The Effects of Low-Dose Lithium Carbonate on the Spermatogenic Parameter in the adults Male Wistar Rats

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Abstract: Lithium and its salt have been frequently used in the treatment of neurodegenerative disorders. Many studies explain side effects of treatment with high-dose of lithium in testes but effect of low-dose on testes was ambiguous. This study examined the adverse effect of low-dose of lithium carbonate on testicular tissue and its effects on the three hormones of LH, FSH and testosterone. Wistar adult male rats with mean weight of 200-250 g were divided into four groups, each group with 6 rats.Wistar adult male rats were treated for 48 days using three doses of10, 20 and 30 mg/kg BW by gavage and control group treated with sterile distilled water (solvent of lithium carbonate). Twenty four (24) hours after the last gavage, blood sample was drawn from the heart and the two testicles were removed from rats' body. Once the tissue was fixed with Bouin's fixative solution and the section slides were stained with hematoxylin and eosin. Hormones were measured using a kit. The results showed that, compared to the control group, taking lithium carbonate in a 48-day period at all three doses resulted in a significant difference in the number of spermatogonia, primary spermatocytes, spermatid and spermatozoa cells and in a specific dose-dependent decrease. Lithium carbonate could reduce the concentration of LH, FSH and testosterone hormones in a dose-dependent manner. Based on the results of this study, it can be concluded that through considerably decreasing testosterone production and preventing normal development of spermatogenic cells, lithium carbonate causes spermatogenic dysfunction through reducing the level of LH and FSH hormones that adjust this process.

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1. Introduction

The use of lithium and its salts to treat mania – depression was first introduced in Australia in 1949. Lithium chloride was registered as a drug for treatment of mania – depression in 1960s.Lithium chloride was commonly used at 900-1200 mg/day doses in Europe and the United States up to 1970. In the European Union classification, lithium carbonate is classified in category 3. According European Union classification, lithium carbonate is identified as R62 that can affect fertility. Long-term use of lithium at therapeutic doses may cause complications such as neurotoxicity (Chen et al., 2004) and hypothyroidism (Henry 2002). The use of lithium and its salts in the high-dose (therapeutic dose) may cause complications such as infertility, nephrogenic diabetes ,nephrotoxicity and damage to urethra cells (Markowitz et al., 2000; Li et al.,2006;Nciri et al., 2008) but few studies available about side effects of low-dose of lithium carbonate on the testes.

Normal function of the reproductive system depends on the harmonious regulatory activities of hypothalamus and anterior pituitary gonadotropin cells and subsequent secretion of steroids and essential molecules in the testicles known as hypothalamic–pituitary–gonadal axis (Cevik et al., 2004). Many studies have proved the reciprocal relationship between pituitary and testicular function and believe that changes in testosterone level and testicular histology during the 48-day of spermatogenesis in rats is associated with the plasma level of LH and FSH (Lee et al., 1975 ; Gholami et al., 2012). Secretion of gonadotropin-releasing hormone (GnRH) from hypothalamus causes an increase in transcription of genes encoding α and β subunits related to LH and FSH hormones and an increase in their secretion from the pituitary (Freeman et al., 2000). The presence of the two LH and FSH gonadotropins in combination is essential for activation of spermatogenesis process as maturation of germinal cells in seminiferous tubules in the presence of FSH and production of testosterone in Leydig cells occur under the influence of LH hormone (Rago, 2008).

Concurrent activity LH and FSH induces the secretion of testosterone and necessary trophic factors on Lydia and Sertoli cells. Therefore, maintaining the normal concentration of the LH and

FSH for initiation of the complete spermatogenesis is necessary (Shalet, 2009). The long term use of high-dose lithium and its salts decrease the level of LH, FSH, testosterone and prolactin hormones. lithium and its salts in high-dose also prevent the production of testosterone through reducing the steroid-producing activity of testicles by decreasing the production of key steroidogenic enzymes delta 5-3 β hydroxysteroid dehydrogenase and 17-betahydroxysteroid dehydroxygenase(Lee et al., 1999; Shupnik and Weck 1998; Sharpe et al.,2003; Stanton et al., 1992). Another study showed a decrease in the level of LH, FSH and testosterone of Wistar rats treated with lithium chloride that returned to normal under a prolactin-compensating treatment.

Disorders in proliferation and differentiation of male germinal cells and changes in spermatogenesis regulatory mechanisms may occur in any stage of the process. (Lithium in the high-dose can reduce the plasma level of testosterone and gonadotropin hormones through both direct and indirect mechanisms. The direct mechanism involves developing germinal cells. The indirect mechanism involves decreasing activities of hypothalamic–pituitary–gonadal axis, and it can also disturb the germinal cell development by influencing spermatogenesis-supporting somatic cells in the testicles (Zarrindast et al., 2006). Lithium carbonate is used in different doses depending on the intensity of the mania-depression disease (Rybakowski, 2012). Low-dose of lithium carbonate used for treatment of neurodegenerative disease (Marmol, 2008). Low-doses of lithium carbonate have been studied in the kidney and heart (Evan, 1972; Nciri, 2008; Vijaimohan, 2010). Side effects of low-dose of lithium carbonate on the testes are known. In this study, the effect of three doses of 10, 20 and 30 mg/kgBw/day of lithium carbonateat histological and cellular modifications of testes and also on the level of LH, FSH and testosterone hormones was examined.

2. Material and Methods

All experiments were performed in accordance with principles of laboratory animal care. In this study, Wistar adult male rats with mean weight of 200-250 g and 7-8 weeks old (provided by Pasteur Institute of Tehran, Iran) were treated for 48 days. The rats were kept and treated at 23-25°C and 60-75% relative humidity for 48 days under 12h/12h dark/light cycles. All the rats received the same potable water and the standard rat feed pellet (supplied by Pars Animal Feed).Every effort was made to minimize the number of animals used and their suffering.

The study groups and preparation of lithium carbonate solution: The rats were randomly divided into 4 groups, each with 6 rats. Group 1 was determined as the control groups and groups 2, 3 and 4, as the experimental groups, received lithium carbonate at doses of 10, 20 and 30 mg/kgBw/day, respectively. In each gavage, 0.5 ml of lithium carbonate solution which was prepared by dissolving the specific amount of white powder of lithium carbonate (Tehran Darou Co. with serial no. 8808) for each group with sterile distilled water was given to the rats.

Blood sampling: rats were anesthetized intraperitoneally by ketamine HCl (80 mg/kg) and xylazine (10 mg/kg) (Pharmacia and Upiohn, Erlangen, Germany) (Gholami et al., 2012) 24 hours after termination of treatment in accordance with the protocols approved by the Lorestan University of Medical Science Animal Care and use committee. Blood sample was drawn from the heart and the testicular tissue was removed immediately.

Hormonal measurement: Blood sample of 4-5 ml was drawn from each rat and collected in glass tubes specific for centrifuges. The blood samples were maintained in the laboratory setting for 1 hour to form clots and then centrifuged for 2 min at 500 rpm. In this way, serum was separated from the blood and surfaced the clot. The serum was removed using a 1000 sampler and transmitted to smaller tubes. These tubes were kept in a freezer at -20°C in order to be used in ELISA kits for measurement of LH, FSH and testosterone hormones (Ghosh et al., 1991a; Ghosh et al., 1990b). Finally, optical absorption of the prepared solutions was recorded according to the kit guidelines at wavelength of 450 nm and concentration of each hormone was recorded in ng/ml.

Preparation of tissue sections: The removed testicular tissue was placed in Bouin's fixative solution for 24 hours after being weighed. Then, paraffin blocks and serial sections of the tissue were prepared and the slides were stained with hematoxylin and eosin.

Histological examination**:** The testicular tissue sections were observed and examined using an ordinary optical microscope (Hm-Lux3, Germany) with 10X, 40X and 100X magnification and the spermatogonia, primary spermatocytes, spermatozoa, Sertoli and Leydig cells were counted in each microscopic field in 10 seminiferous tubules which had a round shape and an appropriate cross-section. All sections selected for cell counting and histological analysis are located in the similar Stage. Morphological changes of the seminiferous tubules and germinal epithelium of the groups were compared with each other. In this study, besides counting cells and comparing mean germinal cell and testicular somatic cell count, the score count method

or Johnson's model was used for the level of spermatogenesis which is the common method for histopathological measurements (Johnson et al., 1980).

Statistical analysis of the results: The results obtained from the control and the experimental groups were analyzed in the form of mean ± SD (standard deviation) values and significant differences among groups were determined using one-way ANOVA and Duncan post hoc test through SPSS software.

3. Results

The relative weight of the testicular tissue had a direct relationship with spermatogenetic function and germinal cell count and is calculated using the following equation: Relative weight of testicular tissue = (mean weight of testes)/body weight × 100

This index was reduced in groups receiving lithium carbonate in that it reached 0.24936 ± 0.021927 g in the group receiving 30 mg/kgBw/day of lithium carbonate while it reached 0.54428 ± 0.02791 in the control group. This difference is Significant ($p<0.001$). This index reached 0.32121 ± 0.13154 in the group receiving 20 mg/kgBw/day of lithium carbonate, which is significantly different from that of the control group ($p<0.01$). Likewise, this index reached 0.42535± 0.04978 in the group receiving 10 mg/kgBw/day lithium carbonate, which has a Significant difference with that of the control group ($p<0.05$) (Figure 1).

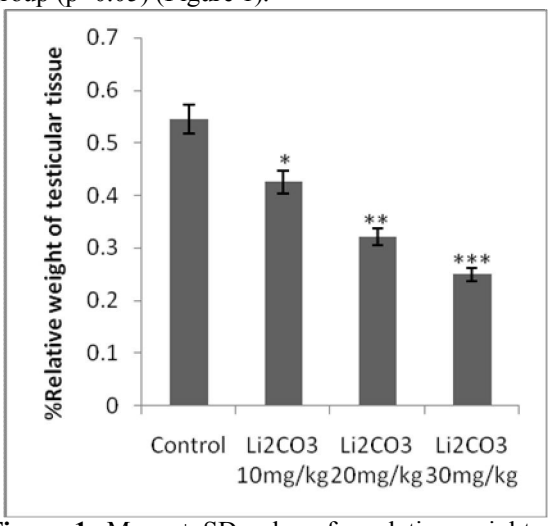


Figure 1: Mean ± SD values for relative weight of the testicular tissue (%). Compared to the control group, the testicular tissue weight in the experimental groups decreased significantly that indicates the destructive effect of lithium on testicular cell density. ***p<0.001, **p<0.01, *p<0.05

Analysis of microscopic images: Johnson's score count: In the method used in this study for histological examination, seminiferous tubules with complete spermatogenesis which were full of mature spermatids and sperms with regular accumulation received 10 score based on the score count for the spermatogenetic level.

According to the score count, density of germinal and somatic cells in seminiferous tubules was based for the scoring and is shown in Table 1. On the basis of the results, the control group scored 10, and the groups receiving 10, 20, and 30 mg/kgBw/day of lithium carbonate scored 8, 4.9 and 3.7, respectively.

Table 1: Johnson's scoring system for the level of spermatogenesis

Groups	Score
Control	**10**
10 mg/kgBW Lithium carbonate	**8**
20 mg/kgBW Lithium carbonate	**4.9**
30 mg/kgBW Lithium carbonate	**3.7**

Cell count and comparisons among control and experimental groups: Through considerable histological changes in testicles, lithium caused a decrease in the number of germ and somatic cells in seminiferous epithelium, an increase in interstitial space, and an increase in the number of cells being destroyed. The decreased number of cell count is shown in the table 2.

The number of spermatogonia near the basal membrane was 86 ±4.42719 ($p<0.01$), 75.16 ±4.44597 ($p<0.05$) and 69.33±6.40833 ($p<0.001$) in groups receiving 10, 20 and 30 mg/kgBw/day of lithium carbonate, respectively, which is significantly different from that of the control group with the count of 119.7±11.9443. Primary spermatocytes in seminiferous epithelium were 295.8±22.89469 ($p<0.01$), 265±15.49193 ($p<0.05$) and 168.5±3.67423 ($p<0.001$) in groups receiving 10, 20 and 30 mg/kgBw/day of lithium carbonate, respectively while that was 357±36.33180 in the control group. Furthermore, the number of spermatids was 108.8±7.5476 ($p<0.01$), 98.5±5.75326 ($p<0.001$) and 74.7±6.9761 ($p<0.001$) in groups receiving 10, 20 and 30 mg/kgBw/day of lithium carbonate, respectively while that was significantly different (1173±12.3288) in the compared control group (Figure 2).

The number of spermatozoids in groups receiving 10, 20 and 30 mg/kgBw/day of lithium carbonate were 1087.16±294.307 ($p<0.01$), 1030.33±70.5256 ($p<0.05$) and 772±61231 ($p<0.001$), respectively, which was significantly different in the compared control group (Figure 3).

Table 2: Mean cell count of the tissue sections: germinal and Sertoli cells per seminiferous tubule and Leydig cells per circumference of seminiferous tubule are explained in the control and experimental groups in the form of mean ± SD. Groups defined with; A: control, B: 10 mg/kgBW Lithium carbonate, C: 20 mg/kgBW Lithium carbonate, D: 30 mg/kgBW Lithium carbonate.

Groups	Spermatogonia (Count± SD)	Primary spermatocyte (Count± SD)	spermatid (Count± SD)	Spermatozoa (Count± SD)	Sertoli (Count± SD)	Leydig (Count±SD)
A	119.6667±11.9443	357.000±36.33180	173.0000±12.3288	2550.5000±123.0670	11.5000±1.04881	8.66±2.42212
B	86.0000±4.42719	295.8333±22.89469	108.8333±7.5476	1087.1667±294.307	10.5000±0.8366	6.33±1.63299
C	75.1667±4.44597	265.0000±15.49193	98.5000±5.75326	1030.3300±70.5256	10.000±1.0236	6.05±0.30767
D	69.3333±6.40833	168.5000±3.67423	74.667±6.9761	772.+-61231	9.3±1.0366	5.47±1.75119

Table 3: Mean values for the level of LH, FSH and testosterone hormones in the control and the experimental groups (Mean ± SD). Groups defined with; A: control, B: 10 mg/kgBW Lithium carbonate, C: 20 mg/kgBW Lithium carbonate, D: 30 mg/kgBW Lithium carbonate.

Groups	Testosterone (ng/ml) ± SD	LH (ng/ml)± SD	FSH (ng/ml)± SD
A	6.200±0.16106	1.4886±0.07137	9.2461±0.16106
B	3.1744±0.33806	0.9089±0.07851	8.0094±0.33806
C	1.6704±0.0618	0.71640±0.07806	5.0633±0.06188
D	1.1839±0.16553	0.5844±0.03184	4.3556±0.01655

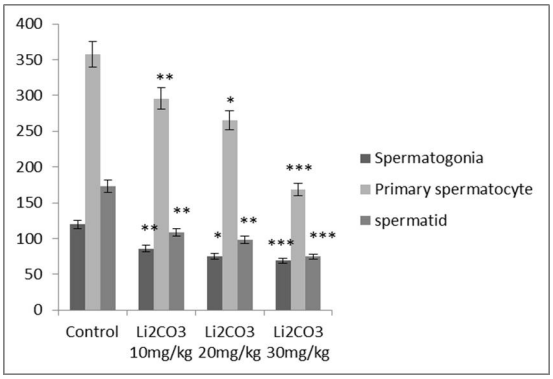


Figure 2: The results of changes in number of Spermatogonia and Primary spermatocyte and Spermatid cells in each seminiferous tubule treated with 10 mg/kg BW, 20 mg/kg BW and 30mg/kg BW doses. Data, mean ± SD, obtained from three various tests.***p<0.001, **p<0.01, *p<0.05.

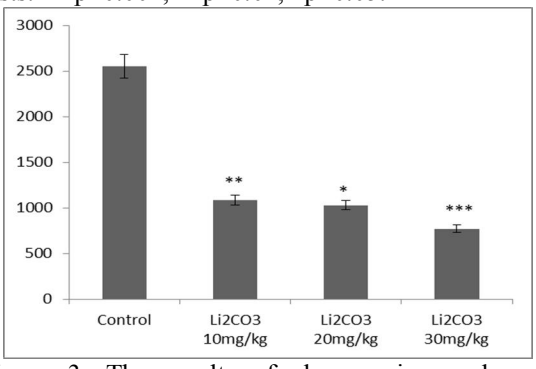
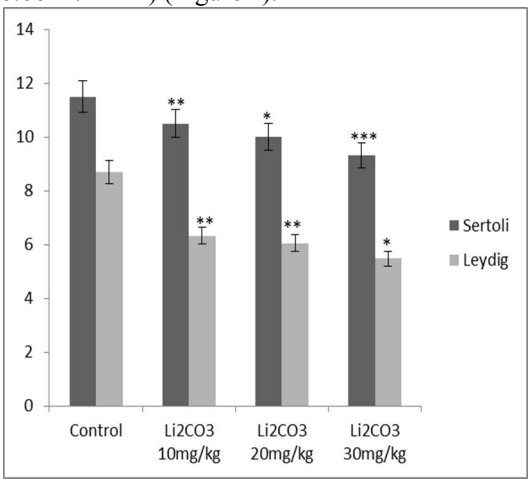


Figure 3: The results of changes in number of Spermatozoa cells in each seminiferous tubule under the treatment with 10 mg/kg BW, 20 mg/kg BW and 30mg/kg BW doses. Data, mean ± SD, obtained from three various tests. ***p<0.001, **p<0.01, *p<0.05

Comparison between groups (10, 20 and 30 mg/kg BW of lithium carbonate) and control showed that the number of Leydig and Sertoli cells were significant. The number of Sertoli cells was 10.5±0.8366 (p<0.01), 10±1.0236 (p<0.05) and 9.3±1.0366 (p<0.001) in groups receiving 10, 20 and 30 mg/kg BW of lithium carbonate, respectively while that was 11.5±1.04881 in the control group. The number of Leydig cells was reduced to 6.33±1.63299 (p<0.01), 6.05±0.30767 (p<0.01) and 5.47±1.75119 (p<0.05) in groups receiving 10, 20 and 30 mg/kg BW of lithium carbonate, respectively as compared with that in the control group (8.66±2.42212) (Figure 4).



Figur4: The results of changes in number of Sertoli and Leydig cells per seminiferous tubule treated with 10 mg/kg BW, 20 mg/kg BW and 30mg/kg BW doses. Data, mean ± SD obtained from three different tests. ***p<0.001, **p<0.01, *p<0.05

Histological changes of lithium carbonate on the number of cells can be seen in Figure 5.In the section B, treated with 10, spermatid and sperm cells are visible but are not seen in section C and D, treated with 20 and 30 mg/kg, respectively. Basement membrane arranged regularly and spermatogonia stem cells, in all groups, be seen on the basement membrane regularly.

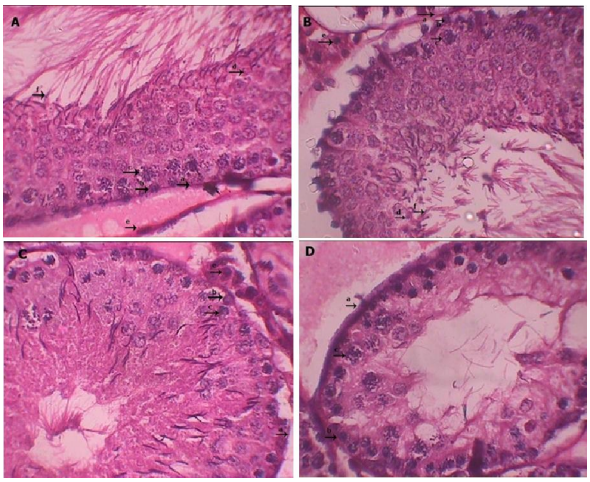


Figure 5: hematoxylin and eosin staining of testes from rats treated with lithium carbonate at dose 10(B), 20(C) and 30(D) mg/kgBW revealed loss of spermatid and cessation of spermatogenesis. Section A related to Control group. a: basement membrane, b: spermatogonia cell, c: primary spermatocyte cell, d: spermatid, e: Leydig cell, f: spermatozoa.

Results of the hormonal measurement: The one-way ANOVA analysis of the hormonal measurement results showed that, compared to the control group, mean ± SD concentration of LH, FSH and testosterone hormones in groups receiving lithium carbonate reduced significantly (Table3).

The lowest level of LH belongs to the group receiving 30 mg/kg BW lithium carbonate (0.584±0.03184 ng/mg, $p<0.001$).Mean ± SD values of LH hormone (ng/ml) in the groups receiving 20 and 10 mg/kg BW of lithium carbonate and the control groups were 0.716±0.07806 ($p<0.05$), 0.908±0.07851 ($p<0.01$) and 1.49±0.07137 ng/ml, respectively (Figure 6).

With regards to FSH, it had such regular decrease in the same order as for LH. So, the lowest rate was recorded for the group receiving 30 mg/kg BW of lithium carbonate as 4.355±0.01655ng/ml ($p<0.001$).Mean ± SD values of FSH hormone (ng/ml) in the groups receiving 20 and 10 mg/kg BW of lithium carbonate were 5.063±0.06188 ($p<0.05$), 8.009±0.33806 ($p<0.01$). This reduction was significant as compared with the control group with 9.246±0.16106 ng/ml of FSH (Figure 7).

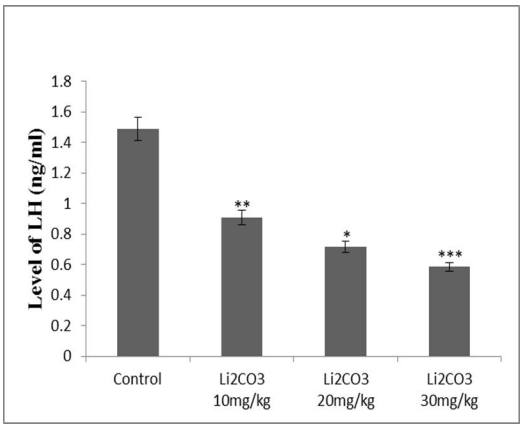


Figure 6: Compared to the control group, Mean ± SD values of LH hormone (ng/ml) in the experimental groups had a significant dose-dependent decrease that showed the reducing effect of lithium on hypothalamus- pituitary –gonad axis.***$p<0.001$, **$p<0.01$, *$p<0.05$

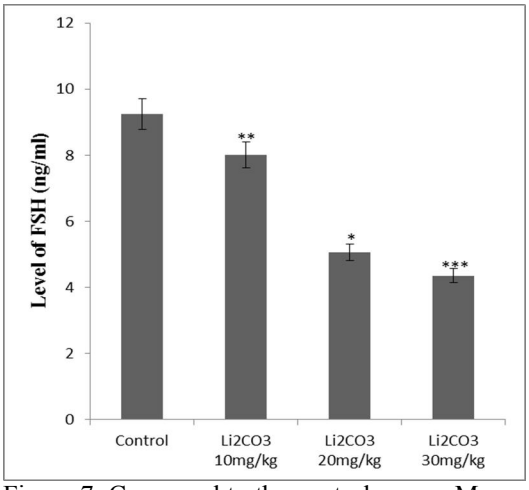


Figure 7: Compared to the control group, Mean ± SD values of FSH hormone (ng/ml) in the experimental groups had a significant dose-dependent decrease that showed the reducing effect of lithium on hypothalamic neurons and pituitary and its indirect effect on gonad.***$p<0.001$, **$p<0.01$, *$p<0.05$

Testosterone level was reduced significantly in the experimental groups as compared with that in the control group. It was 1.183±0.16553 ($p<0.001$), 1.67±0.0618 ($p<0.05$) and 3.17±0.33806 ($p<0.01$) ng/ml in the groups receiving 30, 20, and 10 mg/kg BW of lithium carbonate, and 6.2±0.16106 ng/ml in the control group (Figure 8).

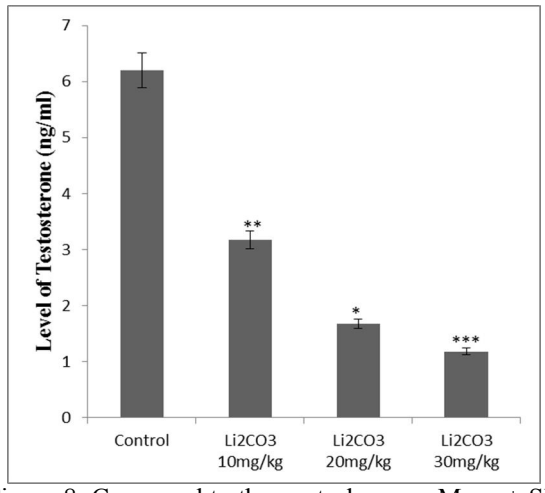


Figure 8: Compared to the control group, Mean ± SD values of testosterone hormone (ng/ml) in the experimental groups had a significant dose-dependent decrease that showed the reducing effect of lithium on hypothalamus- pituitary –gonad axis.***p<0.001, **p<0.01, *p<0.05

Discussions

Long-term treatment with high-dose lithium is associated with the incidence of toxic side effects in many tissues including the reproductive system. A study on lithium carbonate toxicity in rat reproductive system, doses at 500, 800 and 1100 mg/kg of diet were used for 90 days and the obtained results showed a considerable reduction of testicular weight, changes of cell destruction in seminiferous tubules, absence of spermatozoa cells in testicles, epididymis and Vas deferens and lack of secretions in the lumen of the seminal vesicles and prostate. Therefore, hypospermatogenesis and formation of abnormal spermatozoa along with reduced level of testosterone are among the side effects of using high doses of lithium (Zarrindast et al.,2006). Another study examined the weight loss of testicular tissue and other sexual organs, which is largely a representative of dysfunction and extensive changes in the number of developing germinal cells in seminiferous tubules and epididymis (Romera et al., 2002).

A 21-day treatment with lithium carbonate at 35 mg/kgBW dose in an electromicroscopic study showed testicular atrophy, loss of spermatogenic cell adhesion and formation of multiple intercellular spaces in germinal epithelium and also showed that cells in the early development of germinal lineage became inflamed and disintegrated with outward expanding of the nuclear outer membrane. There were round spermatid cells with transformed and abnormal acrosomes and reduced sub-acrosomal space in the above study, and also a large number of destroyed mature spermatids scattered in the sections (Zarnescu and Zamfirescu2006).

The mechanism through lithium controls mania symptoms is mostly attributed to inhibition of Gsk3-β enzyme in WNT signaling pathway. Once the WNT signaling pathway is activated in neurons and the enzyme is inhibited, breakdown of β-catenine molecules will be prevented. The accumulation of β-catenine in cells results in its entrance into the nucleus and involvement in the mechanisms regulating expression of genes (Gould et al., 2007). For instance, neuroprotective effects of lithium are due to the increased expression of bcl-2 (cytoprotective protein) and cause an increase in neural viability and function (N-acetylaspar) in grey matter (Xu et al., 2003). In addition to this mechanism, proximity of lithium ionic radius to those of sodium and potassium and their competition for passing through the plasma membrane cause changes in neuronal membrane potential and disorders in cellular signaling mechanisms such as protein kinaseC, CAMP and CGMP, and prevention of these mediator molecules (Thakur et al.,2003). This ionic competition also prevents the activity of the enzymes such as DNA polymerase and topoisomerase I and II which are activated by cationic cofactors (Ncri et al., 2009). As the synthesis and secretion of LH, FSH and GnRH hormones are possible through this mechanism, lithium can considerably reduce the concentration of these hormones. Lithium at high concentrations and plasma level higher than 2.5 mM causes life-threatening side effects and at plasma level of 6-9.6 mM causes acute toxicity (Suwalky et al., 2007). These concentrations lead to excessive expression of WNT signaling pathway in cells including male and female germinal cells and disturb their development and vital functions. In ovarian follicular cells, lithium inhibits estrogen functioning by deactivating estrogen receptors in their membrane (Medunjanin et al., 2005). In another way, lithium causes an excessive expression of WNT/CTNNB1 signaling pathway in Sertoli and Leydig cells. The accumulated stable form of the product of this pathway modifies the gene expression, stops the cell cycle in G2/M, and induces apoptosis genetically or by inhibiting the destruction. A study showed that igniting this pathway in Sertoli and Leydig cells culture prevents the expression of steroidogenic acute regulatory protein (STAR) and reduces capability of these two types of cells for supporting spermatogenesis and thorough functioning through induction of apoptosis (Boyer et al.,2008). Lithium is influential in a way other than direct targeting testicular tissue cells and modification of intracellular signaling pathways. Lithium affects the testicular somatic cells through reducing the activity of

hypothalamic–pituitary–gonadal axis. This axis stimulates secretion of LH and FSH through synthesizing GNRH hormone which is transmitted from the hypophyseal portal system to the anterior pituitary. These two glycoproteins activate the cyclic AMP (adenosine monophosphate) secondary messenger system in testicular somatic cells and stimulate all their functions including secretion of testosterone. Reduced level of these two glycoproteins in blood results in the reduction of secretory activities of spermatogenesis-supporting cells (Sertoli) and testosterone-producing cells (Leydig) (Rago 2008; Ghosh et al., 1991).

Based on the results of the present study, it can be concluded that through significant decrease in testosterone production and preventing normal development of spermatogenic cells, lithium carbonate causes spermatogenic dysfunction through reducing the level of LH and FSH hormones that adjust this process. Hypospermatogenesis and production of abnormal spermatozoa in treatment with lithium are attributed to the significant reduction of plasma level of testosterone and capability of this ion to pass through blood-testis barrier. Lithium reduces the production of trophic factors and testosterone which are directly involved in protection, differentiation and survival of germinal cells, through either disturbing the function of Sertoli and Leydig cells or induction of apoptosis in these cells. Furthermore, lithium disturbs the natural development and differentiation of spermatogenetic cells through affecting the germinal lineage cells directly. Low-dose of lithium carbonate similar to high-dose may causes side effects at testes such as reduce level of LH, FSH and testosterone, cessation of spermatogenesis and abnormal histological changes in the testes.

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