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# Lithium promotes the production of reactive oxygen species via GSK-3β/TSC2/TOR signaling in the gill of zebrafish (*Danio rerio*)

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## HIGHLIGHTS

- Lithium promotes the production of reactive oxygen species (ROS).
- Lithium decreases the activities of antioxidant enzymes.
- Lithium induces TSC2/TOR signaling by inhibiting GSK-3β.
- Lithium promotes ROS production via GSK-3β/TSC2/TOR signaling.

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## ABSTRACT

In this study, the mechanism that lithium (Li) promotes the production of reactive oxygen species (ROS) via the glycogen synthase kinase-3β (GSK-3β)/tuberous sclerosis complex 2 (TSC2)/target of rapamycin (TOR) signaling was investigated in the gill of zebrafish (*Danio rerio*). After the zebrafish were treated by 25 and 50 mg/L $Li^+$, the mRNA expression of GSK-3β and TSC2 was inhibited, but the expression of TOR was induced in the gill of zebrafish. The levels of hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^{\cdot-}$), and hydroxy radical (·OH) as well as the activity of superoxide dismutase (SOD) were increased, while the activities of catalase (CAT), glutathione peroxidase (GSH-PX), and peroxidase (POD) were decreased by 25 and 50 mg/L $Li^+$ treatments. In the ZF4 cells, the mRNA expression of GSK-3β and TSC2 was inhibited, but TOR expression was induced by 1, 5, and 10 mmol/L $Li^+$ treatments. To further confirm that lithium promoted ROS production via GSK-3β inhibition, GSK-3β RNA was interfered. It was found that the interference of GSK-3β RNA induced the TSC2/TOR signaling. The levels of $H_2O_2$, $O_2^{\cdot-}$, and ·OH were increased, but the activities of CAT, GSH-PX, and POD were decreased by GSK-3β RNA interference. In addition, lithium decreased the mitochondrial membrane potential (MMP) with Rhodamine-123 assay, but increased the levels of ROS by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay. The present results indicated that lithium promoted the ROS production through the GSK-3β/TSC2/TOR signaling in the gill of zebrafish.



## 1. Introduction

Lithium (Li) has various industrial applications, including Li batteries, ceramics, polymers, pharmaceuticals, atomic reactors, and air conditioners as humectants, etc. (Léonard et al., 1995; Scrosati and Garche, 2010; Bonino et al., 2011; Saeidnia and Abdollahi, 2013). With the increasing usage of Li batteries, they are disposed along with other living garbage, which causes potential impact to the environment (Scrosati and Garche, 2010). As a trace element in soil, Li is mainly present in the clay fraction of soil (7–200 μg/g) (Sapek and Sapek, 1983; Bowen, 1966; Weiner, 1991). The level of Li is 1–10 μg/L in the surface water and 0.18 μg/L in seawater (Bowen, 1966; Weiner, 1991). The lithium concentrations in ground water may reach 500 μg/L (Zaldivar, 1989). However, in some natural mineral waters, the level of Li is higher than 100 mg/L (Nevoral, 1988).

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Lithium is a non-essential element for life and has not a known biological use (Léonard et al., 1995). In the human body, lithium can be absorbed from the gastrointestinal tract (Schrauzer, 2002) and excreted primarily through kidneys 24 h later (Freeman and Freeman, 2006). The dietary intake is recommended as 1 mg/day for an adult of 70 kg (Schrauzer, 2002). It is found that the 50% effective concentration ($EC_{50}$) is 33–197 mg/L in *Daphnia magna* (US EPA, 2008). In humans, ingestion of 5 g LiCl results in fatal toxicity (Aral and Vecchio-Sadus, 2008). In addition, lithium affects the physiologic functions and inhibits the functions of multiple enzymes (Moore 1995; Phiel and Klein, 2001). The central nervous system is the primary target organ for lithium toxicity. The renal toxicological symptoms could be induced by lithium (Chmielnicka and Nasiadek, 2003), and the congenital defects to the cardiovascular system were observed in the pregnancy women (Birch, 1988; Ferner and Smith, 1991, 1992).

For living in the water environment, fish are constantly exposed to various pathogens and stress factors, which easily induce inflammatory responses and tissue injuries (Saurabh and Sahoo, 2008; Troncoso et al., 2012). Fish immunity is tightly associated with the antioxidant status of tissues (Tort et al., 2003; Kuang et al., 2012). Reactive oxygen species (ROS), mainly including hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^{\cdot-}$), and hydroxy radical ($\cdot OH$), are products of normal metabolism (Sugiyama, 1994; Qu et al., 2014a). However, under the oxidative stress states, lipid peroxidation and protein oxidation can be induced by ROS, which causes structural and functional dysfunction of fish organs (Cooke et al., 2003; Martinez-Alvarez et al., 2005; Maynard et al., 2009). The antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), and peroxidase (POD), participate in defensing against ROS-mediated cellular injury in fish species (Martinez-Alvarez et al., 2005; Qu et al., 2014b; Wang et al., 2015).

The target of rapamycin (TOR) signaling pathway participates in regulating numerous physiological functions (Inoki et al., 2005b), and TOR has been demonstrated to play a role in the response to environmental stresses (Patel and Tamanoi, 2006). The tuberous sclerosis complex 2 (TSC2) can negatively regulate TOR signaling and inhibit cell growth (Tapon et al., 2001; Tee et al., 2003; Inoki et al., 2002, 2006). Decreasing TOR activity results in the increase of lifespan and the age-related neurodegenerative diseases (Kapahi et al., 2004; Inoki et al., 2005a; Powers et al., 2006).

As a serine/threonine protein kinase, glycogen synthase kinase-3β (GSK-3β) is involved in numerous biological processes, including cell differentiation, cell cycle, and apoptosis (Doble and Woodgett, 2003; Luo, 2009). In the intracellular signaling systems, GSK-3β is also a fascinating enzyme and plays a significant role in regulating TSC2/TOR signaling (Buller et al., 2008). Moreover, GSK-3β is an attractive target for inhibiting TOR signaling, and the activity of GSK-3β can be inhibited by lithium (Terstappen et al., 2006). By targeting GSK-3β, lithium induces apoptosis, cell growth arrest, and terminal differentiation (Fu et al., 2010). However, there is little information on the regulation mechanism whether lithium induces oxidative stress by inhibiting GSK-3β in fish species. In this study, the effect of lithium on ROS generation through GSK-3β/TSC2/TOR signaling was to be investigated in the gill of zebrafish (*Danio rerio*).

## 2. Materials and methods

### 2.1. Animals and experimental conditions

*Danio rerio* were obtained from Zibo (Shandong, China) and transported to Shandong University of China (Zibo, China). Forty-five fish (0.30 ± 0.05 g) were randomly distributed into three 3.0 L glass tanks. Animals were acclimated for 15 days and fed twice daily with a commercial diet with no lithium (Sanyou Beautification Feed Tech Co., Ltd, China). The tanks were maintained with a natural photoperiod and the continuous aeration. The water temperature was 29.0 ± 1 °C, dissolved oxygen was 6–8 mg/L, and the ammonia-nitrogen and nitrite was 0.07–0.1 mg/L. All animal procedures were approved by Shandong University of China's Institutional Animal Care Committee in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of China).

### 2.2. Animals treated by lithium

*Danio rerio* in three tanks were used for Li treatments. The concentration of Li was chosen according to the concentrations of lithium in groundwater and surface water (Zaldivar, 1989; Weiner, 1991; Schrauzer, 2002) as well as the preliminary experiment. The different concentration of LiCl was added and the final concentration of $Li^+$ was 0, 25, and 50 mg/L in three glass tanks, respectively. The concentration of $Li^+$ was detected with a Dionex ICS-2000 ion chromatograph. The exposure system was semi-static and half of the water was replaced every 2 days. Animals were fed twice daily with the commercial diet with no lithium. Two weeks later, the gills of twelve fish were sampled after fish were anesthetized with MS222 (0.1 g/L). Six gill samples were flash-frozen in liquid nitrogen and stored at −80 °C for molecular biology analysis. The other six gill samples were homogenized in cold saline using a glass homogenizer. The homogenates were centrifuged (Eppendorf, Germany) at 4000 × g for 15 min at 4 °C. The supernatants were collected for the biochemical analysis.

### 2.3. GSK-3β RNA interference

Animals and experimental conditions were the same as the Li treatment, and thirty fish (0.3 ± 0.05 g) were randomly distributed into two 3.0 L glass tanks. The open reading frame (ORF) of GSK-3β (NM_131381.1) was cloned and the double-T7-stranded RNA was further synthesized according to the primers listed in Table 1 according to the method of Arockiaraj et al. (2014). Reactions were performed in water at 37 °C in a solution containing 100 mM DTT, 5 × transcription buffer, RNase Inhibitor, 2.5 mM rNTP, T7 RNA polymerase (Takara) and the template. For the experimental group, the double-stranded RNA (dsRNA) was diluted to 8 ng/μL, and one fish received intraperitoneal injection of 25 μL dsRNA (200 ng/per fish). The fish of control group received 25 μL the diethylpyrocarbonate (DEPC) treated water. Three days later, the gills were sampled for the subsequent biochemical analysis and molecular biology analysis, which was the same as Li treatments.

**Table 1**
PCR primers for GSK-3β RNA interference.

| PCR Primers | Forward (5′-3′) | Reverse (5′-3′) |
|---|---|---|
| ORF | CTGGTGAGCAGTAGGGTG | CGGATTCGTTCAAGACAA |
| dsRNA | GATCACTAATACGACTCACTATAGGGCGGCATTCGGCAGCATGAAAG | GATCACTAATACGACTCACTATAGGGGCACGGCTGTGTCTGGGTCCA |