

## Article

# Effects of Lithium Chloride and Nitric Oxide Inhibitor on Orthodontic Tooth Movement in the Rat

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**Abstract:** Orthodontic tooth movement in a rodent model is reduced by lithium chloride (LiCl), a mood-stabilizing agent with antithyroid effects. Considering the established inhibitory effect of N(omega)-nitro-L-arginine methyl ester (L-NAME) on orthodontic tooth movement and the possible role of nitric oxide synthase in LiCl mechanism of action, the question arises if these two mechanisms are synergistic. To answer this question, 70 male Sprague Dawley rats were randomly divided into seven groups: untreated group without any interventions (i), and the orthodontic tooth movement groups receiving daily saline injection (ii), 300 (iii), and 600 mg/kg (iv) of LiCl per os, 10 mg/kg of L-NAME (v) and the combinations of 300 (vi) and 600 mg/kg LiCl (vii) with L-NAME. The first molar was moved towards the incisor with 60 g of mesial tipping force applied by an activated fixed coil spring for two weeks. The resulted distance between the first and the second molar was measured using a feeler gauge. The serum parameters were also determined. We report here that both concentrations of LiCl significantly decreased tooth movement. Even though L-NAME was capable of reducing orthodontic tooth movement, no synergistic effects with LiCl were observed. Moreover, L-NAME had no impact on the robust and significant increase of thyroid-stimulating hormone (TSH) and decrease of triiodothyronine (T3) and thyroxine (T4) in the LiCl treated rats. These findings suggest LiCl significantly decreases the orthodontic tooth movement in rats; however, this ability seems not to be principally mediated through nitric oxide synthase.

**Keywords:** orthodontic tooth movement; lithium chloride; L-NAME; rats; thyroid-stimulating hormone; thyroid hormones



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## 1. Introduction

Orthodontic tooth movement (OTM) is a consequence of the molecular and cellular events that are initiated when a force is applied, causing resorption of the alveolar bone on the pressure side and apposition of bone on the tension side [1,2]. Thus, a biomechanical signal is translated into a biological signal that initiates a cascade of cellular events that finally allows the tooth to move within the mineralized alveolar bone. Similar to the physiological and even pathological systemic bone remodeling where the resorption and formation by osteoclasts and osteoblasts are coupled, this balance is affected by a large spectrum of factors including pharmacological compounds that target the effector cells [3]. Considering that osteoclasts and osteoblasts are also required for OTM, pharmacological compounds targeting remodeling usually also affect OTM [4,5]. Thus, the hypothesis relevant for OTM arises from current knowledge on how pharmacological compounds

change bone remodeling. Among those pharmacological compounds is LiCl, a commonly prescribed medication for mental disorders [6], also affecting bone remodeling—in particular, LiCl can prevent osteoporotic bone loss in ovariectomized rats [7], suggesting that LiCl blocks bone resorption. Local release of lithium from biomaterials can also accelerate bone regeneration through activation of the Wingless (Wnt)/beta ( $\beta$ )-catenin pathway [8]. However, with respect to OTM where bone resorption is blocked by LiCl, the enhanced bone formation is maybe not that relevant.

Consistent with observations in ovariectomized rats, LiCl significantly reduced orthodontic tooth movement in rats [9] and attenuated force-induced root resorption [10]. The molecular mechanism remains unclear but may be attributed to a positive balance of bone turnover that also helped to compensate for bone loss and stimulates osseointegration in ovariectomized rats [11] involving the Wnt/ $\beta$ -catenin and other signaling pathways [12]. Importantly, biomechanical activation of the Wnt/ $\beta$ -catenin pathway in osteocytes involves nitric oxide production [13] and in OTM, these are the osteocytes that become the major promoters to mediate osteoclastogenesis and to regulate bone formation. For example, osteocyte-derived receptor activator of nuclear factor kappa-B ligand (RANKL) is required for OTM in mice [14]. This question arises if the way LiCl reduces orthodontic tooth movement requires nitric oxide production. The support for this hypothesis comes from observations that administration of L-NAME, a general inhibitor of nitric oxide synthases (NOS) activity, significantly reduced tooth movement in a rat model [15–17]. Similar to LiCl, also L-NAME prevented ovariectomy-induced bone loss [18]. Moreover, co-administration of LiCl and L-NAME had additive effects [19]. If, however, LiCl requires the NOS activity to reduce tooth movement was yet unclear.

That LiCl-reduced OTM in rats [9,10] that may be related to lithium ability to cause hypothyroidism [20], indicated by an increased thyroid-stimulating hormone (TSH) and consequently, a decrease in triiodothyronine (T3) and thyroxine (T4) production. Hypothyroidism lowers bone turnover by reducing both osteoclastic bone resorption and osteoblastic activity [21], and decreases bone regeneration [22]. One hypothesis is that LiCl causes hypothyroidism which in turn reduces bone remodeling, and thus also OTM [9,10]. Experimental thyroxine gavage consequently stimulates OTM [23,24]. Indirect support for this hypothesis comes from observations that hypothyroidism affects the development of teeth and craniofacial structure in children and animal models [25,26]. We assume that LiCl mediates at least part of its own activity on OTM via significant lowering of thyroid hormones, while L-NAME and thus nitric oxide have no dramatic impacts on thyroid hormones. Based on this assumption, it is, unlikely that the LiCl-induced reduction of OTM is modulated mainly by L-NAME.

The overall aim of the study was therefore twofold, first to study if the proposed effects of LiCl and L-NAME to reduce OTM are additive or even counteract each other—and if independent, then also TSH, T3, and T4 should be clearly changed by LiCl but not by L-NAME. Our data basically confirm this hypothesis that the effects of LiCl and the effects of L-NAME on OTM are not synergistic, and that LiCl but not L-NAME causes a hypothyroidism.

## 2. Materials and Methods

### 2.1. Animals

Seventy male Sprague Dawley adult rats with an average weight of  $275 \pm 25$  g obtained from the Pasteur Institute of Iran were used in this study. It seems that OTM is efficient [27] and not highly affected by the remodeling and modeling processes occurring during growth cycle in adult rats [28]. Animals were kept in the plastic cages accessing a standard 12-h light-dark cycle. A diet of soft laboratory pellet was used for the feeding to minimize animal discomfort and the possibility of intraoral appliances displacement. Animals had free access to water and pellet. The study was performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The rats were randomly divided into seven study groups (N = 10 in each group);

Untreated animals were left without any interventions (i). The control group had OTM and received daily i.p saline (ii). The Li300 and Li600 groups had OTM and received 300 and 600 mg/kg LiCl per os (Sigma, St. Louis, MO, USA), respectively (iii, iv). The concentrations used and the routes of the application in rats were established by us [29–31] and others [10], even though daily i.p administration of LiCl is also effective to reduce OTM [9]. L-NAME group in which the animals received 10 mg/kg daily i.p of N-nitro-L-arginine methyl ester (L-NAME (v); nitric oxide synthase inhibitor; Sigma, St. Louis, MO, USA) as we have established in OTM [16], Li300-L-NAME and Li600-L-NAME groups with OTM (vi; vii). All injections were administered starting from the first day of appliance insertion to the 14th day.

## 2.2. Orthodontic Treatment

Each rat was anesthetized with an intraperitoneal injection of Ketamine hydrochloride 100 mg/kg body weight (Pharmacia & Upjohn, Erlangen, Germany) and Xylazine HCl 5 mg/kg body weight (Rompoun, Bayer, Leverkusen, Germany). The setting of orthodontic appliances was similar to what was presented by King and Fischischweiger [32]. Applying a 5 mm length of 0.006 × 0.022" Nitinol closed-coil spring (3M/Unitek Hi-T II. Monrovia, CA, USA) running between the right upper first molar and incisor, orthodontic force was applied. The spring was fixed in place via 0.010" steel ligature wires (Dentaureum, Ispringen, Germany) surrounding the molar tooth and incisor. A cervical groove was designed on the incisor area and the ligature wire was seated. A self-cured composite resin (Bisfil™ 2B, Bisco, Schaumburg, IL, USA) was used to secure the wire on both incisors. According to the Heller and Nanda's method [33], activation of the spring was done to provide 60 g of force. Injections were done during the two weeks, while appliances were regularly exerting the force. Fourteen days following appliance insertion, animals were sacrificed using an overdose of ether and then decapitated. Immediately, before blood clot formation, trunk blood was collected for serum preparation for the later analysis of the thyroid hormones. Prepared serum was stored at −70 °C until required.

## 2.3. OTM Measurement

Using a Feeler Gauge (Mitutoyo Co, Kawasaki-Shi, Japan), orthodontic tooth movement was measured directly in the mouth to reveal the distance between the first and second right molars that was zero initially. To have the opportunity of re-measurement and to prevent any potential relapse of tooth movement before removing the appliances, an additional silicone impression (Zermack, Mahl, Germany) was taken and poured with ultra-strength dental stone (Velmix, Gildand, Germany). Re-measurement was performed using the same Feeler Gauge on the plaster replica that confirmed the first direct measurement. The same operator performed all the measurements.

## 2.4. Serum Analysis

The hormonal assay which was conducted in Pasteur Institute of Tehran, Iran, benefited from the radioimmunoassay (RIA) to determine serum levels of T3, T4, and TSH based on the methods recently described [34,35]. In brief, the blood samples that were collected from the hearts of anesthetized rats, were centrifuged at 5000 rpm for 20 min at 4 °C and the sera were collected and frozen at −20 °C for future analyses. Calorimetric competitive enzyme immunoassays were used for the serum T3 and T4 analyses by using individual enzyme-linked immunosorbent assay kits. All the procedures for T3, T4, and TSH levels measurements were performed based on the instructions of manufacturers.

## 2.5. Statistical Analysis

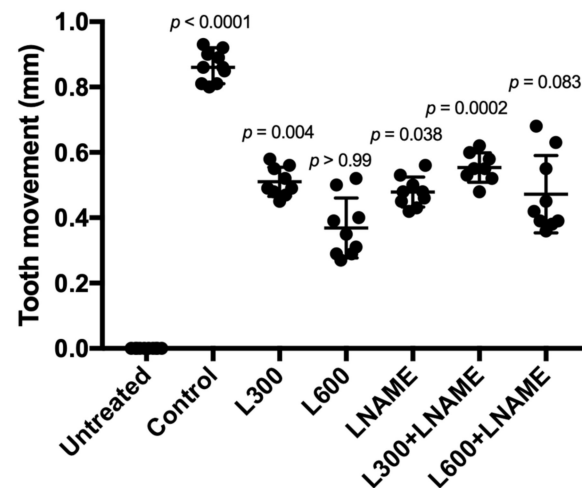
Prism 7e (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. The Kruskal–Wallis test followed by Dunn's test for multiple comparisons was applied to analyze the data. Statistically, a *p*-value less than 0.05 was considered significant. The *p*-values refer to the normal and not the OTM rats. We also include the *p*-values

of comparing LiCl with LiCl plus L-NAME in the text. Data are shown as dot blot with median and 95% confidence interval (CI). Sample size was calculated based on our previous studies showing that 8–12 animals are sufficient to reach a significance on OTM by LiCl [9] and L-NAME [16].

### 3. Results

#### 3.1. Orthodontic Tooth Movement

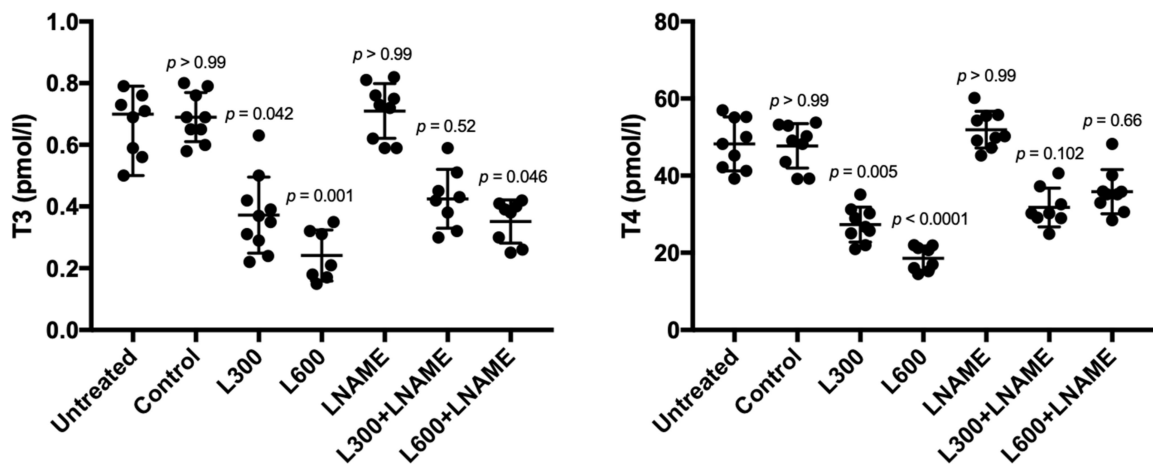
Compared to the untreated contralateral first molar (M1), the tooth movement of control rats were in median 0.86 mm (min 0.81; max 0.92; Figure 1). Both concentration of LiCl, namely, 300 mg/kg and 600 mg/kg lowered the tooth movement to 0.49 mm (min 0.47, max 0.56;  $p < 0.0001$ ) and 0.35 mm (min 0.29, max 0.50;  $p = 0.004$ ), respectively. Likewise, 10 mg/kg daily i.p injection of L-NAME decreased tooth movement 0.48 mm (min 0.43, max 0.53;  $p = 0.038$ ). Based on the multiple comparison, L-NAME had no considerable impact on changing the tooth movement in the presence of 300 mg/kg and 600 mg/kg LiCl with 0.55 mm (min 0.48; max 0.62;  $p > 0.99$ ) and 0.42 mm (min 0.38; max 0.63;  $p > 0.99$ ), respectively. These findings show that LiCl and L-NAME independently and non-synergistically reduce OTM.



**Figure 1.** Orthodontic tooth movement. The orthodontic force was applied to move the right upper first molar towards the incisor. Fourteen days following appliance insertion orthodontic tooth movement was measured directly in the mouth to reveal the distance between the first and second right molars and confirmed on a plaster replica. Multiple comparisons were based on Kruskal–Wallis test and Dunn’s correction.  $p$ -values show the changes compared to the normal non-OTM group. Comparison of L300/L600 with L300/L600 + LNAME are all  $p > 0.99$  and thus not shown in Figure 1. Data are shown as dot blot with median and 95% CI.

#### 3.2. Thyroid Hormone Levels

Daily application of LiCl but not L-NAME ( $p > 0.99$ ) significantly lowered the serum levels of T3 and T4 (Figure 2). Compared to the untreated rats with a T3 of 0.7 pmol/L in median (min 0.5, max 0.79), the daily gavage of 300 mg/kg and 600 mg/kg LiCl decreased T3 to 0.36 pmol/L (min 0.24; max 0.50;  $p = 0.042$ ) and to 0.21 pmol/L (min 0.15; max 0.35;  $p = 0.001$ ), respectively. The T3 levels were lightly reversed by the presence of L-NAME to 0.43 pmol/L (min 0.3; max 0.59) and 0.39 pmol/L (min 0.25; max 0.42); however, not significantly (both  $p > 0.99$ ).

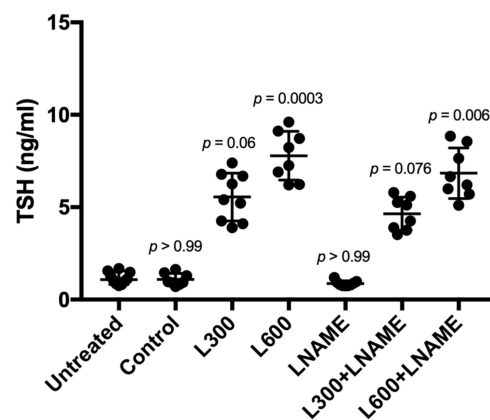


**Figure 2.** Thyroid hormones levels. Fourteen days following orthodontic tooth movement, trunk blood was collected for serum preparation. Prepared serum was stored at  $-70^{\circ}\text{C}$  until measured by radioimmunoassay. Multiple comparisons were based on Kruskal–Wallis test and Dunn’s correction.  $p$ -values show the changes compared to the normal non-OTM group. Comparison of L600 with L600 + LNAME is  $p = 0.15$  but for consistency not shown in Figure 2. Data are shown as dot blot with median and 95% CI.

The same was true for T4; here, 300 mg/kg and 600 mg/kg LiCl decreased T4 from 48.23 pmol/L (min 41.25; max 55.26) to 26.69 pmol/L (min 21.95; max 31.25;  $p = 0.005$ ) and 18.84 pmol/L (min 14.52; max 21.99,  $p < 0.0001$ ), respectively. Again, L-NAME partially reversed T4 lowering effects of LiCl to 30.23 pmol/L (min 24.89; max 40.66,  $p > 0.99$ ) and 35.17 pmol/L (min 30.55; max 40.12,  $p = 0.149$ ) but not significantly. Taken together, LiCl but not L-NAME significantly reduced T3 and T4 levels and the decreases were not significantly reversed by L-NAME.

### 3.3. TSH

As shown in Figure 3, TSH levels in the OTM treated controls significantly increased from median 1.09 ng/mL (min 0.82; max 1.56) to 5.4 ng/mL (min 4.11, max 6.78,  $p = 0.06$ ) and 7.75 ng/mL (min 6.2; max 9.61,  $p = 0.0003$ ) with 300 mg/kg and 600 mg/kg LiCl, respectively. Even though L-NAME had no impact on basal TSH levels, the effects of LiCl were slightly but not significantly ( $p > 0.99$ ) reversed to 4.69 ng/mL (min 3.52; max 5.8) and 6.43 ng/mL (min 5.11; max 8.85), respectively.



**Figure 3.** Thyroid-stimulating hormone (TSH). Fourteen days following orthodontic tooth movement, trunk blood was collected for serum preparation. Prepared serum was stored at  $-70^{\circ}\text{C}$  until measured by radioimmunoassay. Multiple comparisons were based on Kruskal–Wallis test and Dunn’s correction.  $p$ -values show the changes compared to the normal non-OTM group. Comparison of L300/L600 with L300/L600 + LNAME are all  $p > 0.99$  and thus not shown in Figure 3. Data are shown as dot blot with median and 95% CI.

#### 4. Discussion

Orthodontic tooth movement is an established clinical approach to bring teeth in the desired position, a therapy that uses forces to initiate cellular events culminating in the resorption and formation on the pressure and tension site, respectively [1,2]. However, it is a clinical challenge to predict the speed of tooth movement, and ideally even to find therapeutic approaches to speed up the process. Progress in clinical orthodontics is based on the fundamentals of preclinical research where the mechanisms of OTM are evaluated in the light of pharmacological therapies; some therapies are maybe not intentionally affecting bone remodeling, but as a side effect, they do. One example depicted in the present study is LiCl, a commonly prescribed drug for patients suffering from mental disorders [6]. Based on the knowledge that LiCl prevents bone resorption in ovariectomized osteoporotic rat models and thus bone resorption [7], it is not surprising that LiCl reduces OTM in rat models [9], observation that are supported by the findings reported here. Further, in support of what we report here are observations showing blocking of nitric oxide by means of the inhibitor L-NAME not only reduced ovariectomy-induced osteoporotic bone loss in the rat model [18] but also had a blocking effect on OTM [15,16]. Overall, the findings we have are supporting previous knowledge and providing a solid basis for the actual research hypothesis we have proposed.

Based on the previous observations, we have now asked if the inhibition of OTM induced by LiCl and by L-NAME are synergistic or even dependent on each other. The support for this assumption comes from the findings that co-administration of LiCl and L-NAME have additive effects on reducing morphine withdrawal syndromes in mice [19]. There is thus a reason to ask if LiCl requires the NOS activity to reduce tooth movement. However, we have to reject this hypothesis because the suppression of OTM induced by LiCl and by L-NAME was not synergistic and also without any kinds of dependency. In support of these new observations are the data from changes in thyroid hormones levels observed with LiCl but not with L-NAME, resulting in a robust and significant increase of TSH and decrease of T3 and T4 in the LiCl-treated rats only. Thus, it seems that the antithyroid effects of LiCl are independent of NOS activity. Considering that 5-thyroxine [23] and L-thyroxin [24] stimulated OTM and thyroid hormones excess accelerates bone turnover with predominant bone resorption [36], the known LiCl-mediated reduction of thyroid hormones [37] maybe culminates in the reduced tooth movement. Even the therapeutic effects of LiCl in mania and depression are thought to be mediated mainly via thyroid hormones [38]. In support of previous observations, numerous studies have confirmed a particular association between serum TSH levels and obesity [39]. Again, our findings nicely match those of others, adding the novelty that the reduction of OTM induced by LiCl may be mediated via thyroid hormones but not nitric oxidase activity.

The study has limitations. Rat model not necessary represents the complex situation in patients undergoing OTM and it does not provide the real clinical scenario of the patients suffering from mental disorders under the treatment with LiCl [6]. Moreover, the clinical relevance of our findings with L-NAME should be interpreted with care as L-NAME is an experimental antihypotensive agent that has not yet received the FDA approval. Nevertheless, it is important to understand the molecular mechanism of OTM to take advantage of this knowledge for future stimulation of this process. Another limitation is that we have no robust information on the resorption of the tooth roots and how LiCl and L-NAME change this osteoclastic event. This should be considered in future studies.

Future research should further investigate if it is the diminished thyroid hormones levels that are responsible for the decreased OTM in response to LiCl treatment. These studies should focus on how thyroid hormones change the sensitivity of osteocytes to drive osteoclastogenesis and thus bone resorption—particularly, because RANKL, the essential mediator of osteoclastogenesis, is expressed by osteocytes and allows OTM to occur [14]. Theoretically, TSH and T3/T4 might have reciprocal actions together lowering the RANKL expression by the osteocytes or maybe making the osteocytes less responsive to biomechanical forces. Hence, the present study paves the way for future research to reveal

the molecular mechanisms of how LiCl reduces OTM possibly without NOS activation. Moreover, interesting, and somehow enigmatic, is the molecular mechanism by which L-NAME reduced OTM. It might as well mean that NOS production in osteocytes or maybe blood vessels is required to stimulate the expression of RANKL by the osteocytes and consequently allow OTM to occur. What is possible is that maybe the LiCl-induced changes of the thyroid hormones and the L-NAME-induced reduction of NOS are independent but maybe culminate to target the same cells, namely, the osteocytes, and by that lowering the ability of the osteocytes to push osteoclastogenesis and OTM.

Within the limitation of this study, we can conclude that lithium chloride significantly reduces orthodontic tooth movement in rats. It seems this effect is not principally mediated through the nitric oxide synthase.

**Author Contributions:** R.T. contributed to data acquisition, interpretation of data, drafted manuscript, critically revised manuscript, and gave final approval. F.J. contributed to conception, design, critically revised manuscript, and gave final approval. A.R.D. contributed to the protocol design, interpretation of data, critically revised manuscript, and gave final approval. R.G. contributed to interpretation of data, drafted manuscript, critically revised manuscript, gave final approval. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the local ethical committee at the Tehran University of Medical Sciences; IR.TUMS.MEDICINE.REC.1390.146.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data sets used and/or analyzed during the current study are available from the corresponding author or reasonable request.

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