt form of lithium (Li_2CO_3) was used at first for curing gout but after that was used also for bipolar depression disorders [2]. Other uses of lithium carbonate are treatment of cluster headache, skin disorders, depression treatment, schizophrenia, aggression and attention deficit disorder
The distribution of lithium in the human body is almost uniform and can be found in tissues such as the brain, kidneys, thyroid, bone, liver and muscle cells. This element is distributed widely in central neural system and is related to some of neural transmitters. For example, it reduces the release of norepinephrine and increases the serotonin synthesis [3]. About 95% of lithium carbonate is excreted in urine, about 1% in stool and 4-5% in sweat [4].
Lithium seems to be effective on mood, so that in case of inadequate absorption of lithium, people quickly become angry and react [5]. Lithium is also important to enhance the transfer of two important nutrients in the brain: folate and vitamin B12. Transportation of these matters is co inhibited by lithium deficiency and can be reversed with lithium supplementation. Since vitamin B12 and folate affect ethical parameters, stimulating the transportation of these vitamin to brain cells can be another mechanism of anti-depressant and anti-aggressive actions of lithium [6].
Existence of factors such as sodium and caffeine in diet increase excretion of lithium and then increase our necessity to this essential nutrient. In addition to these factors, stress also affects the body's need for lithium [6]. Lithium also regulates different neurotrophins including brain-derived neurotrophic factor or BDNF, nerve growth factor (NGF), neurotrophin -3 or NT3 and also receptors of these growth factors in brain [7].
Lithium also significantly protects nerve cells against glutamate, heart attacks and apoptosis through various neurotoxins by regulating and inhibiting NMDA receptors [7]. Lithium is useful in repairing brain damages from stroke, Alzheimer and Parkinson by stimulating stem cells [7],[8].
Nanotechnology is one of the areas of technology that has emerged from the convergence of physics and chemistry, and is growing rapidly around the world. Also, the application of nanoparticles in different branches of medicine and basic sciences has grown more than before. This technology has become a promising strategy for creating new materials with more properties and potential applications in treating illnesses. [9]. The small size of these particles increases surface space, solubility and mobility which give them unique chemical, physical and biological properties. Nanoparticles pass biological barriers easily, so that have more access to tissues, cells, and biological molecules in body. As a result, use of these materials can harm the health of human and other animals which are exposed to them and cause damages such as tissue breakdown and impaired function of various tissues [10].
Nowadays in various communities, a growing concern has been created about harmful effects of nanoparticles on reproduction system, fertility, growth and development of fetus. Production of sexual cells that are functional and quality damaged can lead to congenital malformations, fetal death, or incidence of cancer [11]. Considering the importance of reproduction and close linkage between the pituitary- thyroid axis with reproduction system, knowing the factors which affect hypothalamus-pituitary-gonadal axis and pituitary- thyroid axis is important especially for young adults. Also, billions of people are exposed to lithium due to using lithium in phone batteries. So, current study was carried out to study the perinatal effects of lithium carbonate nanoparticles on pituitary-gonadal and pituitary-thyroid axes of rats' female progenies.

Materials and methods

The experimental study was carried out in research animal house of Islamic Azad University (Falavarjan Branch). Forty female Wistar rats in the weight range of 200-220 g were divided into four groups: control, placebo (0.5 ml of distilled water), 1.26 and 1.90mg/kg/bw doses of lithium carbonate nanoparticles. Furthermore, eight male rats were used for mating. Rats were kept under 12:12 hours photoperiod, 25℃ temperature, and 40-50% of humidity for 14 days to adapt to environment. Samples had free access to food and water. Control group did not receive any treatment. Placebo group received 0.5 ml of normal saline daily and experimental groups received 1.26mg/kg and 1.90mg/kg of lithium carbonate nanoparticles intraperitoneal daily for 20 days. Lithium carbonate was prepared from Merck, Germany and was converted to nanoparticles by *Nanozino Company* using crystallization method .
To synchronize the ovulation time of rats, 100 micro gram of estradiol valerate was dissolved in 0.2 ml of olive oil and injected intramuscular using Insulin syringe.
After 42 hours, 50 microgram of progesterone was injected intramuscular. Six hours later, vagina smear was prepared and *Marcondes* method was used to identify the stages of the Estrous cycle [12]. In this method, each stage of Estrous cycle is identified b Based on the ratio of three types of epithelial, horny and leukocyte cells in vagina smear.
Microscopic studies showed that menstrual cycles were identical in the estrus phase. Then, every five female rats were kept in one cage with a male rate for mating. By observing vagina plaque, zero day of pregnancy was determined, male rats were separated, and every ten rats were kept in a cage. At the end of lactation, male and female progenies were separated and kept until maturity (two month). Then, female progenies were separated from each group and blood samples were taken from the heart. Samples were centrifuged for five minutes by 3000 cycle/ minute and kept under -20℃ to measure hormone amounts.
Thyroid and ovaries were separated, tissue slides were prepared and colored using hematoxylin-eosin method to count ovarian follicles. Physical dissector technique [13], was used to count follicles.

Then thyroid glands of animals were resected. After obtaining the tissue sections, they were stained with hematoxylin-eosin and counted their thyroid follicles. Regarding that there is existing a big axis along with a small axis in each follicle of thyroid tissue (figure 1). The short and long diameters of colloid spaces of each follicle were measured by Streolight software in a way that the long diameter is perpendicular on small. Then, in order to convert these direct measurements into the diameters (d) of a presumptive circle with a similar area, following formula was used [14].

$$d=\sqrt{a \times b}$$

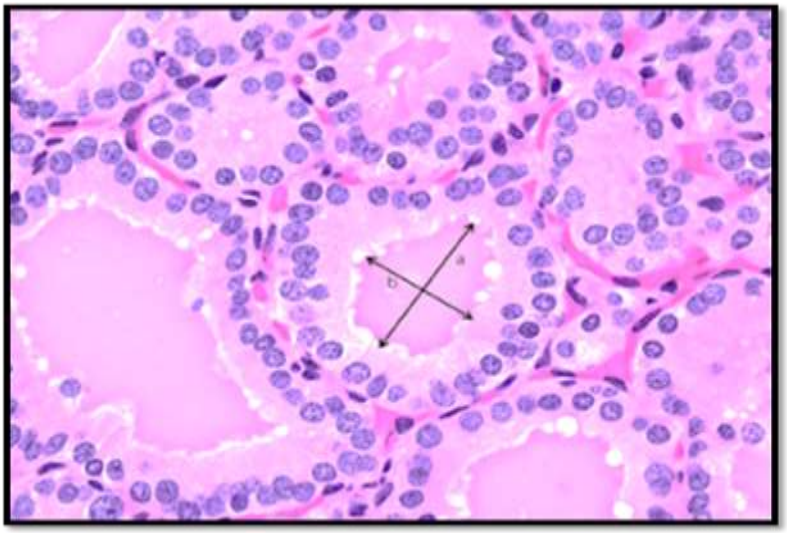

Fig 1. Light photomicrograph of thyroid tissue and presence of big and small axis a,b

Then, in order to compensate the effects of sectioned tissue (which looks like a circle) on the measured diameter which was less than expected. Abercrombie method was used. Hence, more precise diameter is calculated . Then mean of diameter (D) was estimated using the following formula.

$$Đ=d\times 4/\pi$$

Then mean correction of diameter was computed in order to determine the colloid space supposing that transverse sections are equal to a circle with the same space and then space of colloid space was computed using the following formula [14].

$$Vcol=\frac{\pi\times Đ^3}{6}$$

Also, in order to determine the nuclear-cytoplasm ratio, first 50 follicles in each sample belonging to each rat were investigated and then external and internal borderlines around of each follicle were manually drawn , using Streolight software (fig2). In this stage, cytoplasm region was computed by manually subtraction ofthis area occupied by each nucleus using the above software(although the number of nucleus in each follicle ranges 25-68, on average 44 nuclei were measured) then these nuclear regions were added and subtracted from the main area, computing the area of cytoplasmic region(Ac). Also, nuclear – cytoplasmic ratio was determined using the following formula [14].

$$N/C \text{ ratio} = \frac{A_n}{A_{nc}-A_n}$$

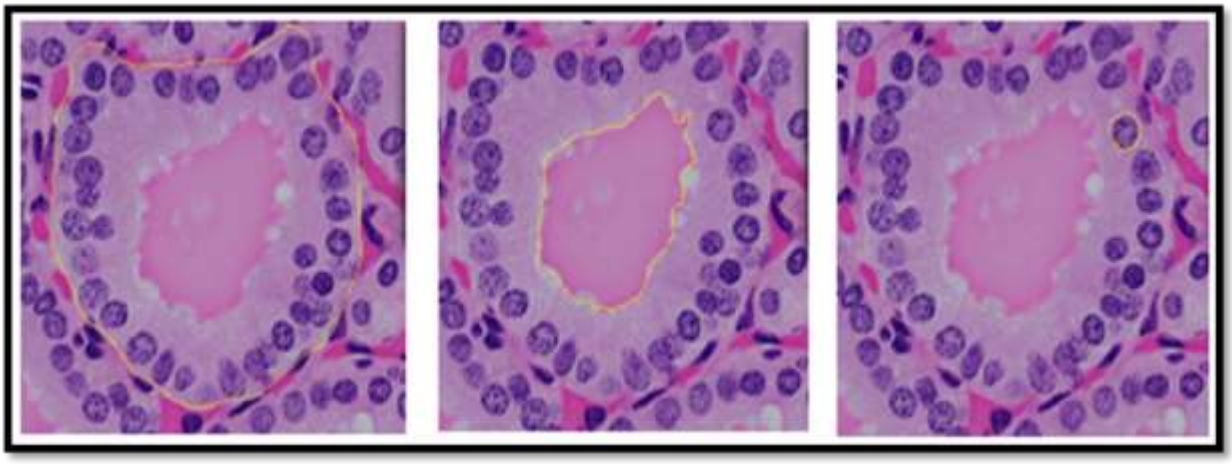

Fig 2. Light photomicrograph of thyroid tissue, external and internal lines and the area occupied by nuclei

The amounts of estrogen and progesterone hormones were measured using Elisa Machine-*State Fax 2100* , and FSH, LH and T3, T4, and TSH hormones were measured using electrochemiluminescence-*SIMENS*.
Obtained data were analyzed using one-way analysis of variance in SPSS program. Means were compared using Tukey HSD test at 5% probability level.

Results

Results showed that control and placebo groups were not significantly different in sexual and thyroid hormones and tissue changes of thyroid (table 1 and table 2) whereas both doses of nanoparticles reduced FSH, LH, estrogen, progesterone, T3, T4 significantly and increased TSH significantly ($P<0.01$).
Furthermore, nanoparticles in both doses reduced pre-antral and antral follicles and the number of corpus luteum, increased corrupted follicles, increased colloid volume significantly, and reduced total number of follicles and the number of active thyroid follicles, the nuclear-cytoplasmic ratio (N / C) of follicular cells and epithelial height of the thyroid follicles significantly (table 3 and table 4)(Figure 1 and Figure 2).

Table 1. Comparison of serum levels of FSH, LH, Estrogen and Progesterone hormones in groups treated with lithium carbonate nanoparticles with respect to to control group (mean ± standard mean error)

Groups	FSH(IU/dl) Mean ±SD	LH(IU/dl) Mean ±SD	Estrogen (ng/dl) Mean ±SD	Progesterone (ng/dl) Mean ±SD
Control	0.920 ±0.087	0.856±0.049	57.19±3.95	2.69±0.37
Placebo	0.942±0.088	0.880±0.052	57.14±3.89	2.62±0.37
Group1	0.618±0.081**	0.534±0.054**	45.31±4.06**	1.76±0.26**
Group2	0.424±0.066**	0.414±0.062**	40.46±1.96**	1.38±0.20**

**** signs significant difference at p≤0.01 compared to control**

Table 2. Comparison of serum levels of thyroid hormones T3, TSH and T4 in groups treated with lithium carbonate nanoparticles with respect to to control group (mean ± standard mean error)

Groups	TSH(IU/ml) Mean ±SD	T3(pg/ml) Mean ±SD	T4 (ng/ml) Mean ±SD
Control	1.285±0.089	1.309±0.089	2.072±0.206
Placebo	1.310±0.070	1.296±0.089	2.202±0.215
Group1	1.542±0.086**	0.605±0.077	0.958±0.149**
Group2	1.519±0.138**	0.548±0.102**	0.941±0.135**

**** signs significant difference at p≤0.01 compared to control**

Table 3. Comparison the number of ovarian follicles in the groups treated with lithium carbonate nanoparticles when compared to control (mean ± standard mean error)

Groups	number of atresia follicles	number of primordial follicles	Number of pre-antral follicles	Number of antral follicles	Number of yellow bodies
Control	4.990±0.303	4.000±0.316	5.000±0.216	4.710±0.247	4.730±0.156

Placebo	4.720±0.429	3.830±0.211	4.740±0.236	4.960±0.222	5.090±0.152
Group1	6.330±0.343**	3.190±0.255**	3.660±0.263**	3.520±0.204**	3.100±0.182**
Group2	7.240±0.309**	2.690±0.251**	3.180±0.248**	2.840±0.245**	3.150±0.242**

**** signs significant difference at p≤0.01 compared to control**

Table 4. Comparison the number of thyroid follicles, epithelium height of follicles, nuclear-cytoplasmic ratio of follicle cells and volume of colloid space of colloids in the groups treated with lithium carbonate nanoparticles when compared to control (mean ± standard mean error)

Groups	The total number of follicles Mean±SD	The total number of active follicles Mean±SD	The height of follicular epithelium (µm) Mean±SD	Nuclear-cytoplasmic ratio of follicular cells Mean±SD	The amount of space colloids (3µm) Mean±SD
Control	20.710±0.610	15.400±0.429	7.680±0.415	0.541±0.029	1440.362±36.641
Placebo	21.200±0.547	15.730±0.457	7.860±0.320	0.560±0.021	1442.621±36.182
Group1	17.860±0.748**	12.720±0.454**	6.180±0.367**	0.471±0.023**	1715.633±19.304**
Group2	16.950±0.812**	12.130±0.305**	5.550±0.430**	0.397±0.024**	1825.214±22.609**

**** signs significant difference at p≤0.01 compared to control**

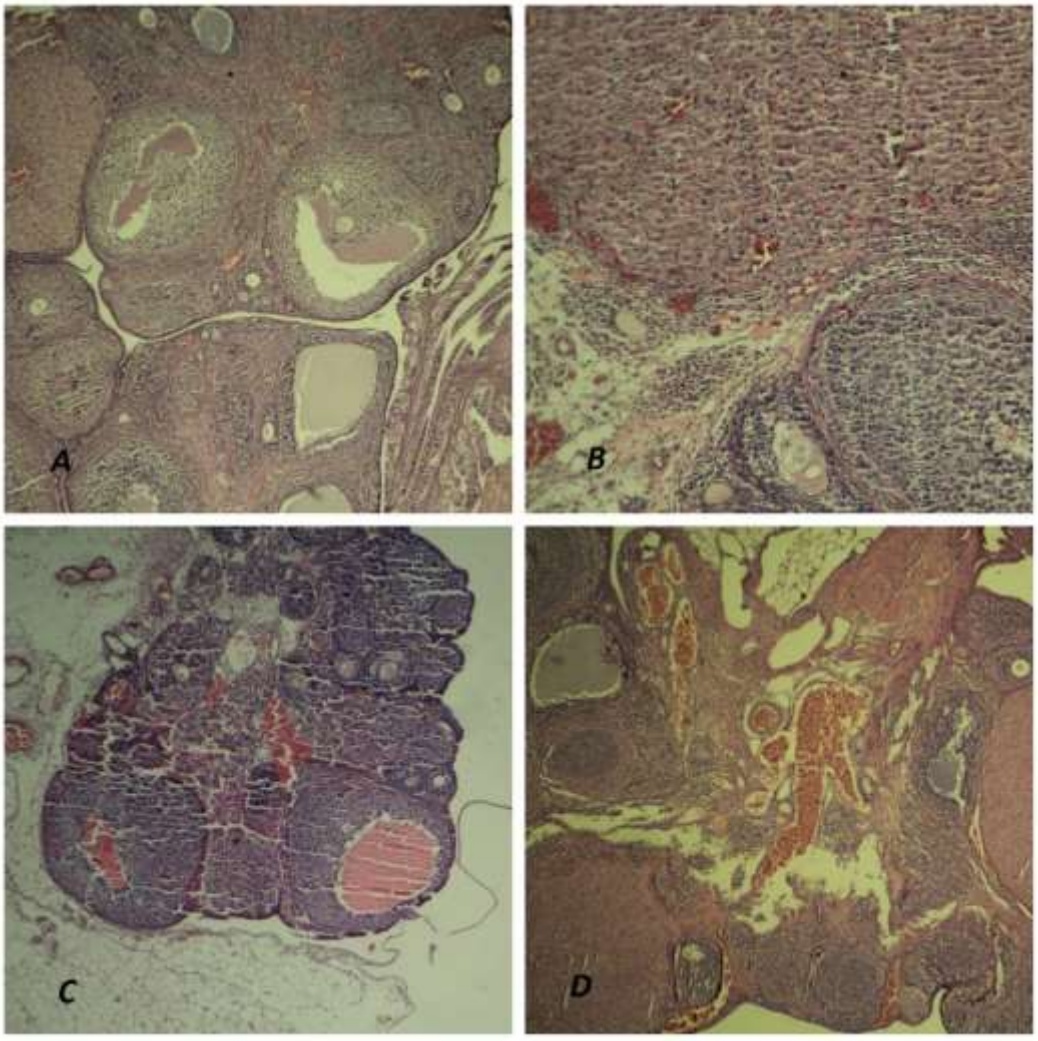

Fig 3. Light photomicrograph of ovarian tissue in (A) control group , (B) pelacebo, (C) group receiving (1.26mg / kgbw) nanoparticles of lithium carbonate and (D) group receiving (. 1.90mg / kgbw) nanoparticles of lithium carbonate. Tissues have been stained with Hematoxylin - Eosin magnification × 100

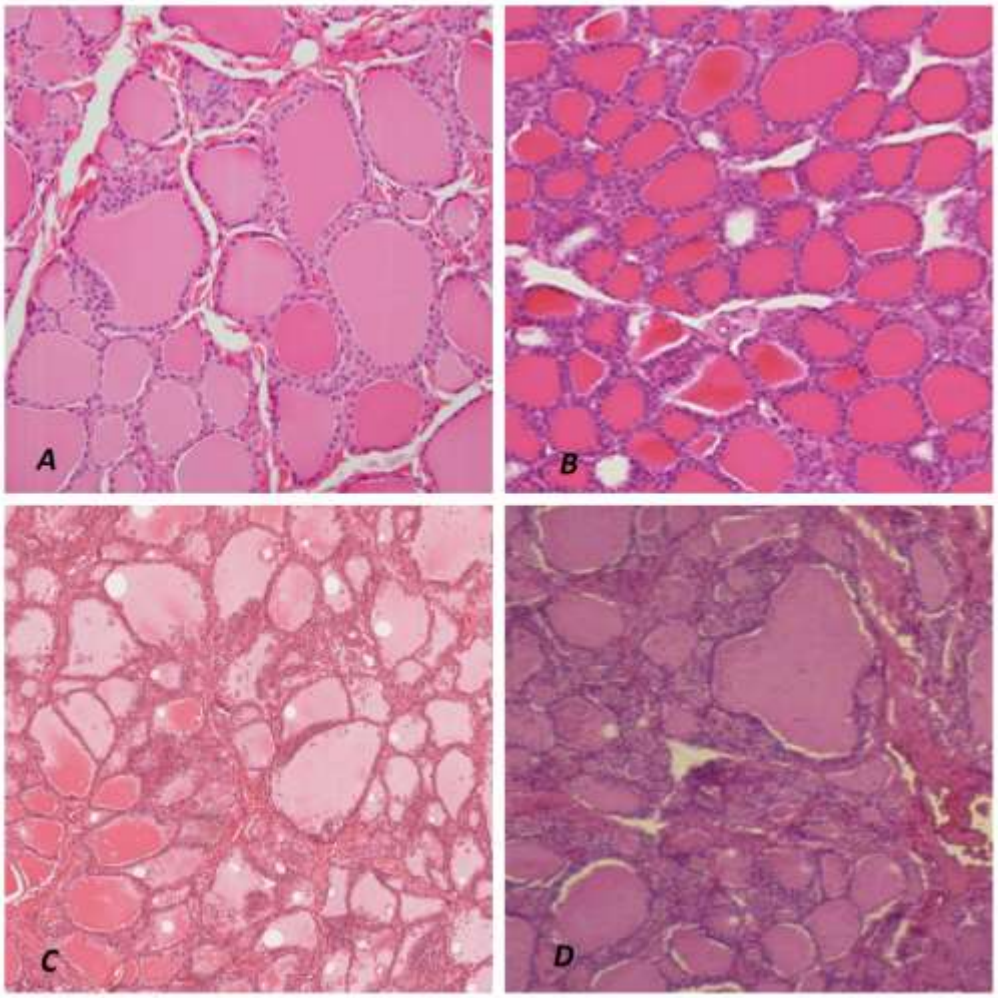

Fig 3. Light photomicrograph of thyroid tissue in (A) control group , (B) pelacebo, (C) group receiving (1.26mg / kgbw) nanoparticles of lithium carbonate and (D) group receiving (. 1.90mg / kgbw) nanoparticles of lithium carbonate. Tissues have been stained with Hematoxylin - Eosin magnification × 100

Discussion

According to results, nanoparticle of lithium carbonate reduced FSH, LH, estrogen and progesterone significantly. Also, histological study of ovarian tissue showed reduction of pre-antral and antral follicles and the number of corpus luteum, increased corrupted follicles.

Female reproductive performance is regulated by interaction of hypothalamic, anterior pituitary and ovaries hormones. FS FSH is secreted in response to gnadotropin-releasing hormones (GnRh) from the anterior pituitary basophilic cells and stimulates follicles by producing estrogen. Therefore, FSH reduction will lead to estrogen reduction and delayed follicle growth.

In agreement with this study, [15] reported that lithium had undesired effects on rat ovaries by interfering ovulation[15]. It has been determined that lithium enforces most of its effects by controlling Glycogen syntheases kinase-3 (GSK-3β) enzyme which is a key molecule in main pathway of *wnt* gene expression [16]. Wnt-family are secreted glycoproteins and have several rich and protected cysteine sections which play roles in various processes such as fetal implications, determination of cells' polarity and determining the specialty of cells.

The importance of Wnt pathway in maturity of female organs and gonads is proven [17].Since expression of various molecules of this pathway have been reported in rat's ovaries, it seems that Wnt pathway plays important role in follicle maturity [18].

Apparently, lithium interferes follicle production by interfering GSK-3B and also reduces ovarian follicles via inducing apoptosis by disrupting sodium, magnesium and other cations.

showed that nanoparticles are able to cross the placenta using endocytosis. Placental damages of nanoparticles may potentially lead to anomalies and retardation of fetal growth and development [19] showed that transferring of nanoparticles through placenta depends on size and area of them. In another study, [20] reported that gold nanoparticles can cross placental barrier. The important issue is the size of nanoparticles, so that small particles are transferred better than larger particles with similar

chemical compound. They showed that by injecting gold nanoparticles to pregnant rat, smaller particles (1.14 diameter) were observed in placenta much more than larger ones (1.8 nanometer) .

It has been reported that nanoparticles can induce harmful effects on mice reproduction and also growth and development of fetus in vivo and in vitro. Exposure to nanoparticles leads to the placement of nanoparticles in the fetus. Produced oxidative stress in fetus, is dominant teratogen factor caused by nanoparticles [21]

[22] reported negative effects of cadmium oxide nanoparticles inhalation on pregnant mice. So that, these nanoparticles reduced the weight of the placenta and the length of the fetus, and caused delay in the development of the fetus, as well as decline in growth during infancy.

[23] showed that titanium oxide nanoparticles caused ovarian injury, accumulation of these matters in ovaries, imbalance of sexual hormones, fertility reduction, and oxidative stress in mice. Ramezani et al. (2016) studied the effects of nanoparticles on ovarian histology and reported that nanoparticles reduced the number of follicles and corpus luteums by disturbance in cell oxidative pathways and also increased atretic follicles by producing oxygen free radicals and destroying microfilaments. This condition can have a negative effect on the fertility of female rats [24].

In a study about the effect of silver nanobioxide on ovarian tissue, large amounts and long-term use of silver nanobioxide had toxic effects on ovary and chronic exposure to silver nanoparticles may cause damage to the reproductive system in women. [25]. In recent years, many reports have showed that nanoparticles affect ovulation by activating caspase pathways and creating oxidative stress [25].

Exposure of mother to nanoparticles causes embryo disorder in two ways: nanoparticles are transferred to fetus by blood circulation and there cause ROS; or nanoparticles produce ROS in mother's body and affect fetus by producing inflammatory cytokines. Since ROS is not stable enough to be transferred via cells, ROS in mother, may cause fetal dysfunction indirectly [26].Increased ROS leads to the reaction of biological macromolecules, the disruption of intracellular homeostasis due to the onset of apoptosis, and ultimately leads to maternal and fetal toxicity [27].

In current study, T3 and T4 hormones were reduced and TSH was increased significantly. Also, histological studied showed reduction in total number of follicles and active follicles, epithelial height of follicles and nuclear-cytoplasmic ratio of follicular cells and also increased colloid volume.

On the contrast, [28] reported that chronic use of lithium reduced serum amount of TSH and increased T3 and T4 hormones which is probably because of different behavior of nanoparticles in proportion to original composition of that material. Results of another study ,[29]. showed that lithium decreased thyroid weight and TSH and increased thyroxin and tri-iodothyronine.

[30] reported that lithium reduced thyroid hormones especially T4 and long-term use of this drug caused hypothyroidism which is in agreement with our results.

It has been reported that people who use medicines containing lithium carbonate for long time suffer from disorders such as goiter, hypothyroidism, myxedema with decreased T3 and T4 hormones, as well as decreased iodine reuptake [31]. In a study, lithium caused hypothyroidism by intensified parathyroid hyperplasia and multi-glomerular disease[32].

Lithium is controlling factor of thyroid gland which can lead to appearance of clinical hypothyroidism and goiter, and also increase the level of antithyroid antibodies and inhibit iodine absorption in the thyroid, iodization of tyrosine and release of thyroid hormones. Furthermore, lithium increases serum level of TSH which causes hypothyroidism finally [33].

[34] reported that nanoparticles of iron oxide reduced serum concentration of TSH significantly and increased T3 hormone significantly but did not have significant effect on T4. In another study, various concentrations of iron oxide nanoparticles showed opposite effects on T4 hormone [35].

[36]. announced that TiO_2 nanoparticles caused considerable tissue changes in thyroid and reduced also T3 serum level.

[37],reported that Zinc nanooxide reduced thyroid activity, T3 and T4 hormones even in low doses.

Nanoparticles studies show interfering effects of these materials on cells as oxidative responses and in all studies, direct relationship between ROS production and oxidative responses with destruction of involved cells have been proven [37]. In current study also lithium carbonate reduced total follicles and active follicles dose dependently. Therefore, reduction in the number of active follicles explain decreased thyroid activity and serum level of T3 and T4 hormones. Increase in TSH hormone can be due to negative feedback of thyroid hormones. Also, as it is obvious, lithium increases serotonin [3]. Considering that serotonin increases TSH [39], it is possible that lithium carbonate nanoparticles, like lithium, increase TSH by increasing serotonin.

Previous studies have shown that lithium with different mechanisms leads to thyrotoxicosis in bipolar patients treated with lithium. Also, lithium treatment is the reason of hypothyroidism in these patients. Subclinical hypothyroidism, goiter and elevated thyroid antibody in patients treated with lithium shows a dramatic increase and controls secretion of thyroid hormones [40].

Results of current study and most of conducted studies about lithium effects on thyroid performance shows similar effects of lithium carbonate nanoparticles. Studies have shown that nanoparticles aim mitochondrion via unknown mechanisms and increase oxidative stress [41]. Increased oxidative stress reduces cellular anti-oxidants. Also, ROS (reactive oxygen species) which is produced during oxidative stress inside the cell can increase activity and phosphorylation of Mitogen Activated Protein Kinase (MAPK) and induction of extracellular signal regulated Kinase (ERK). Since activity of MAPK family leads to activation of transcription factors and the activity of these factors leads to a change in the expression of the genes involved

in inflammation, apoptosis, proliferation and differentiation [41], tissue changes of thyroid by lithium nanoparticles can be explained.

Conclusion

Results of this study showed destructive effects of these nanoparticles on performance of pituitary gonadal axis and pituitary-thyroid axis in female progenies, but since there are contraries about bioadaption of nanoparticles because of their special specifics such as size, shape, concentration of used nanoparticles, type of compound and so on, it is suggested that further studies be conducted about immunological and molecular reactions and cytotoxicity of this substance in in vivo models.

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