Cardiac chronotropic hypo-responsiveness and atrial fibrosis in rats chronically treated with lithium

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ABSTRACT

Lithium is a widely used mood-stabilizing agent; however, it causes a variety of cardiovascular side effects including sinus node dysfunction. In this study we explored the potential adverse effects of lithium on cardiac chronotropic responsiveness, atrial tissue histology and gene expression in rats that were chronically treated with therapeutic doses of lithium. Male Wistar albino rats were given lithium chloride (2.5 g/kg) orally for 2 or 3 months. Following treatment, the atria were isolated and spontaneously beating rate and chronotropic responsiveness to β-adrenergic stimulation was evaluated in an organ bath. Development of cardiac fibrosis was examined by histological methods. The expression of atrial Col1a1 (collagen I, alpha 1) and β-arrestin2 was also assessed using quantitative RT-PCR. Treatment with lithium induced a significant hypo-responsiveness to adrenergic stimulation ($P < 0.001$) and caused fibrosis in the atrial tissue of treated rats. In addition, the expression of atrial Col1a1 mRNA was significantly increased in atrial tissues of lithium-treated animals, while β-arrestin2 mRNA expression did not show a significant difference compared with control animals. Altogether, these findings indicate that cardiac chronotropic hypo responsiveness and associated cardiac fibrosis are side effects of chronic lithium treatment. Moreover, it seems that lithium treatment does not influence β-arrestin2 mRNA expression.

1. Introduction

Lithium has a long history of application as a first-line agent in the treatment of acute mania and prophylaxis of bipolar affective disorder (Belmaker, 2004). One to three percent of the world's population suffers from bipolar disorder and lots of affected individuals are on lithium therapy (Chiu et al., 2013). Despite its efficacy, different side effects including heart-related problems have been reported for lithium. Indeed, diverse cardiac abnormalities are known to be caused by lithium, including cardiac conduction defects (sinus node dysfunction, sinus bradycardia, premature atrial and ventricular contractions, atrioventricular nodal block, cardiac dysrhythmias), electrocardiographic (ECG) alterations, congenital heart disease and myocarditis (Anantha Narayanan et al., 2015; Brady and Horgan, 1988; Moltedo et al., 2002; Wellens et al., 1975).

Multiple molecular targets have been identified for lithium;

however, the exact mechanism of its actions remains unclear. Several studies have proposed that lithium exerts some of its biochemical and behavioral effects through modulation of GPCR signaling, including effects on G protein levels or G protein coupling (Lenox and Hahn, 2000; Farhy Tselnicker et al., 2014). Regulation of β-arrestin-mediated effects, activity of PKC isozymes, and direct inhibition of glycogen synthase kinase-3 (GSK-3) and inositol monophosphatase have also been reported (Lenox and Hahn, 2000; O'Brien et al., 2011).

There is a proven link between chronic use of lithium and development of renal interstitial fibrosis (Walker et al., 2013). Development of fibrosis is known to cause organ dysfunction in a variety of diseases. Indeed, renal fibrosis that is caused by lithium is known to mediate, at least in part, lithium's nephrotoxic effects. Despite lithium's cardiac side effects, it is not known whether this drug can cause similar alterations in heart tissue. Herein, we investigated cardiac chronotropic responsiveness following prolonged treatment of rats with lithium.

Experiments were performed to examine potential development of cardiac fibrosis in lithium-treated animals. This was followed by examining the expression of collagen and β-arrestin2 genes in isolated heart tissues.

2. Materials and methods

All materials and chemicals were purchased from Sigma (Pool, UK), unless otherwise stated.

2.1. Animals

Thirty-one male Wistar albino rats weighing 250–280 g were obtained from Department of Pharmacology in Tehran University of Medical Sciences. Animals were housed in a controlled environment (24 ± 2 °C, 50 ± 5% humidity) on a 12:12 h light/dark cycle with free access to food and water. With a light/dark cycle of 12 h; at a temperature of 22 °C. All animal procedures were in accordance with 'Guide for the Care and Use of Laboratory Animals' (NIH US publication No 85-23, revised 1985) recommendations. Animals were randomly assigned to the following groups: (I) saline for 2 months, control); II) lithium chloride (2.5 g/kg, for 2 months); (III) lithium chloride (2.5 g/kg, for 3 months). Dose of lithium was chosen based on its therapeutic dose in manic disorder in rat. To confirm the achievement of therapeutic plasma concentrations of lithium between 0.6 and 1.5 mmol/l (Walker et al., 2013), we measured plasma lithium level after 1, 2 and 3 months of treatment. Also time intervals (2 or 3 months) were chosen based on the comparative studies between human and rat and the maximum time we were able to keep animals with minimum mortality. Lithium chloride was dissolved in sterile saline and all the groups received either the drug or saline by gavage once daily.

2.2. Preparations of isolated atria

In order to study the chronotropic responsiveness to adrenergic stimulation, the spontaneously beating right atria were used. On day 61, after thoracotomy under deep anesthesia (ketamine 100 mg/kg and xylazine 8 mg/kg, intraperitoneally), the hearts were quickly excised ($n = 10$, 5 rats of each control and 2-mo lithium treated groups) and placed in a dissection dish filled with cold oxygenated physiological salt solution of the following composition (mM):NaCl,112.0; KCl, 5; CaCl$_2$, 1.8; MgCl$_2$, 1.0; NaH$_2$PO$_4$, 0.5;KH$_2$PO$_4$, 0.5; NaHCO$_3$, 25.0; glucose, 10.0; and EDTA, 0.004. Then, the right atria were carefully dissected and mounted vertically under isometric tension of 1 g force in a 20-ml organ bath chamber (Power lab system, AD Instrument, Australia).The organ bath chamber was filled with physiological salt solution which was gassed continuously with 95% O$_2$ and 5% CO$_2$ (pH 7.4) and maintained at 37.0 ± 1 °C. Before evaluation of the spontaneous contractions, an equilibration period of 30 min was considered. The signals were digitized and displayed on a PowerLab system. The responsiveness of isolated atria to adrenergic stimulation was assessed by addition of cumulative concentrations of isoproterenol, a full agonist for β-ARs, (10_10 to 10_5 M) to the organ bath (Jazaeri et al., 2013).

2.3. Histologic assessment of atria

Isolated atria were fixed in 10% neutral buffered formalin overnight at room temperature ($n = 6$, 3 rats of each control and 2-mo lithium treated groups). Following the initial fixation, tissues were progressively dehydrated with increasing concentrations of ethanol, and embedded in paraffin. Four micrometer sections were stained with hematoxylin and eosin (H&E) for histomorphological characterization or with Masson's trichrome for visualizing collagenous and elastic tissue. Stained sections were examined and scored by two pathologists using a light microscope under double blind conditions.

2.4. Real-time PCR

2.4.1. RNA extraction and reverse transcription

Animals' atria were isolated as described previously and immersed in liquid nitrogen ($n = 12$, 4 rats in each group).Total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Germany) following the manufacturer's directions. Genomic DNA was removed using DNase (QIAGEN, Germany).The concentration and purity of RNA was determined using spectrophotometer by measuring the absorbance at 260 nm and ratio at 260 nm/280 nm (nucleotide/protein), respectively. The isolated RNA had absorbance 260 nm/280 nm ratios greater than or equal to 1.8. To produce first-strand cDNA, DNase-treated RNAs were reverse transcribed using a Prime Script RT reagent Kit (TAKARA, Japan) based on manufacturer's protocol.

2.4.2. Quantitative real time polymerase chain reaction

Quantitative RT-PCR (qRT-PCR) was performed with a Rotor-Gene machine for evaluation of β-arrestin2 and collagen type 1, alpha1 (COL1A1) expressions in atria in control and lithium treated rats. Oligonucleotide primers used for PCR amplification were as follows: Rat β-arrestin2, Forward: 5′-CGTGGTGCTTGTGGACCCTG-3′; Reverse: 5′-CCTGGTAGGTGGCGATGAAC-3′. Rat collagen type 1, alpha1 (COL1A1), Forward: 5′-CTTCGTGTAAACTCCCTCCATCC-3′; Reverse: 5′-AAGTCCATGTGAAATTGTCTCCCA-3′. Rat GPDH, Forward: 5′-TGCACCACCAACTGCTTAG-3′; Reverse: 5′-GGATGCAGGGATGATGTTC-3′.

Total volume of the reaction was 10 μl which comprised of 0.2 μmol of each forward and reverse primers, 5 μl of SYBR Premix Ex Taq 2X (TAKARA) and 1 μl of cDNA template. The holding stage (95 °C for 5 s) was followed by the cycling stage (denaturation 10 s at 95 °C, annealing 20 s at 60 °C, and extension/elongation 30 s at 72 °C) and the number of cycles was 40. For analyzing data, we used the competitive critical threshold (ΔΔCT) method in which the RNA of β-arrestin2 as well as COL1A1 were adjusted to RNA of GPDH.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). The results were expressed as mean ± SEM. Chronotropic study was analyzed using Student's *t*-test. P values < 0.05 were considered statistically significant.

3. Results

3.1. Measurement of plasma levels of lithium

To confirm that treated animals have reached therapeutic plasma concentrations of lithium (between 0.6 and 1.5 mmol/l) (Walker et al., 2013), we measured plasma lithium levels after 1, 2 and 3 months of treatment. The results were as follows (values are mean ± SEM): 1 month: 0.65 ± 0.06 mmol/lit; 2 months: 0.7 ± 0.1 mmol/lit; 3 months: 0.67 ± 0.07 mmol/lit.

3.2. Analysis of chronotropic responses following lithium treatment

We first examined the potential effects of lithium on cardiac chronotropic responses using isolated rat atria. Our data showed that chronotropic responses to isoproterenol, a β-adrenergic agonist, were significantly impaired in isolated atria following 2 months lithium treatment ($P < 0.001$, Fig. 1a). Moreover, a significant difference in the EC50 of isoproterenol between lithium treated atria (logEC50 = −7.501 ± 0.05) was observed compared with control atria (logEC50 = −8.966 ± 0.09). However, there was no significant difference in the maximum response (Rmax) to isoproterenol in lithium treated group in comparison with control group (Fig. 1a).

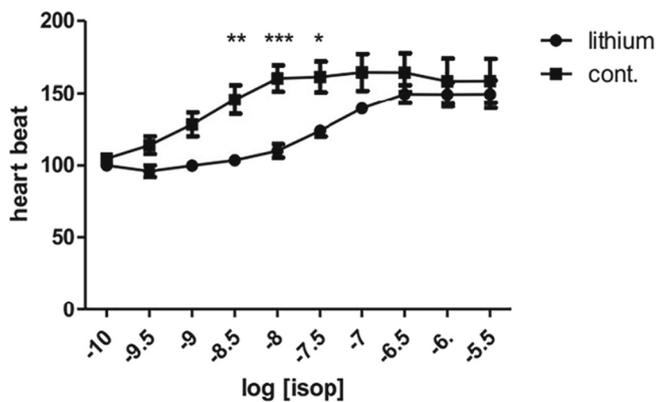Moreover, chronotropic responsiveness data were iteratively fitted

Fig. 1a. Concentration-dependent responsiveness of isolated right atria to iso-proterenol (β-adrenergic stimulation) in control or 2 months lithium-treated rats. Chronotropic studies were obtained on spontaneously beating atrium. Results are shown as mean ± SEM. 5 rats were used in each group. (*$P < 0.05$ comparison between atrial responses in concentration $10^{-7.5}$ M of isoproterenol in two groups, **$P < 0.01$ compared between atrial responses in concentration $10^{-8.5}$ M of isoproterenol in two groups, ***$P < 0.001$ compared between atrial responses in concentration 10^{-8} M of isoproterenol in two groups, Student's *t*-test).
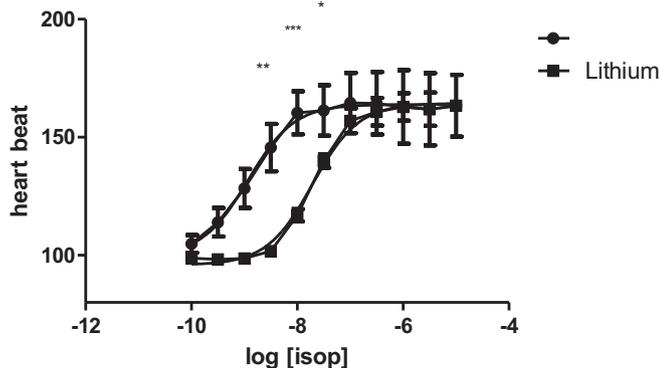

Fig. 1b. Concentration-dependent responsiveness of isolated right atria to iso-proterenol (β-adrenergic stimulation) in control or 2 months lithium-treated rats. Chronotropic studies were obtained on spontaneously beating atrium. Results are shown as mean ± SEM. 5 rats were used in each group. (*$P < 0.05$ comparison between atrial responses in concentration $10^{-7.5}$ M of isoproterenol in two groups, **$P < 0.01$ compared between atrial responses in concentration $10^{-8.5}$ M of isoproterenol in two groups, ***$P < 0.001$ compared between atrial responses in concentration 10^{-8} M of isoproterenol in two groups, Results obtained from an iterative fitting procedure to the Hill equation in a nonlinear model).

to the Hill equation, obtaining curve parameter estimates for mid-point location (EC50), upper asymptote of the observed maximal effect and Hill slope. GraphPad Prism software was used to fit the data. Fig. 1b shows the sigmoidal curve that is obtained with the Hill model.

3.3. Development of atrial fibrosis in lithium-treated rats

Considering the impaired chronotropic responsiveness to β-adrenergic agonist in lithium-treated animals, we then examined the effects of treatment on development of cardiac fibrosis and inflammation using two different histological methods, H&E and Masson's trichrome staining (Fig. 2). No evidence of fibrosis or inflammation was seen in stained sections from control animals (Fig. 2A). However, animals that had received lithium for 2 or 3 months showed profound changes in cardiac histology. The most prominent change in tissue histology was development of fibrosis (excess fibrous connective tissue), as seen in the areas indicated by the arrows in Fig. 2 B and C. Masson's trichrome

staining better reveled the extent and location of these fibrotic changes. The histopathological features of 3 observed sections in each group were as follows; in 3 months lithium treatment group: minimal interstitial fibrosis, mild focal interstitial fibrosis, negative for fibrosis; in 2 months lithium treatment: mild focal interstitial fibrosis, subendocardial fibrosis, negative for fibrosis (Fig. 2).

3.4. Atrial Col1a1 and β-arrestin2 expression following lithium treatment

Development of fibrosis is generally associated with increased activity of fibroblasts and enhanced expression of extracellular matrix (ECM)-related genes. Collagen type 1, a major ECM component, is composed of three polypeptides which are encoded by collagen type 1 alpha 1 (Col1a1) and alpha 2 (Col1a2) genes. To examine whether histological fibrosis is associated with gene expression alterations we evaluated the expression of Col1a1 gene in isolated rat atria. Real-time PCR analysis of mRNA levels showed significant upregulation of Col1a1 in the atria of lithium-treated rats, both in 2-months and 3-months treated groups, compared with controls (Fig. 3a). Moreover, considering that beta-adrenergic receptor activity is regulated by beta-arrestin molecules, we asked whether lithium treatment might have affected beta-arrestin gene expression. Real-time PCR analysis for β-arrestin2 mRNA did not show any significant differences in expression in atria of lithium-treated rats compared with control animals (Fig. 3A). While we did not analyze β-arrestin2 protein levels, nonetheless these findings diminish the likelihood of β-arrestin2 involvement, at least at the transcriptional level, in the observed effects.

4. Discussion

The mood stabilizer lithium is primarily used in the management of bipolar disorder, a chronic mental illness and an important cause of disability worldwide (Licht, 2012). Prolonged treatment with lithium is associated with various side effects including renal damage as well as cardiac problems. In the current study, we investigated the effects of lithium treatment on heart's chronotropic response as well as atrial tissue histology and expression of relevant genes. Our results demonstrated a significant decrease in beta-adrenergic response in rats that had received a therapeutic dose of the drug over a period of 2 to 3 months. Histological analyses showed atrial fibrosis in treated animals. Molecular assays revealed enhanced levels of collagen, but not beta-arrestin, transcripts in cardiac tissues obtained from treated animals.

Sinus node dysfunction and sinus bradycardia have been reported following long-term use of lithium, both in therapeutic and toxic serum levels (Moltedo et al., 2002; Wellens et al., 1975; Freeman and Freeman, 2006; Oudit et al., 2007; Livingstone and Rampes, 2006). Various intrinsic and/or extrinsic factors have been suggested to be involved in lithium-induced sinus node dysfunction, including degree of cardiac sympathetic and parasympathetic tone, lithium-induced blockade of cardiac sodium channels and decreased intrinsic sinus rate due to age-dependent occurrence of interstitial fibrosis (Oudit et al., 2007). In the current study, following experiments that revealed decreased beta-adrenergic response in lithium-treated animals, we examined the effects of lithium on development of interstitial fibrosis in atrial tissue. This research direction was partly based on the results of studies performed in the context of lithium induced nephropathy. Indeed, several studies have shown that long-term lithium treatment can lead to development of renal interstitial fibrosis, both in animal models and in human cases (Walker et al., 2013; Marti et al., 2016; Rao et al., 1981). This drug-induced interstitial fibrosis is believed to interfere with renal tubular function, leading to the widely recognized lithium side effect, i.e. renal damage. Likewise, cardiac fibrosis might have pathological outcomes, predisposing individuals to cardiac arrhythmia due to diminished conduction velocity, conduction blocks, or impaired cardiomyocyte-fibroblast couplings (Lin et al., 2016). Sinoatrial node
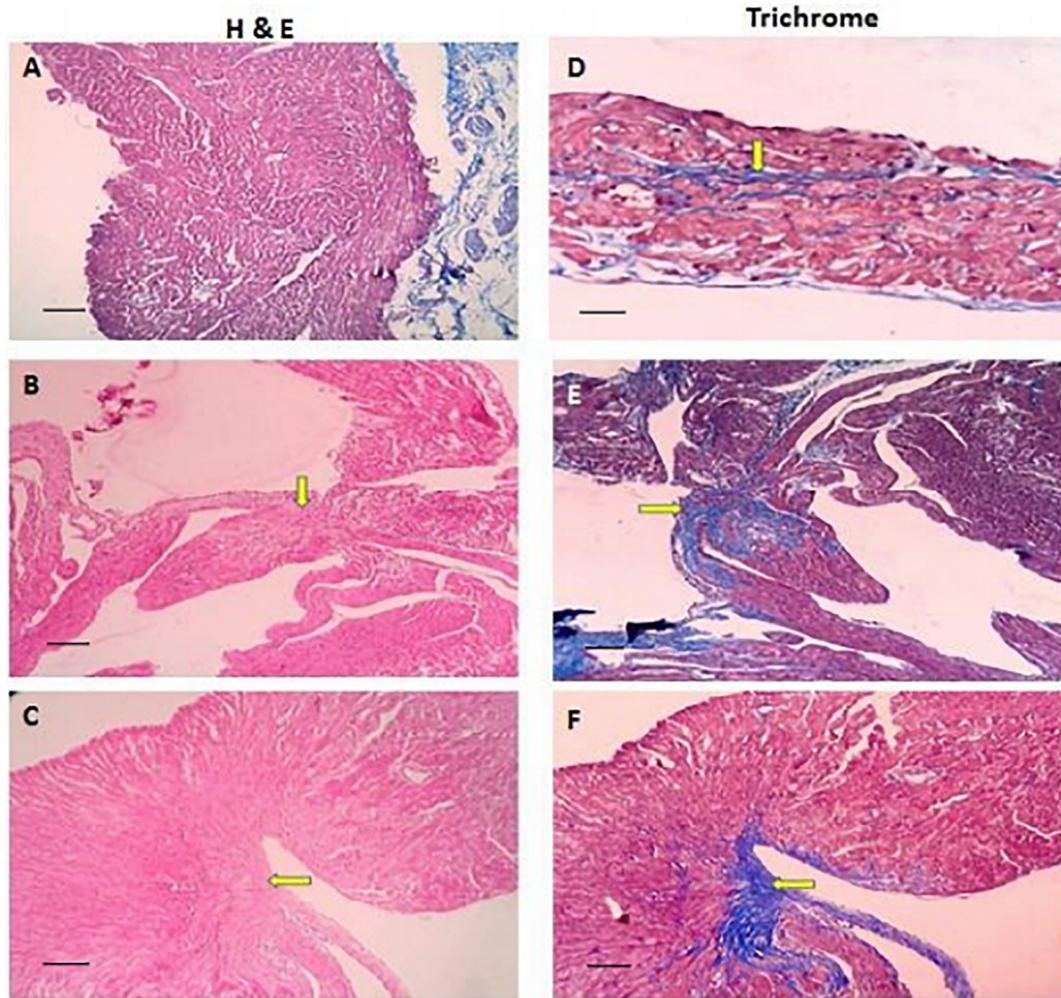
Fig. 2. Histological assessment of atrial tissue. Photomicrographs (40×) are shown for sections of the atria isolated from control or lithium treated rats. Atrial sections are stained with Heamatoxylin & Eosin (A, B, and C) or Masson's trichrome (D, E and F), which stains collagen (for fibrosis). Note the absence of fibrosis in control group (A). B, C, D, E and F show the histopathological features after 2 or 3 months of receiving lithium. B: Mild focal interstitial fibrosis. C: Subendocardial fibrosis. D: Minimal interstitial fibrosis. E: Mild focal interstitial fibrosis. F: Subendocardial fibrosis. Arrows indicate the fibrotic region. 3 rats were used in each group. The scale bar represents 100 μm.

(SAN) which is composed of clusters of specialized cardiomyocytes is enmeshed within strands of connective tissue, mostly a combination of collagen, elastin and fibroblasts. Development of fibrosis within the SAN is known to affect SAN's function and it might lead to tachycardia-bradycardia arrhythmias and cardiac arrest (Csepe et al., 2015). Our histological analyses showed the occurrence of fibrosis in atrial tissue of lithium-exposed animals. Our histological study was descriptive, nonetheless it could clearly display the presence of fibrosis in the H&E and specific stains, enabling us to provide a qualitative comparison between groups of animals. Consistent with qualitative histology, quantitative real-time PCR analyses on cardiac tissues revealed statistically significant upregulation of collagen (Col1a1) gene in lithium-treated hearts.

The observed association between reduced chronotropic response and atrial fibrosis suggests, but it does not confirm, a causal link between these two phenomena. Indeed, it is possible that lithium might have led to altered chronotropic response and atrial fibrosis through independent mechanisms. Establishment of a cause and effect relation between these phenomena would require beta-adrenergic response analysis in lithium-treated animals in the presence of an intervention that blocks the induction of fibrosis. Nonetheless, pervious reports indicating that intranodal fibrosis is associated with lower heart rates lend some support, albeit indirect, to a link between these two effects

(Csepe et al., 2015).

It has been reported that lithium exerts some of its effects through direct targeting of G protein-coupled receptor (GPCR) signaling. Previous studies have shown that beta-arrestin2 is required for the regulation of Akt and GSK3 phosphorylation by lithium (O'Brien et al., 2011). Chronic or acute exposure to lithium destabilizes the signaling complex comprised of β-arrestin2, Akt, and PP2A that normally promotes Akt dephosphorylation/inactivation. In theory, these signaling pathways can influence both chronotropic and pro-fibrotic effects of lithium. However, our findings indicated that chronic lithium treatment does not affect β-arrestin2 mRNA expression in cardiac tissues of rats. While we did not measure β-arrestin2 protein levels or the activity downstream kinases, it is likely that altered expression of β-arrestin2 is not a major player in cardiac effects induced by lithium treatment.

5. Conclusion

This study indicates that cardiac chronotropic hypo-responsiveness is a consequence of chronic lithium treatment and that lithium treatment leads to atrial fibrosis associated with enhanced expression of Col1a1 gene in atrial tissue. Moreover, it seems that β-arrestin2 expression is not a key player in lithium-induced cardiac effects.
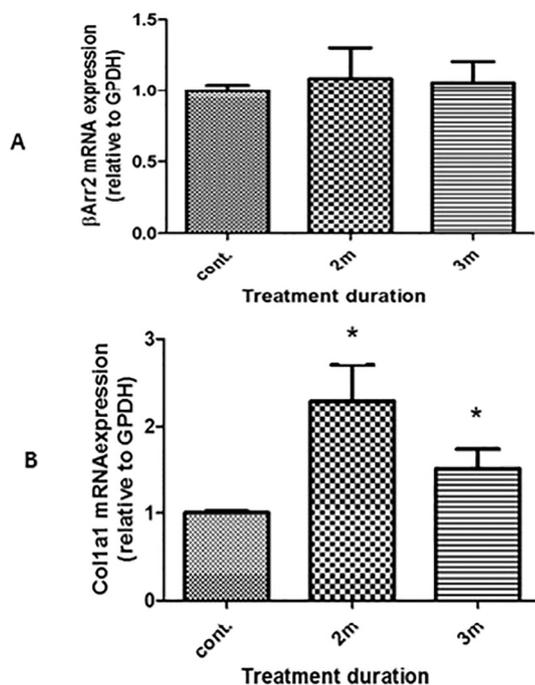
Fig. 3. mRNA expression levels of Col1a1 (A) and β-arrestin2 (B) normalized against the expression of Gapdh in the atria of lithium-treated rats vs control group. 4 rats were used in each group (*$P < 0.05$, compared with control).

Acknowledgements

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