



## Dietary overload lithium decreases the adipogenesis in abdominal adipose tissue of broiler chickens

Shiping Bai<sup>\*,1</sup>, Shuqin Pan<sup>1</sup>, Keying Zhang, Xuemei Ding, Jianping Wang, Qiufeng Zeng, Yue Xuan, Zuowei Su

Animal Nutrition Institute, Sichuan Agricultural University, Chengdu 611130, Sichuan, China

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### ABSTRACT

To investigate the toxic effects of dietary overload lithium on the adipogenesis in adipose tissue of chicken and the role of hypothalamic neuropeptide Y (NPY) in this process, one-day-old male chicks were fed with the basal diet added with 0 (control) or 100 mg lithium/kg diet from lithium chloride (overload lithium) for 35 days. Abdominal adipose tissue and hypothalamus were collected at day 6, 14, and 35. As a percentage of body weight, abdominal fat decreased ( $p < 0.001$ ) at day 6, 14, and 35, and feed intake and body weight gain decreased during day 7–14, and day 15–35 in overload lithium treated broilers as compared to control. Adipocyte diameter and DNA content in abdominal adipose tissue were significantly lower in overload-lithium treatment than control at day 35, although no significant differences were observed at day 6 and 14. Dietary overload lithium decreased ( $p < 0.01$ ) transcriptional expression of preadipocyte proliferation makers ki-67 (KI67), microtubule-associated protein homolog (TPX2), and topoisomerase 2- $\alpha$  (TOP2A), and preadipocyte differentiation transcriptional factors peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ), and CCAAT/enhancer binding protein (C/EBP)  $\alpha$  mRNA abundance in abdominal adipose tissue. In hypothalamus, dietary overload lithium influenced ( $p < 0.001$ ) NPY, and NPY receptor (NPYR) 6 mRNA abundance at day 6 and 14, but not at day 35. In conclusion, dietary overload lithium decreased the adipogenesis in abdominal adipose tissue of chicken, which was accompanied by depressing transcriptional expression of adipogenesis-associated factors. Hypothalamic NPY had a potential role in the adipogenesis in abdominal adipose tissue of broilers with a short-term overload lithium treatment.

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### 1. Introduction

Lithium has become widely used in the treatment of bipolar affective disorders in human (Belmaker et al., 1991). Epidemiological evidence indicated that lithium had a protective function in atherosclerotic heart disease (Correa and Strong, 1972). The potential protective function of lithium was related to its involving the neurotransmitter signaling (Belmaker et al., 1991; Chen et al., 1999), and/or lipids metabolism (Voors, 1969). Fleischman et al. (1974) reported that serum free fatty acids increased, whereas liver total lipids decreased in rats with increasing dietary lithium from 0 to 800 mg Li/kg. Recently, *in vitro* study indicated lithium stimulated lipolysis, and increased adipocyte specific gene peroxi-

some proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) mRNA abundance in mature adipocytes (Holowachuk, 2007). The preadipocytes conversion to adipocytes was inhibited by lithium treatment through dephosphorylation of CCAAT/enhancer binding proteins (C/EBP)  $\alpha$  (Ross et al., 1999). However, to date the effects of lithium on the adipogenesis in adipose tissue of broilers has not been reported.

The neuropeptide Y (NPY) was considered to be involved in the pathogenesis of affective disorders, and acute or chronic treatment with lithium had been shown to increase hippocampal NPY expression in rats (Weiner et al., 1992; Husum et al., 2000). The NPY was potently orexigenic, and promoted adipogenesis in chicken adipose-derived cells *in vitro* (Zhang et al., 2015; Shipp et al., 2016). Thus, we hypothesized that the NPY system may have a potential role in the toxic effects of lithium on the adipogenesis in broiler chicken.

The objective of this study was thus to evaluate the morphological, biochemical and molecular changes in adipose tissue of chickens after dietary overload lithium treatment, and to determine the potential role of hypothalamic NPY in this process.

\* Corresponding author at: Institute of Animal Nutrition, Sichuan Agricultural University, Huimin Road 211#, Chengdu 611130, Sichuan, China.

E-mail addresses: [shipingbai@sicau.edu.cn](mailto:shipingbai@sicau.edu.cn), [shipingbai@aliyun.com](mailto:shipingbai@aliyun.com) (S. Bai).

<sup>1</sup> These authors contributed equally to this work.

## 2. Materials and methods

### 2.1. Birds and experimental design

All animal protocols were approved by the Animal Health and Care Committee in Sichuan Agricultural University. A total of 3 batches of male local broilers (Daheng strain), obtained from a local hatchery, were used in this study with one batch per experiment. Birds (1-day-old) were randomly allotted into control and overload-lithium treatments with 6 replicate cages (100 × 100 × 60 cm) per treatment, and there were 15 birds per cage in Experiment 1 and 2, and 10 birds per cage in Experiment 3. Two dietary treatments included the basal diets added with 0 (control) or 100 mg Li/kg from lithium chloride (overload-lithium). Lithium chloride (LiCl) (purity: 99.9%) was purchased from Sigma-Aldrich (USA) and this reagent could be completely dissolved in water. Birds were housed in electrically heated and thermostatically controlled cages with *ad libitum* access to feed and water. The ambient temperature was gradually decreased from 32 °C on day 1 to 25 °C on day 14 by 0.5 °C per day, and was kept at 25 °C after 14 days of age. The basal diets (ME = 12.59 MJ/kg; crude protein = 21.50%; methionine = 0.50%; lysine = 1.10%; calcium = 1.00%; non-phytate phosphorus = 0.45%) were formulated to meet the requirements of chickens during the experimental period (35 days). The concentration of lithium was chosen based on previous studies in mice and poultry in which the dose was not lethal or toxic to the animals, but a treatment effect was observed (Scott et al., 1973; Clement-Lacroix et al., 2005). The control and overload lithium diets contained no detectable Li (0.008 mg/kg) and 97.66 mg Li/kg respectively by analysis using atomic absorption spectrometer (ContrAA 700, Analytik Jena Company, Germany).

### 2.2. Experiment 1: the performance and abdominal adipose tissue development

On day 6, 14, and 35, body weight and feed consumption for each replicate cage were measured. Body weight gain and feed intake per body weight gain (g:g) were calculated from day 1 to 6, 7 to 14, and 15 to 35 ( $n=6$ ). Two birds per replicate cage were randomly selected, weighted and euthanized for samples collection on day 6, 14 and 35. For the first bird per replicate, abdominal adipose tissue depot (attached to the gizzard) was collected and weighed. And then, the weight was converted into a percentage of the chick's body weight ( $n=6$ ). For another bird per replicate, about 5 mL blood was taken from the brachial vein after the 12-h feed withdrawal. Blood samples were collected in heparinized tubes, and then were centrifuged (3000g for 20 min at 4 °C) to collect plasma ( $n=6$ ). The concentrations of blood glucose, plasma triglycerides, and total cholesterol were analyzed using enzymatic determination method with commercial kits (Sigma-Aldrich Inc., China). Plasma lipoproteins including very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) were analyzed using a spectrophotometer (Multiskan<sup>®</sup> Spectrum, Thermo Fisher Scientific, Finland) with the colorimetric kits (Nanjing Jiancheng Biotechnology Co., China).

### 2.3. Experiment 2: DNA content, histology, and glycerol-3-phosphate dehydrogenase specific activity in abdominal adipose tissue

On day 6, 14, and 35, three birds per replicate cage were randomly selected and euthanized for samples collection. For the first bird per replicate, abdominal adipose tissue was collected, weighted, and then pre-mixed ( $n=6$ ). Genomic DNA was extracted from about 100 mg of pre-mixed adipose tissue using a TRIzol reagent (Invitrogen, USA). The DNA concentration was measured

by UV spectrophotometry (Nanodrop 2000, Thermo Scientific, USA) and the total DNA content in abdominal adipose tissue was calculated.

For the second bird per replicate, abdominal adipose tissue was collected, rinsed in phosphate-buffered saline, submerged in neutral-buffered formalin and incubated overnight at 4 °C ( $n=6$ ). Samples were then dehydrated in a graded ethanol series, paraffin embedded, sectioned at 5 μm, mounted and stained with hematoxylin and eosin (Sigma-Aldrich Inc., China). For each tissue, fat was sectioned at three locations, with three sections mounted per slide/location. Images were captured with a Nikon Eclipse 80i microscope and DS-Ri1 color camera, and images analyzed using NIS-Elements Advanced Research Software (Nikon, Japan). Three images were captured on each section, and the density and area of all adipocytes within the field of an image were measured under 20× magnification. The threshold method was used to count adipocytes (Bai et al., 2015). Adipocytes were treated as binary objects with the restriction that measurements must exceed 20 μm<sup>2</sup>. The mean area and equivalent diameter of each adipocyte and the total adipocyte numbers as well as the total area were recorded. Adipocyte density and the size distribution pattern for each image were also determined.

For the third bird per replicate, abdominal adipose tissue was collected and glycerol-3-phosphate dehydrogenase (G3PDH) activity was determined as described by Bai et al. (2015). Briefly, about 0.2 g of sample was transferred to a 10 mL tube containing 5-mL ice-cold lysis buffer (50 mM Tris-Cl, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 7.5) on ice. Tissues were homogenized on ice with a Polytron homogenizer for 30 s at 300 × rpm. This was repeated in 30 s pulses alternating with 30 s on ice twice. The lysates were then centrifuged at 12,000 × g at 4 °C for 30 min, and the supernatant used for measuring G3PDH activity and for determining total protein concentration. The G3PDH activity was measured for each sample in duplicate in assay buffer (100 mM triethanolamine-HCl, 2.5 mM EDTA, 0.12 mM NADH, 0.2 mM dihydroxyacetone phosphate (DHAP), 0.1 mM β-mercaptoethanol, pH 7.5) in a total reaction volume of 200 μL in UV transparent plates (Corning, USA) using a spectrophotometer (Thermo Scientific, USA). Absorbance was measured at 340 nm for 20 cycles at 25 °C and the maximum slope calculated from the absorbance data. Protein concentration was quantified with Bradford reagent (Sigma-Aldrich, USA) using an Infinite M200Pro multi-mode plate reader and Magellan software (Tecan, USA). The maximum slope was normalized to the protein concentration to calculate specific activity, expressed as μmol/min mg.

### 2.4. Experiment 3: genes expression analysis

On day 6, 14, and 35, one bird per replicate cage were randomly selected and euthanized for samples collection. The hypothalamus was collected as described by Huang et al. (2010), and abdominal adipose tissue was collected as described above. Collected tissues were snap-frozen in liquid nitrogen. The total RNA was extracted from frozen samples with the TRIzol reagent (Invitrogen, USA). The total RNA samples were evaluated for integrity by agarose-formaldehyde gel electrophoresis and concentration and purity assessed by spectrophotometry at 260/280/230 nm with a spectrophotometry (Nanodrop 2000, Thermo Scientific, USA). First-strand cDNA was synthesized from 200 ng total RNA with a SuperScript Reverse Transcript Kit (Invitrogen, USA). Reactions were performed under the following conditions: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min. Primers for qPCR (Table 1; reported in Bai et al. (2015) and Zhang et al. (2015)) were validated for amplification efficiency before use. Real-time PCR was performed in duplicate in 10 μL volume reactions that contained 5 μL SYBR Green PCR Mix (TaKaRa, China) and 3 μL of 10-fold

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