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# GSK-3β participates in the regulation of hepatic lipid deposition in large yellow croaker (*Larmichthys crocea*)

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**Abstract** In this study, the participation of glycogen synthase kinase-3β (GSK-3β) in the lipid deposition was investigated in the liver of large yellow croaker (*Larmichthys crocea*) by LiCl treatment. It was found that the expression of GSK-3β and peroxisome proliferator-activated receptor-γ (PPARγ) was inhibited, but the expression of β-catenin was induced by LiCl treatment. Furthermore, the gene expression and activity of fatty acid synthetase (FAS) and lipoprotein lipase (LPL) in the liver was inhibited by LiCl treatment. The content of total cholesterol (TC), triglyceride (TG), and non-estesterified fatty acid in the liver, as well as TC, TG, and low-density lipoprotein cholesterol in plasma, was decreased by LiCl treatment. However, high-density lipoprotein cholesterol in plasma was increased, and the number of lipid droplets in the liver was decreased by LiCl treatment. The results indicate that GSK-3β/β-catenin may participate in regulating LPL and FAS through PPARγ in the liver of large yellow croaker, which will lead to the inhibition of hepatic lipid deposition.

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## Introduction

The partial or total replacement of fish oil by vegetable oils is widespread in fish feed production. However, the vegetable ingredients and high level of lipids in fish diets easily induce high lipid deposition in the hepatic adipose tissue (Caballero et al. 2002, 2004). Excessive accumulation of lipid will cause production losses, flesh quality alteration, and lipoid liver disease (Seierstad et al. 2005, 2009; Benedito-Palos et al. 2008; Saera-Vila et al. 2009). In recent years, the regulation of lipid metabolism has become a notable point of interest in aquaculture research (Leaver et al. 2008; Zhang et al. 2014).

Fish is a potential model for studying lipid metabolism because it has relatively high lipid accumulation in the tissues of liver and muscle. In fish, both the liver and muscle are also main sites of lipid synthesis and storage (Robinson and Mead 1973; Sheridan 1988). Hepatic lipid deposition is related to the balance of fatty acid oxidation, synthesis, and transport. It is known that fatty acid synthetase (FAS) and lipoprotein lipase (LPL) participate in the regulation of hepatic lipid deposition (Richard et al. 2006; Nilsson-Ehle et al. 1980). The transcriptional factor peroxisome

proliferator-activated receptor-γ (PPARγ) also participates in hepatic lipid deposition by orchestrating the gene transcription of the enzymes involved in lipid metabolism (Spiegelman and Flier 2001).

In mammals, the canonical Wnt/β-catenin pathway has negative effects on adipogenesis (Bennett et al. 2002; Prestwich and Macdougald 2007). This signaling pathway is controlled by intracellular β-catenin, which is a multifunctional protein involved in cell adhesion and transcriptional regulation (Ross et al. 2000). In this signaling pathway, glycogen synthase kinase-3β (GSK-3β) is an attractive target for inhibiting β-catenin signaling. If there are no Wnt signals, β-catenin is phosphorylated by GSK-3β and leads to ubiquitin-mediated degradation (Aberle et al. 1997; Huelsken and Behrens 2002). However, Wnt ligands could bind to a receptor complex and cause the inactivation of GSK-3β and accumulation of cytosolic β-catenin. Consequently, cytoplasmic β-catenin translocates into the nucleus and induces target gene expression (Huelsken and Behrens 2002). The transcriptional factors such as PPAR isotypes are necessary for the transition of preadipocytes into adipocytes (MacDougald and Lane 1995). In 3T3-L1 preadipocytes, β-catenin inhibits adipocyte differentiation by inhibiting the expression of the master adipogenic transcription factor PPARγ (Ross et al. 2000; Huelsken and Behrens 2002; Moldes et al. 2003). In fish species, PPARα, PPARβ, and PPARγ have been found in the Atlantic salmon (*Salmo salar*) (Ruyter et al. 1997), European sea bass (*Dicentrarchus labrax*) (Boukouvala et al. 2004), and gilthead sea bream (*Sparus aurata L*) (Leaver et al. 2005; Diez et al. 2007). However, to our knowledge, there are no reports on lipid metabolism regulated by GSK-3β/β-catenin signaling in fish species.

For the function of GSK-3β in fish hepatic lipid deposition remains unknown, it is essential to investigate whether GSK-3β/β-catenin signaling is involved in lipogenesis. The results will be helpful to explain the mechanism of lipid deposition in fish liver. GSK-3β is an attractive target for inhibiting β-catenin signaling, and lithium could inhibit the activity of GSK-3β kinase, stabilize β-catenin, and thereby cause the activation of β-catenin signaling (Terstappen et al. 2006). The aim of this study was to investigate the effect of GSK-3β on hepatic lipid deposition in large yellow croaker (*Larmichthys crocea*) by LiCl treatment.

## Materials and methods

### Animals and experimental conditions

All animal procedures were approved by Ocean University of China's Institutional Animal Care Committee in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of China). Large yellow croakers were obtained from a commercial farm (Ningbo, China) and transported to the indoor facilities in the experimental station. At the beginning of experiment, 90 fish (average body weight 50.56 ± 0.25 g) were randomly distributed into six 400-L circular fiberglass flat-bottom tanks (three tanks for each treatment). During this period, animals were fed ad libitum by hand once daily with a commercial diet (Qihao Biotech Co. Shandong, China) and acclimated for 15 days before experiments. All rearing tanks were maintained with continuous aeration and a natural photoperiod. The temperature of water ranged from 17.5 to 18.5 °C, and salinity ranged from 30 to 32 ‰.

### Injection experiment

Animals were injected intraperitoneally with 50.0 mg/ml LiCl (according to 0.1 mg/g body mass) three times a week. LiCl was purchased from Sigma (St. Louis, MO) and dissolved in 0.7 % sodium chloride. The animals injected with 0.7 % sodium chloride were treated as the control. Three tanks were randomly used for LiCl treatment and control group, respectively.

### Sample collection

Five fish from each tank were randomly sampled and immediately anesthetized a week later. The liver samples were immediately frozen in liquid nitrogen and then stored at −80 °C until analysis. Blood samples of another three fish were taken from the caudal vein with heparinized syringes to obtain plasma after centrifugation for 10 min at 1400×*g*.

### Biochemical analysis

Liver samples in each tank were homogenized in ice-cold 0.7 % sodium chloride. Following centrifugation at 1400×*g* for 10 min, the supernatants were collected and maintained at −80 °C. The activity of LPL and

FAS as well as the protein concentration of liver supernatants was measured according to the analytical procedures specified in the commercially available kits (Kits for LPL activity and protein concentration were purchased from Nanjing Jiancheng Bioengineering Institute, China; Kit for FAS was purchased from Beijing Solarbio Technology Co., LTD, China).

The content of non-estesterified fatty acid (NEFA) in liver was measured according to the procedures of the commercially available kits (Nanjing Jiancheng Bioengineering Institute, China). In addition, the content of total cholesterol (TC) and triglyceride (TG) in liver, as well as the content of TC, TG, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) in plasma, was measured with BS180 Auto detector (BS-400, Mindray, China) using the matching commercial reagents and kits (Mindray Bio Medical Co., Ltd., China).

### RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was extracted from tissues using Trizol reagent (Invitrogen, USA) and electrophoresed on a 1.2 % denaturing agarose gel to test the integrity. Then, 3.0 μg total RNA was subjected to reverse transcription with EasyScript First-Strand cDNA Synthesis SuperMix kit (TransGen, Beijing, China), and SYBR® Premix Ex Taq™ II was used to quantify genes' expression (TransGen, Beijing, China). The primer sequences for reference gene (β-actin), LPL, FAS, PPARγ, GSK-3β, and β-catenin were designed following the published sequences and are listed in Table 1. Real-time PCR was carried out in a quantitative thermal cycle (Mastercycler® ep realplex; Eppendorf, Germany). The real-time PCR program was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. The reaction was carried out with three duplicates for each sample. Gene expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001).

### Western blot analysis

Protein samples of liver were prepared, and Western blot analysis was performed according to the method of Dai et al. (2013). Nitrocellulose membranes were incubated with rabbit polyclonal antibody anti-β-catenin (1:500; Santa-Cruz Biotechnology), rabbit polyclonal antibody anti-p-β-catenin (Ser33) (1:500; Santa-Cruz Biotechnology), goat polyclonal antibody anti-GSK-3β (1:500; Santa-Cruz Biotechnology), goat polyclonal antibody anti-p-GSK-3β (Ser9) (1:500; Santa-Cruz Biotechnology), goat polyclonal antibody anti-PPARγ (1:500; Santa-Cruz Biotechnology), and goat polyclonal antibody β-actin (1:1000; Santa-Cruz Biotechnology), respectively. Then, HRP-conjugated secondary antibody (1:3000; Santa-Cruz Biotechnology) was incubated and visualized by chemiluminescence using an echochemiluminescence (ECL) detection kit (Nanjing Jiancheng Bioengineering Institute, China). Protein bands were visualized with ECL detection system, and the expression levels of these proteins were evaluated using ImageJ software (NIH Image, Bethesda, MD, USA).

### Immunohistochemistry

The paraffin sections (5.0 μm thick) of liver were rehydrated in sodium phosphate buffer (+0.3 % Triton X-100, PBST). To reduce endogenous peroxidase activity, the sections were pretreated in 1 % $H_2O_2$ for 10 min. Then, the sections were incubated with goat serum for 15 min and goat polyclonal antibody anti-p-GSK-3β (Ser9) (diluted 1:500 in Tris–HCl and bovine albumen) for 4 h at room temperature. Rinsed with PBST, the specimens were incubated with rabbit anti-goat IgG conjugated to horseradish peroxidase for

**Table 1** Real-time quantitative PCR primers for lipid deposition genes and β-actin of large yellow croaker

| Target gene | Forward (5′–3′) | Reverse (5′–3′) | Size (bp) | GenBank |
|---|---|---|---|---|
| GSK-3β | ACACTACAGCCGAGCCAAAC | AGATCCCAAACGAGTGGATG | 101 | KT865607 |
| β-catenin | GAGGGTGTTCGTATGGAGGA | GCAACTGGACAAAGAGTGGA | 124 | KT865608 |
| PPARγ | CAGTGGCAGCGTTAAACATCG | GAAGAAACCCTTACAGCCCTCA | 98 | Wang et al.(2012) |
| LPL | GCGGAAACACAGACCTTCAT | AGTCGGCACACGCTCATAG | 105 | JQ327827 |
| FAS | AGGTCATCCTTGCTGCCTATT | AGACACTCCTCCCAGGTCAA | 100 | JX456351 |
| β-actin | CTACGAGGGTTATGCCCTGCC | TGAAGGAGTAACCGCGCTCTGT | 107 | GU584189 |

50 min at room temperature. Then, the slides were treated according to the procedures described by reagents kit of ABC staining system. A negative control was provided by incubation with PBST instead of primary antibody. Finally, the slides were dyed with hematoxylin and analyzed under light microscope.

### Oil red O staining

The liver was fixed for 24 h in a solution of 4 % paraformaldehyde in 0.1 mol/L sodium phosphate buffer (PBS, pH 7.4) at 4 °C. The liver was sectioned at a thickness of 20 μm in a cryostat (Leica CM1850, Germany) and mounted on glass slides. Then, the sections were stained with Oil red O kit following standard procedure (Nanjing Jiancheng Bioengineering Institute, China). Finally, the slides were re-dyed with hematoxylin and analyzed under light microscope. Three images were randomly selected to count the number of lipid droplets.

### Statistical analysis

Data are presented as mean of three tank replicates and reported as mean values ± standard error (SE). Statistical analysis was performed by Student's *t* test, and the difference was considered significant at the level of $P < 0.05$. The statistical analyses were performed using SPSS 16.0 (SPSS Inc., 2005, USA).

## Results

### Effect of LiCl on the gene expression of GSK-3β, β-catenin, and PPARγ

The results indicated that LiCl treatment significantly inhibited the mRNA expression of GSK-3β in the liver of large yellow croaker (Fig. 1a). However, the mRNA expression of β-catenin was significantly induced by LiCl treatment (Fig. 1b). Furthermore, compared to the control, the mRNA expression of PPARγ was significantly inhibited by LiCl treatment (Fig. 1c).

### Effect of LiCl on the protein expression of p-GSK-3β, p-β-catenin, and PPARγ

Compared to the control, LiCl treatment significantly induced the level of p-GSK-3β (Ser9) in the liver of large yellow croaker (Fig. 2a). Moreover, the result of immunohistochemistry also indicated that there were more GSK-3β proteins phosphorylated by LiCl treatment (Fig. 3b). Nevertheless, LiCl treatment significantly inhibited the protein expression of p-β-catenin (Ser33) as well as the expression of PPARγ (Fig. 2b, c).

### Effect of LiCl on the gene expression and activity of LPL and FAS

Compared to the control, LiCl treatment significantly inhibited the mRNA expression of LPL and FAS in the liver of large yellow croaker (Fig. 4a, b). Accordingly, the activity of LPL and FAS was significantly inhibited by LiCl treatment (Fig. 4c, d).

### Effect of LiCl on the liver and blood lipid index

The results indicated that LiCl treatment significantly decreased the content of TC, TG, and NEFA in the liver of large yellow croaker (Fig. 5a, b, c). In addition, the content of TC, TG, and LDL-C was significantly decreased by LiCl treatment in the plasma of large yellow croaker (Fig. 6a, b, d). However, the content of plasma HDL-C was significantly increased by LiCl treatment (Fig. 6c).

### Effect of LiCl on lipid droplets

The results of Oil red O staining indicated that there were more lipid droplets in the liver of control group (Fig. 7a). Nevertheless, fewer lipid droplets were observed in the group of LiCl treatment (Fig. 7b). The result of statistical analysis showed that the number of lipid droplets was significantly decreased by LiCl treatment in the liver of large yellow croaker (Fig. 7c).

## Discussion

In this study, the mRNA expression of GSK-3β was inhibited, but the mRNA expression of β-catenin was induced by LiCl treatment in the liver of large yellow croaker. Moreover, it was found that the level of p-GSK-3β was induced by LiCl treatment. For the activity of GSK-3β was negatively related to the level of p-GSK-3β, GSK-3β activity would be inhibited. However, the protein expression of p-β-catenin was

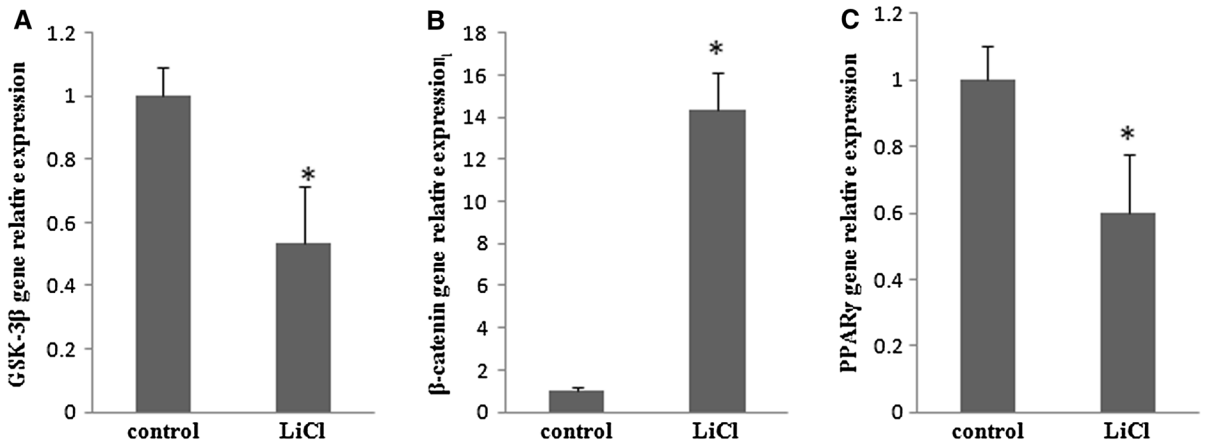


**Fig. 1** Effect of LiCl on mRNA relative expression of GSK-3β (**a**), β-catenin (**b**), and PPARγ (**c**) in the liver of large yellow croaker. Statistical significance by Student's *t* test is indicated by *asterisk* ($P < 0.05$, $n = 3$). *Bars* are mean ± SE

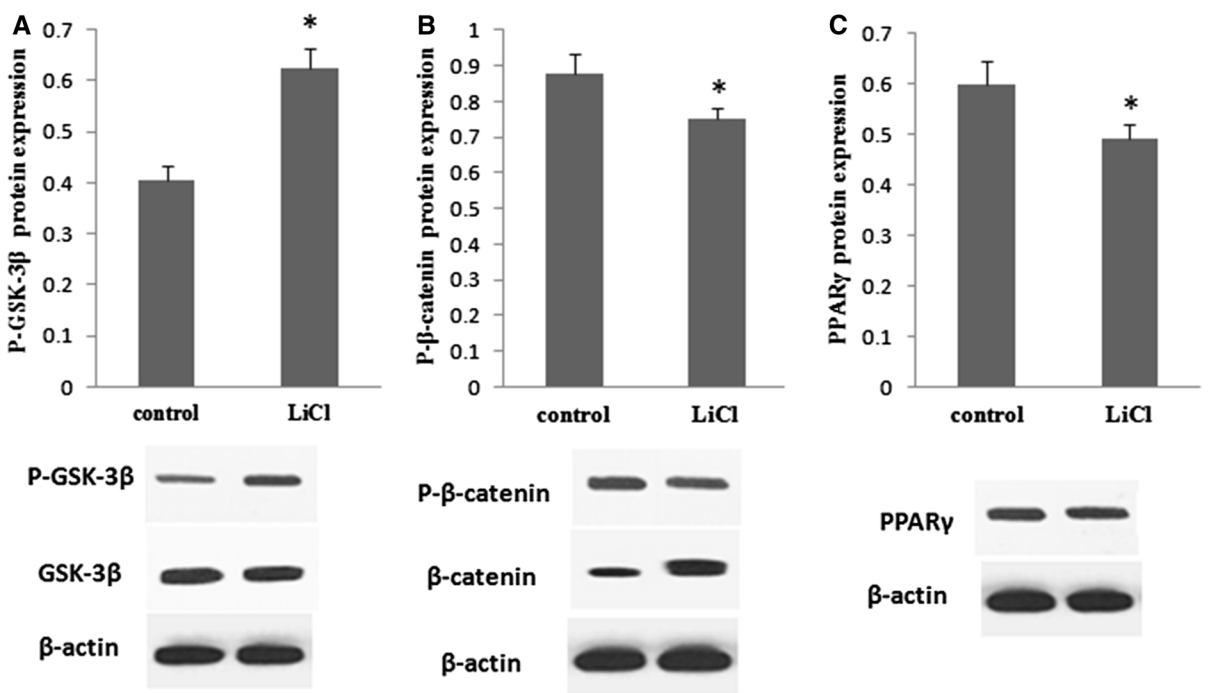


**Fig. 2** Effect of LiCl on protein expression of p-GSK-3β (**a**), p-β-catenin (**b**), and PPARγ (**c**) in the liver of large yellow croaker. Statistical significance by Student's *t* test is indicated by *asterisk* ($P < 0.05$, $n = 3$). *Bars* are mean ± SE

inhibited by LiCl treatment, which would inhibit β-catenin degradation and lead to the cytoplasmic accumulation of β-catenin. These observations suggest that, as in mammals (Terstappen et al. 2006), LiCl is an effective activator of β-catenin via GSK-3β inhibition in large yellow croaker. The defining event in canonical Wnt signaling is the cytoplasmic accumulation of β-catenin. In this process, GSK-3β, an attractive target for inhibiting β-catenin signaling, plays a significant role in regulating β-catenin accumulation (Aberle et al. 1997; Huelsken and Behrens 2002). The results of this study show that GSK-3β inhibition activates β-catenin expression in the liver of large yellow croaker by LiCl treatment.

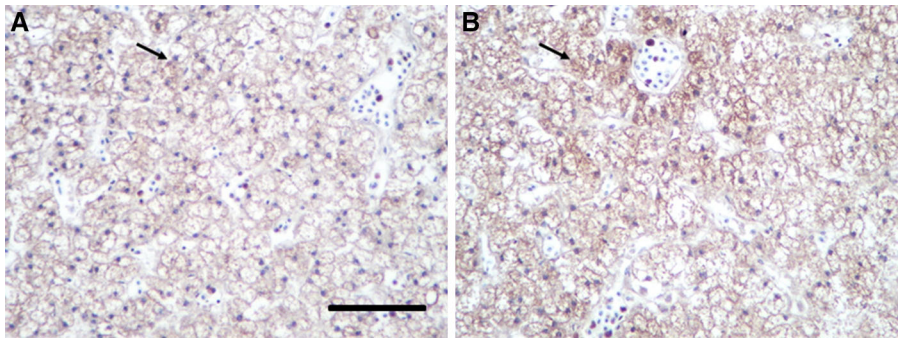


**Fig. 3** Immunohistochemical localization of p-GSK-3β in the liver of large yellow croaker. **a** control; **b** LiCl treatment. *Arrow* indicates p-GSK-3β immunoreactivity. *Bar* 45 μm

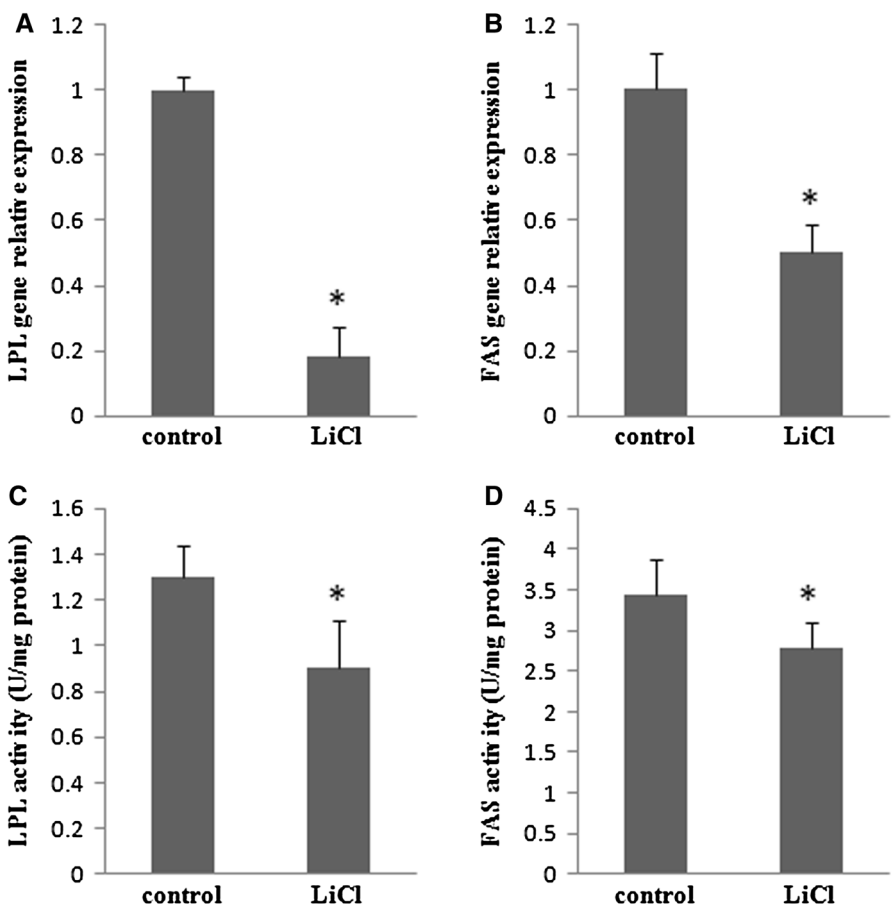


**Fig. 4** Effect of LiCl on the LPL mRNA relative expression (**a**), FAS mRNA relative expression (**b**), LPL activity (**c**), and FAS activity (**d**) in the liver of large yellow croaker. Statistical significance by Student's *t* test is indicated by *asterisk* ($P < 0.05$, $n = 3$). *Bars* are mean ± SE

The differentiation of preadipocytes is important for adipocyte function, and sequential activation of various transcription factors is involved in this process (Rosen and MacDougald 2006; Farmer 2006; Rosen and Spiegelman 2000). As a canonical Wnt signaling molecule, Wnt10b specifically activates canonical Wnt/β-catenin signaling and triggers β-catenin/LEF/TCF-mediated transcriptional programs (Barbolina et al. 2011; Wend et al. 2012). Moreover, β-catenin maintains preadipocytes in an undifferentiated state and blocks adipocyte differentiation (Lefterova and Lazar, 2009; Christodoulides et al. 2006; Ahn et al.

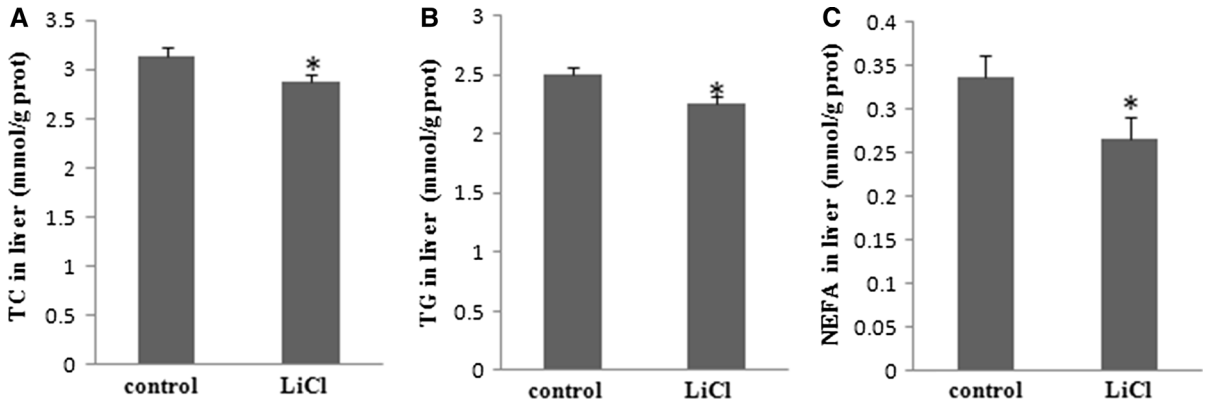


**Fig. 5** Effect of LiCl on TC (**a**), TG (**b**), and NEFA (**c**) in the liver of large yellow croaker. Statistical significance by Student's *t* test is indicated by *asterisk* ($P < 0.05$, $n = 3$). *Bars* are mean ± SE

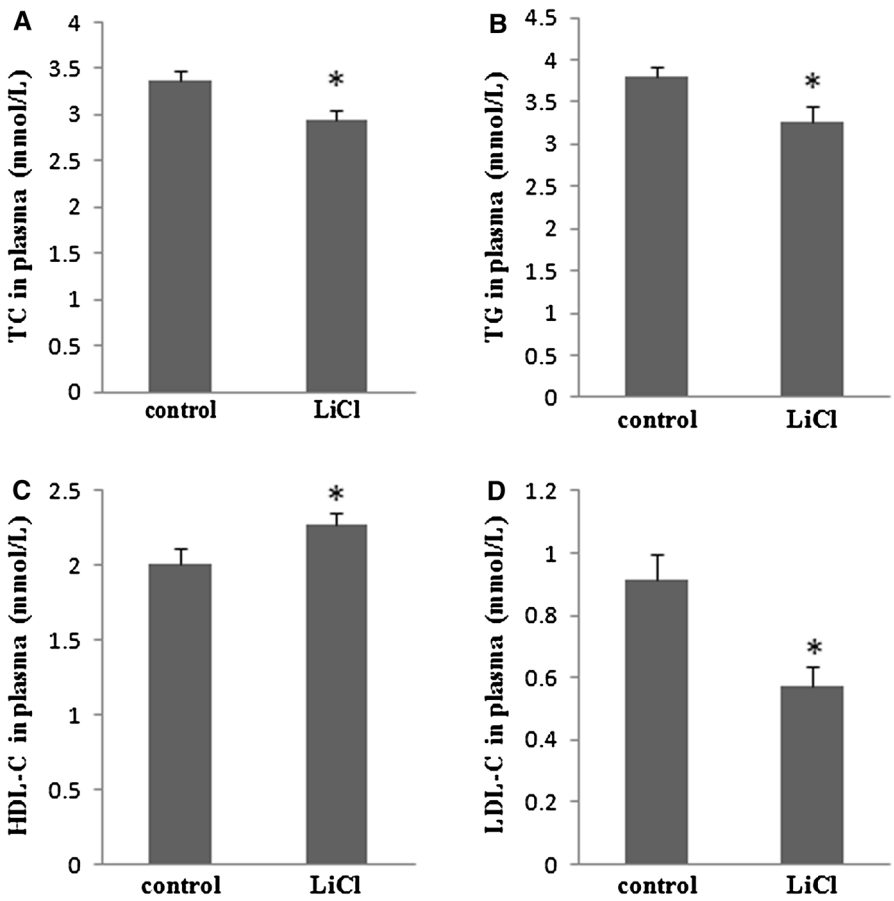


**Fig. 6** Effect of LiCl on TC (**a**), TG (**b**), HDL-C (**c**), and LDL-C (**d**) in the plasma of large yellow croaker. Statistical significance by Student's *t* test is indicated by *asterisk* ($P < 0.05$, $n = 3$). *Bars* are mean ± SE

2010). In mammals, β-catenin blocks the induction of master adipogenic transcription factor PPARγ (MacDougald and Mandrup 2002; Kawai et al. 2007). In this study, it was found that LiCl treatment also inhibited the expression of PPARγ. Since lithium is an effective activator of the canonical GSK-3β/β-catenin signaling (Terstappen et al. 2006), β-catenin may

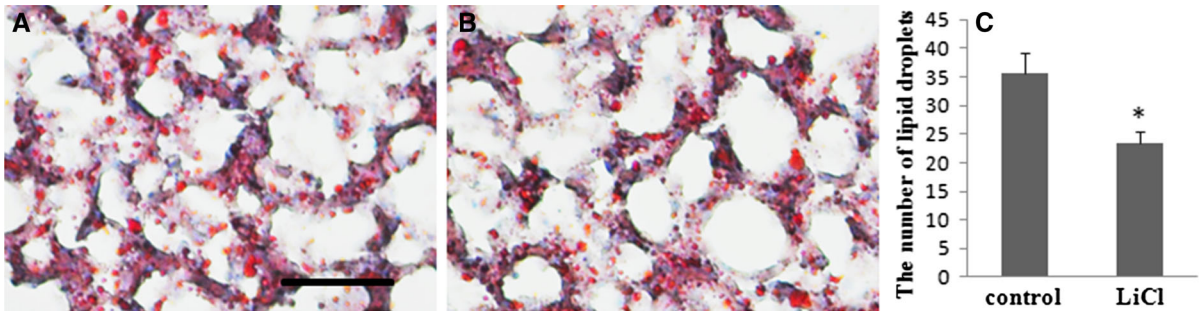


**Fig. 7** Effect of LiCl on lipid droplets in the liver of large yellow croaker. **a** control; **b** LiCl treatment; **c** lipid droplet number. Statistical significance by Student's *t* test is indicated by *asterisk* ($P < 0.05$, $n = 3$). *Bars* are mean ± SE. *Bar* 45 μm

participate in the process of inhibiting PPARγ in the liver of large yellow croaker.

In fish, liver is one of the main sites for lipid synthesis and storage (Robinson and Mead 1973; Sheridan 1988). Lipid accumulation results from the balance between the synthesis of fatty acids and fat catabolism. FAS is the main lipogenic enzyme producing fatty acids (Richard et al. 2006), and LPL hydrolyzes triacylglycerols in plasma lipoproteins and supplies free fatty acids for storage or oxidation in adipose tissue and other tissues (Nilsson-Ehle et al. 1980). The fatty acids released by LPL will be taken up by adipocytes and accumulated in the form of lipid droplet. As a key enzyme in lipid deposition, LPL gene in red sea bream (*Pagrus major*) and rainbow trout (*Oncorhynchus mykiss*) has been analyzed (Oku et al. 2002; Lindberg and Olivecrona 2002).

In the current study, LiCl treatment inhibited the gene expression and activity of LPL and FAS in the liver of large yellow croaker. The content of TC, TG, and NEFA in the liver, as well as TC, TG, and LDL-C in the plasma, was decreased, while the plasma HDL-C was increased by LiCl treatment. Furthermore, the number of lipid droplets was significantly decreased by LiCl treatment in the liver of large yellow croaker. The results indicate that the activity of LPL and FAS influences the liver and blood lipid indexes, which may lead to the decrease in the number of lipid droplets. It has been found that PPARγ participates in hepatic lipid deposition by orchestrating the gene transcription of the enzymes involved in lipid metabolism (Spiegelman and Flier 2001). Since LiCl treatment induced GSK-3β/β-catenin signaling and inhibited the expression of PPARγ, GSK-3β/β-catenin may participate in regulating LPL and FAS through PPARγ in the liver of large yellow croaker.

In conclusion, the mechanism that GSK-3β participates in the hepatic lipid deposition was investigated in the liver of large yellow croaker by LiCl treatment. It was found that the expression of GSK-3β and PPARγ was inhibited, but β-catenin expression was induced by LiCl treatment. Furthermore, the gene expression and activity of LPL and FAS was inhibited by LiCl treatment. The liver and blood lipid indexes as well as the number of lipid droplets were influenced by LiCl treatment in the liver. The results indicate that GSK-3β/β-catenin may participate in regulating LPL and FAS through PPARγ in the liver of large yellow croaker, which will lead to the inhibition of hepatic lipid deposition. However, the lipid deposition is a complex and interactional process, and the detailed mechanism on lipid deposition regulated by GSK-3β/β-catenin signaling needs to be researched in future.

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