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## ORIGINAL ARTICLE

# Disrupting effects of lithium chloride in the rat ovary: Involves impaired formation and function of corpus luteum

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### KEYWORDS

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**Abstract** Lithium is an effective drug for the treatment of bipolar disorder. Evidence suggests that lithium induces side effects on the reproductive system. We have investigated the effect of lithium chloride (LiCl) on the progesterone synthesis, the main steroid produced by corpus luteum (CL), and steroidogenic acute regulatory protein (StAR) expression, the primary mechanism of the control of CL steroidogenesis. Immature female *Wistar* rats (25-day-old) were injected with lithium chloride (2.0 mg/kg/day) or sterile distilled water for 15 days. All rats were induced with injection of pregnant mare's serum gonadotrophin (PMSG) on the 13th day of experiment and followed by human chorionic gonadotropin (hCG) 48 h later. The last injection of LiCl was at 12 h post-hCG injection. Blood and ovaries were collected at 4 h interval from 12 to 24 h post-hCG injection. Serum levels of progesterone were measured by ELISA and CL formation was determined by histological analysis. Then, StAR protein and gene expression were examined using immunohistochemistry and polymerase chain reaction. Results showed the severe changes in CL formation, progesterone secretion and StAR expression in LiCl-treated rats during luteinization. It is concluded that the CL formation and the critical step of progesterone synthesis were affected by LiCl in gonadotropin-induced rat ovary.

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## 1. Introduction

After LH surge and ovulation, luteinization of the follicle cells occurs and the corpus luteum (CL) rapidly develops to become

a highly active steroidogenic tissue (1). The reprogramming of follicular cells into luteal cells requires severe changes in the expression of steroidogenic enzymes and the type of steroid produced. In rodents, CL is a substantial site of progesterone biosynthesis (2). The movement of cholesterol from the outer to the inner mitochondrial membrane is a rate limiting step in progesterone synthesis in the ovary. This translocation is mediated by steroidogenic acute regulatory protein (StAR), a phosphoprotein expressed in steroidogenic cells (3). The expression of both mRNA and protein of StAR was reported in rat (4) and human CL throughout the luteal phase (5). StAR expression coincides with the capacity of steroidogenesis and

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its expression can be used as a functional marker of CL development in rat (6).

Lithium chlorides (LiCl) is an effective drug for the treatment of bipolar disorders, a central nervous system (CNS) disease (7), however evidence suggests that metabolism, neuronal communication, cell proliferation and cell fate determination have been affected by lithium in various organisms (8). Also, lithium induced side effects on the adult male rat reproductive system (9). Ghosh et al. (1990) (10) and Sheard et al. (1997) (11) reported that lithium treatment results in marked diminution in the plasma levels of gonadotropins, prolactin and testosterone in rats and humans. In addition, lithium administration significantly decreased ovarian steroidogenic enzymes and folliculogenesis in the adult female rats (12,13). Recently we showed that the serum level of progesterone and transcript levels of key steroidogenic enzymes were altered in the gonadotropin-stimulated rats following single injection of LiCl (14). In this study, we have examined whether reproductive toxicity of lithium is associated with alterations in progesterone synthesis and the expression of StAR in the gonadotropin-induced rat following two weeks of LiCl treatment.

## 2. Materials and methods

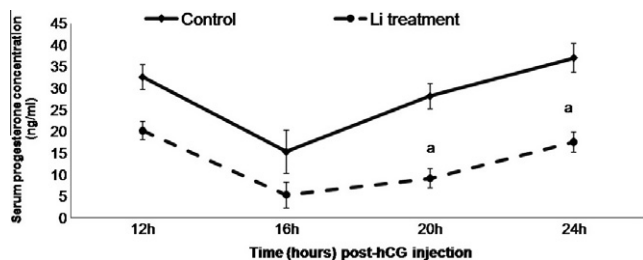
### 2.1. Animals

Immature (25-day-old) female albino rats of the *Wistar* strain were used. All rats were housed in Plexiglas cages and kept under controlled temperature ( $22 \pm 2^\circ\text{C}$ ) and 12/12-h light–dark cycle. Animals were allowed free access to rat chow and water. The procedures were performed in accordance with institutional guidelines for animal care and use. The Animal Ethics Committee of the Department of Studies in Zoology, University of Mysore, Manasagangotri approved the experimental protocol.

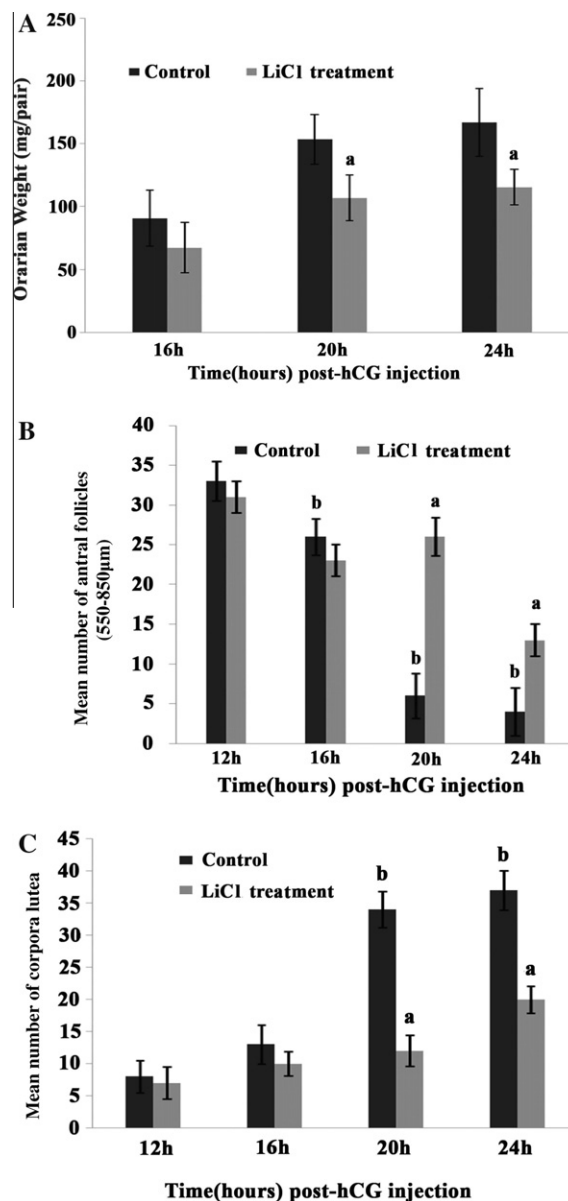
### 2.2. Experimental design

Immature female *Wistar* rats (25-day-old) were injected intraperitoneally (i.p.) with 2.0 mg/kg/day of lithium chloride (Sigma, Germany) or sterile distilled water (0.5 ml) for 15 days.

The dose of LiCl was selected on the basis of the human therapeutic dose. Then, all rats were treated with single i.p. injection of 10 IU pregnant mare's serum gonadotrophin



**Figure 1** Serum concentrations of progesterone were determined in gonadotropin-induced rats after treatment with LiCl or distilled water (control) by ELISA. The results represent the means  $\pm$  SEM of groups of eight rats. (a): Significantly different from respective control group,  $P < 0.05$ .



**Figure 2** Effect of LiCl on the ovarian weight (A), number of antral follicles (550–850  $\mu\text{m}$ ) (B) and corpora lutea (C) in ovaries of gonadotropin-induced rats after treatment with either LiCl or distilled water (control). The results represent the means  $\pm$  SEM of groups of eight rats. (a): Significantly different from respective control group, and (b): significantly different from control group at 12 h,  $P < 0.05$ .

(PMSG) on the 13th day of experiment to induce follicular maturation and followed by single i.p. injection of 10 IU human chorionic gonadotrophin (hCG) (Intervet Inc., Germany) 48 h later to induce ovulation. The last injection of lithium chloride (LiCl) or distilled water was at 12 h post-hCG injection. Rats injected only with distilled water and gonadotropins served as control group. In this model, ovulation approximately occurred at 12–14 h post-hCG injection (the oocytes were observed by applying gentle pressure to both ends of the ampulla, and placed on a slide under the stereomicroscope). All animals were killed by spinal dislocation at 4 h

interval from 12 to 24 h post-hCG injection. Blood samples were collected by cardiac puncture. Serum was separated by centrifugation and stored at  $-20^{\circ}\text{C}$  until used for subsequent determination of hormone levels. The ovaries were rapidly removed, washed in the cold saline solution and weighed. One ovary from each rat was fixed in Bouin's solution for histological studies, and the other was snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for RNA extraction.

### 2.3. Hormone assay

Serum levels of progesterone were determined by ELISA using a commercial kit (Progesterone/Progesteron, IBL, Germany) according to the manufacturer's protocols.

### 2.4. Histology

The paraffin sections ( $7\ \mu\text{m}$ ) of the entire ovaries fixed in Bouin's solution were prepared and stained with hematoxylin and eosin. Then, a number of antral follicles (showing the nucleolus of the oocyte and  $550\text{--}850\ \mu\text{m}$  in diameter) and CLs were counted under the light microscope to evaluate CL formation.

### 2.5. Immunohistochemistry

In this study, the pattern of StAR expression was examined using immunohistochemistry (IHC). The tissue sections of ovaries collected at 20 h post-hCG injection were prepared and immunolabeled using the labeled streptavidin biotin (LSAB) methods. Briefly, paraffin sections ( $5\ \mu\text{m}$ ) were dewaxed in graded alcohols and xylene, thereafter the endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. For antigen retrieval, the slides were first incubated in sodium citrate buffer for 10 min in a microwave oven (600 W), followed by 5% non fat-milk in phosphate-buffered saline (PBS) containing 3% Triton-X100 for one hour (at room temperature) and washed ( $3 \times 5\ \text{min}$ ) with PBS. Then the

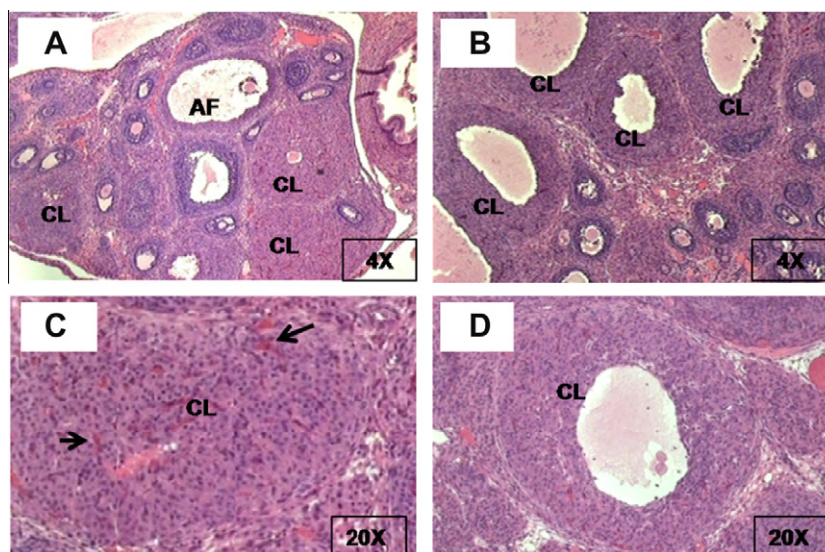
slides were incubated with anti-StAR antibody (Abcam, Germany) diluted 1:100 and kept overnight at  $4^{\circ}\text{C}$ . Then, after washing in PBS ( $3 \times 5\ \text{min}$ ), the slides were incubated with biotinylated polyclonal antibodies (anti-IgG Biotinylated) (LSAB, DAKO) for 30 min, washed again ( $2 \times 10\ \text{min}$ ) in PBS, and subsequently incubated with a streptavidin/peroxidase-conjugated antibody (HRP-Avidin) for 30 min. The sections were then developed with 3,3-diaminobenzidine (DAB), giving a brown product.

### 2.6. RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen, Germany) and the reverse transcription (RT) reaction was performed with QuantiTect Reverse transcription kit (Qiagen, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out with 2 ml of cDNA preparation, using specific primers for StAR as follows: sense  $5'\text{-CATCCAGCAAGGAGAGGAAG-}3'$  and antisense  $5'\text{-CGTGAGTTTGGTCTTTGAGG-}3'$ . The conditions used for PCR were as follows:  $94^{\circ}\text{C}$  for 2 min, 23-cycles ( $94^{\circ}\text{C}$  for 30 s,  $64^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s), and then  $72^{\circ}\text{C}$  for 10 min. Also the amount of quantified mRNA was normalized by Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal control using the following primers: sense  $5'\text{-CAAGGTCATCCATGACAACCTTTG-}3'$ , antisense  $5'\text{-GTCCACCACCTGTGCTGTAG-}3'$ . The PCR condition used for GAPDH was  $94^{\circ}\text{C}$  for 3 min, and 25cycles of  $94^{\circ}\text{C}$  for 30 s,  $58^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 45 s. The cDNA amplified was electrophoresed in 1% agarose gel and stained with ethidium bromide. The band image was quantified using Image J software (version 1.43).

### 2.7. Statistical analysis

Experiments were repeated two times with eight animals in each treatment and time point. One-way ANOVA was used to ana-



**Figure 3** Histological analysis of gonadotropin-induced rat ovaries after treatment with either distilled water (A and C) or LiCl (B and D). The ovaries were collected at 24 h post-hCG injection and tissue sections were stained with hematoxylin and eosin. Figure shows corpus luteum (CL) and antral follicle (AF). Magnification is shown within the selected squares. Arrows indicate blood vessels.

lyze differences between groups, with  $P < 0.05$  considered statistically significant. Tests were performed using SPSS software (Software program, version 13, SPSS, Inc., Chicago, USA).

### 3. Results

#### 3.1. Progesterone serum levels

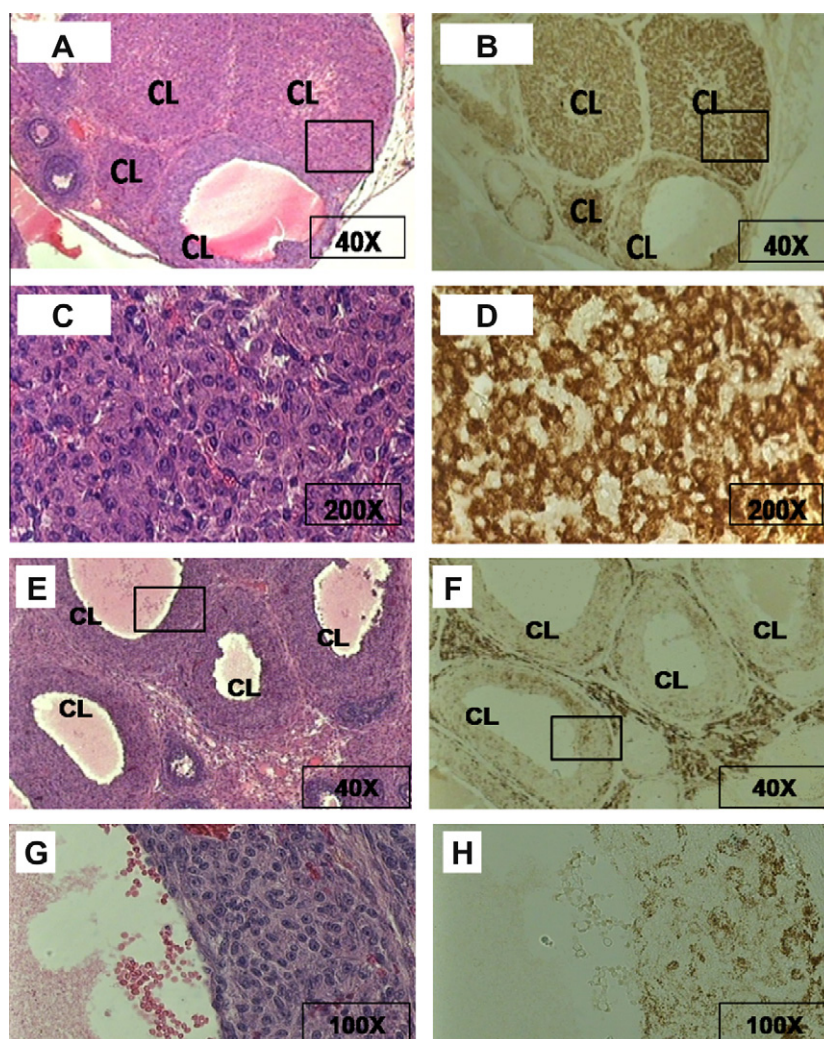
The serum levels of progesterone were evaluated in gonadotropin-induced rats that received either LiCl or distilled water (control). Following hCG injection, the increase in plasma progesterone concentration was observed at 12 h, and this pre-ovulatory peak was followed by a decrease at 16 h and then increased over the time (at 20 and 24 h) in control (Fig. 1). However, significant decreases were observed in serum progesterone concentration following LiCl treatment. Progesterone values were 32.6% and 47.4% of the control group at 20 and 24 h, respectively (Fig. 1).

#### 3.2. Ovarian weight

The average of ovarian weight in all groups is reported in Fig. 2A. Results showed that the ovarian weight in LiCl-treated animals was significantly lower than control at 20 and 24 h post-hCG injection. The values of weights were 69.9% and 68.9% of the control group at 20 and 24 h, respectively.

#### 3.3. Corpus luteum formation

Histological analysis of the ovaries indicated the follicular development and CL formation. Results showed significant increase in the numbers of antral follicles with a 4.3- and 3.2-fold at 20 and 24 h in LiCl-treated group in comparison with control (Fig. 2B). In contrast to the follicles, significant decreases ( $P < 0.05$ ) in the number of corpora lutea was observed, with a 2.8- and 1.8-fold, at 20 and 24 h respectively, in LiCl-treated group in comparison with control (Fig. 2C).



**Figure 4** Immunolocalization of StAR protein in the ovary of gonadotropin-induced rat after treatment with either distilled water (A,B,C,D) or LiCl (E,F,G,H). Tissue sections of ovary collected at 20 h were stained with either hematoxylin and eosin (A,C,E,G) or immunostained with StAR antibody (B,D,F,H). Figure shows corpus luteum (CL). Images denoted by the boxes are shown at higher magnification in the lower panels. Magnification is shown within the selected squares.

In addition, photomicrographs of the ovaries taken at 24 h post-hCG injection showed normal morphology of the CL in control group (Fig. 3A,C), while the CL impaired morphology with a space within the CL was observed in the ovaries of LiCl-treated rats (Fig. 3B,D).

### 3.4. StAR protein expression

The StAR protein expression was detected in the paraffin sections of ovaries by IHC. Results showed intense and moderate expression of StAR protein (brown color) within the luteal cells in the ovary collected at 20 h post-hCG injection in the control group (Fig. 4A,B,C,D). Also, as shown in Fig. 4E,F,G,H, the intensity of brown color was decreased in the CL of LiCl-treated group as compared with control at the same time. Therefore, IHC analysis showed that the expression of the StAR protein was markedly decreased in the luteal cells of the LiCl-treated rat ovary in comparison with control.

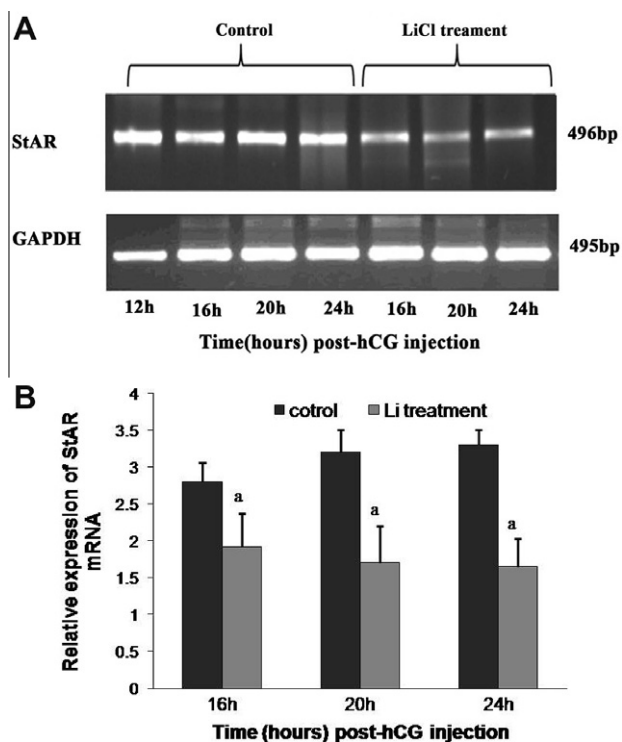
### 3.5. StAR mRNA expression

In order to provide insights into how LiCl may affect the progesterone production, the expression of StAR mRNA was determined by semi-quantitative RT-PCR. Results showed that the StAR expression was totally high after ovulation and dur-

ing luteinization (at 16, 20 and 24 h) in control (Fig. 5A). While, significant decreases were observed after LiCl treatment. The values of StAR expression were 68.5%, 53.1% and 50% of the control group at 16, 20 and 24 h, respectively (Fig. 5B).

## 4. Discussion

In this study, the rapid increase in the ovarian weight and the number of corpora lutea observed between 16 and 20 h post-hCG injection suggests “the critical time” of luteinization between 4 and 8 h post ovulation in the gonadotropin-induced immature rat. Also, we observed the impaired morphology of CL and significant decreases in the ovarian weight and the number of corpora lutea in LiCl-treated groups. Previous study indicated that the ovarian weight and folliculogenesis significantly decreased with LiCl treatment in adult female rats (13). In this study however folliculogenesis has not been affected by LiCl, the number of CL was significantly decreased following LiCl treatment. Therefore, it is conceivable that ovulation that occurred after gonadotropin administration could be affected by LiCl which resulted in disrupted ovulation and CL formation. Also, our result showed that the CL formation is accompanied by a dramatic increase in the serum level of progesterone and the expression of *StAR* gene in gonadotropin-induced rats. In rodents, serum levels of progesterone depend on the amount of progesterone synthesized by the luteal cells (2). Also, it is demonstrated that StAR plays a key role in the steroidogenic process in rat (12). Our results showed significant decreases in the progesterone secretion during luteinization in LiCl-treated rats that could be due to the effects of Li on the formation of CL and StAR expression. LiCl could interfere with the development of CL through several secondary mechanisms. Previous studies indicated lithium-induced adverse effects on the estrous cyclicity and a significant suppression of the pre-ovulatory surge of luteinizing hormone (LH) in Li-treated mice (15). Because LH increases mRNA and protein concentrations of StAR in luteal cells (16,17), it is conceivable that LiCl might influence the LH secretion and lead to a decrease in the luteal StAR expression in rat ovary that results in decreased progesterone production. Furthermore, the Wnt signaling pathway is a network of proteins best known for their roles in embryogenesis and cancer (18,19). Although, the role of Wnt proteins in luteinization is not yet clear, evidence from other studies suggests that Wnt signaling pathway is involved in follicular development (20,21), luteal cell formation and function in the rodent (22). Mechanisms of the canonical Wnt pathway include a series of events that occur when Wnt proteins bind to cell-surface receptors of the Frizzled family, causing to activate disheveled (DSH) family proteins and ultimately resulting in GSK-3 $\beta$  activity inhibition and change in the  $\beta$ -catenin levels that reaches the nucleus (23,24). Previous study indicated that  $\beta$ -catenin represses luteinization in mice (25). It has been reported that LiCl inhibits GSK-3 $\beta$  activity and mimics Wnt/ $\beta$ -catenin signaling (26–28). Consistent with this finding that LiCl mimics Wnt/ $\beta$ -catenin signaling, the effects of LiCl treatment on the progesterone secretion and StAR expression could be through the activation of Wnt signaling pathway during steroidogenesis in the CL. Therefore, LiCl-treated rat may provide a unique model to further identify the role of Wnt signaling pathway in the ovary steroidogenesis.



**Figure 5** Semi-quantitative RT-PCR assay was performed for StAR mRNA analysis in gonadotropin-induced rat ovary after treatment with either LiCl or distilled water (control). A: PCR products were subjected to 1% agarose gel electrophoresis and stained with ethidium bromide for visualization. B: Relative levels of mRNA are expressed as the ratio of the target genes relative to GAPDH in each sample. The results represent the means  $\pm$  SEM of groups of eight rats. (a): Significantly different from respective control group,  $P < 0.05$ .

## 5. Conclusions

In summary, we concluded that the effect of LiCl on the rat ovary is reflected in the reduction of serum progesterone concentration during the luteal phase which could be attributed to the interference in the CL formation and steroidogenesis as evident from the decreased number of CL and disrupting StAR protein and mRNA expression.

## Conflict of interest

There are no conflict of interest.

## Acknowledgment

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