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Original Research Article

Double-edged effect of sodium citrate in Nile tilapia (*Oreochromis niloticus*): Promoting lipid and protein deposition vs. causing hyperglycemia and insulin resistance

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ABSTRACT

Citrate is an essential substrate for energy metabolism that plays critical roles in regulating glucose and lipid metabolic homeostasis. However, the action of citrate in regulating nutrient metabolism in fish remains poorly understood. Here, we investigated the effects of dietary sodium citrate on growth performance and systematic energy metabolism in juvenile Nile tilapia (*Oreochromis niloticus*). A total of 270 Nile tilapia (2.81 ± 0.01 g) were randomly divided into three groups (3 replicates per group, 30 fish per replicate) and fed with control diet (35% protein and 6% lipid), 2% and 4% sodium citrate diets, respectively, for 8 weeks. The results showed that sodium citrate exhibited no effect on growth performance ($P > 0.05$). The whole-body crude protein, serum triglyceride and hepatic glycogen contents were significantly increased in the 4% sodium citrate group ($P < 0.05$), but not in the 2% sodium citrate group ($P > 0.05$). The 4% sodium citrate treatment significantly increased the serum glucose and insulin levels at the end of feeding trial and also in the glucose tolerance test ($P < 0.05$). The 4% sodium citrate significantly enhanced the hepatic phosphofructokinase activity and inhibited the expression of pyruvate dehydrogenase kinase isozyme 2 and phosphor-pyruvate dehydrogenase E1 component subunit alpha proteins ($P < 0.05$). Additionally, the 4% sodium citrate significantly increased hepatic triglyceride and acetyl-CoA levels, while the expressions of carnitine palmitoyl transferase 1a protein were significantly down-regulated by the 4% sodium citrate ($P < 0.05$). Besides, the 4% sodium citrate induced crude protein deposition in muscle by activating mTOR signaling and inhibiting AMPK signaling ($P < 0.05$). Furthermore, the 4% sodium citrate significantly suppressed serum aspartate aminotransferase and alanine aminotransferase activities, along with the lowered expression of pro-inflammatory genes, such as *nfκb*, *tnfα* and *il8* ($P < 0.05$). Although the 4% sodium citrate significantly increased phosphor-nuclear factor-kB p65 protein expression ($P < 0.05$), no significant tissue damage or inflammation occurred. Taken together, dietary supplementation of sodium citrate could exhibit a double-edged effect in Nile tilapia, with the positive aspect in promoting nutrient deposition and the negative aspect in causing hyperglycemia and insulin resistance.

1. Introduction

Citrate is an important metabolic intermediate product of the tricarboxylic acid (TCA) cycle in mitochondria with two main sources: (1) in mitochondria, citrate can be produced by citrate synthase (CS) which catalyzes the synthesis of oxaloacetate and acetyl coenzyme A (AcCoA); (2) it is carried from the circulatory system into the cytoplasm via a sodium-dependent transport carrier (named SLC13A5) on the plasma membrane. Citrate is metabolized by two routes: (1) to generate α-ketoglutarate for the TCA

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cycle in the mitochondria by isocitrate dehydrogenase (IDH); or (2) to produce AcCoA in the cytoplasm by ATP-citrate lyase (ACLY). The latter step is the main source of cytoplasmic AcCoA for de novo lipogenesis (DNL), cholesterol and protein acetylation modifications (Infantino et al., 2019; Mosaoa et al., 2021). In addition to its well-known role as a metabolic intermediate, studies in mammals have also revealed that citrate in the cytoplasm is involved in several biological processes, such as glycolysis (Marinho-Carvalho et al., 2009; Sola-Penna et al., 2010), gluconeogenesis (Wang and Dong, 2019), inflammation, cancer, insulin secretion and histone acetylation (Iacobazzi and Infantino, 2014), suggesting that citrate can be involved in metabolic pathways in various manners.

In mammals, Branco et al. (2021) found that adding 40 mg/kg citrate to the normal diet resulted in glucose intolerance and insulin resistance in mice, along with liver lipid deposition and inflammatory damage. However, the addition of 1% citrate to the diet of mice alleviated a variety of metabolic disorders caused by high-fat diet, such as glucose intolerance, abnormal hepatic lipid deposition and inflammation (Fan et al., 2021). Similarly, the inclusion of citrate in a high-fat diet activated AMP-activated protein kinase (AMPK) signaling and inhibited mechanistic target of rapamycin (mTOR) signaling, resulting in a low-energy metabolic state that increased lifespan and egg production in *Drosophila* (Fan et al., 2021). Other studies in *Drosophila* have shown that mutating the cytosolic citrate transporter (*Indy*/*SLC13A5*) improved metabolic capacity and extended lifespan (Schwarz et al., 2015; Wang et al., 2009). These results suggest that the supplementation of exogenous citrate is important for healthy metabolism in organisms, which is of increasing interest in mammals.

Yet, few studies have been conducted on citric acid/sodium citrate effects in fish. Studies have shown that dietary citric acid supplementation significantly improved growth performance and intestinal digestibility, and enhanced the absorption and deposition of metal ions and phosphorus in fish (Khajepour and Hosseini, 2012a, 2012b; Li et al., 2009; Sarker et al., 2007). However, the results of studies on different fish species are contradictory. Studies on juvenile yellowfin seabream (*Acanthopagrus latus*) found that the addition of 0.5% sodium citrate improved growth performance and promoted protein and lipid deposition (Sotoudeh et al., 2020). A similar growth-promoting effect appeared on goldfish (*Carassius auratus*) with a dietary dose of 1% to 2% sodium citrate (Azari et al., 2021). Nevertheless, a study on hybrid tilapia (*Oreochromis* sp.) found that the supplementation of 2% to 4% sodium citrate reduced growth performance and nutrient deposition, along with liver and intestine damage (Romano et al., 2016), suggesting sodium citrate could be toxic in some fish species like tilapia. It is noteworthy that only a few studies have been designed to examine the effect of dietary citrate supplementation on metabolic processes of nutrients in fish, which largely hinders the understanding of sodium citrate function and its possible application in aquaculture. Therefore, more mechanistic investigations related to the metabolic regulation of sodium citrate on nutrients utilization, especially the potential toxic effects in fish are necessary.

In the present study, we chose Nile tilapia, a globally-farmed fish, as a fish model to investigate the precise effects of dietary sodium citrate on systemic nutrient metabolism. In order to avoid possible disturbance from dietary ingredients, we used gelatin and casein to prepare purified diets. During an 8-week feeding trial, the juveniles Nile tilapia were fed a control diet (Control, protein approximately 35%, lipid approximately 6%) and the control diet supplemented with 2% and 4% sodium citrate, respectively. After the trial, the growth performance, biochemical indicators and gene/protein expressions involved in energy metabolism were systemically analyzed. Our study could provide more in-depth information in understanding the roles of citrate in the regulation of nutrient metabolism in fish, and is helpful to precisely use citrate in aquafeeds as a potential feed additive.

2. Materials and methods

2.1. Animal ethics

All the experimental procedures were performed under the Guidance of the Care and Use of Laboratory Animals in China and approved by the Committee on the Ethics of Animal Experiments of East China Normal University (Approval ID: F20200101). All animal experiments complied with the ARRIVE guidelines.

2.2. Fish, diets, and sampling

Juvenile Nile tilapia were purchased from Huihai Aquaculture Company (Guangzhou, Guangdong, China). These fish were acclimated in 50 L tanks for 2 weeks before the start of the experiment and fed a commercial diet (≥35% total protein and ≥5% total lipids; Tongwei Co., Chengdu, China). Three isonitrogenous and isolipid experimental diets (35.56% total protein, and 6% total lipid) containing 0% (control group), 2% (2% sodium citrate group) and 4% sodium citrate (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) (4% sodium citrate group) were used, respectively. These sodium citrate doses were chosen according to the literatures in which 2% to 4% sodium citrate could change growth or metabolic progresses in goldfish or tilapia (Azari et al., 2021; Romano et al., 2016). Details of the experimental diets are shown in Table 1. Gelatin and casein were used as protein sources, and soybean oil and corn starch were used as lipid source and carbohydrate source, respectively. After acclimation, 270 Nile tilapia of similar mean weight (2.81 ± 0.01 g) were selected and randomly assigned to 9 tanks (0.3 m × 0.3 m × 0.3 m, 30 fish per tank, 3 tanks per diet). During the feeding period, the fish were hand-fed twice daily (08:30 and 17:00) at a feeding rate of 4% body weight. The amount of feed intake was recorded to determine feed conversion ratio ($n = 3$). All fish were weighed every two weeks and the daily feeding mass was adjusted accordingly. During the feeding period, water temperature was 27 to 29 °C, dissolved oxygen was 5.6 to 6.6 mg/L, pH was 7.4 to 7.9, while ammonia nitrogen was less than 0.02 mg/L.

After the 8-week feeding trial, all fish were fasted overnight, then counted and weighed to determine weight gain rate (3 tanks per treatment, $n = 3$). Then, six fish per group were anesthetized with MS-222 (20 mg/L) and sacrificed to collect tissue samples. Blood samples (pooled sample per tank, $n = 3$) were drawn from the tail vein of the fish using a 1-mL sterile syringe (Klmedical, China), and the serum was separated after centrifugation at 1200 × *g* at 4 °C for 15 min. The obtained serum samples were stored at −80 °C until analysis. The individual fish weight, body length, carcass, viscera, mesenteric fat and liver weight were measured to calculate condition factor, carcass ratio, viscerosomatic index, hepatosomatic index and mesenteric fat index ($n = 3$ for all indexes). Afterwards, liver and muscle samples were collected and immediately frozen in liquid nitrogen and stored at −80 °C for further analysis (pooled sample per tank, $n = 3$). Six fish per group were further collected for whole-body composition analysis (pooled sample per tank, $n = 3$).

2.3. Glucose tolerance test

Twenty-four Nile tilapia from each group were starved overnight, anesthetized with MS-222 (20 mg/L), and then injected intraperitoneally with D-glucose (500 mg/kg BW, 20% in 0.85% NaCl) (Sigma, USA). Blood from Nile tilapia was collected at 0, 0.5, 1

Table 1
Ingredients and nutritional composition of the experimental diets (% dry matter).

Item	Control	2% Sodium citrate	4% Sodium citrate
Ingredients			
Casein	36	36	36
Gelatin	9	9	9
Corn starch	30	30	30
Soybean oil	6	6	6
Vitamin premix[1]	0.4	0.4	0.4
Mineral premix[2]	0.5	0.5	0.5
Choline chloride	0.3	0.3	0.3
Butylated hydroxytoluene	0.02	0.02	0.02
Dimethyl-beta- propiothetin	0.05	0.05	0.05
Calcium dihydrogen phosphate	1	1	1
Cellulose	16.73	14.73	12.73
Sodium citrate	0	2	4
Proximate composition			
Total protein	35.56	36.2	35.7
Total lipid	6.2	5.9	6.1

[1] One kilogram of vitamin premix contained: vitamin A 150 mg, vitamin D_3 1.25 mg, vitamin E 2500 mg, vitamin K_3 1000 mg, vitamin B_1 5000 mg, vitamin B_2 5000 mg, vitamin B_6 5000 mg, vitamin B_{12} 5 mg, inositol 25,000 mg, pantothenic acid 10,000 mg, choline 100,000 mg, niacin 25,000 mg, folic acid 1000 mg, biotin 250 mg, vitamin C 10,000 mg.
[2] One kilogram of mineral premix contained: calcium carbonate 314,000 mg, potassium dihydrogen phosphate 469,300 mg, magnesium sulfate heptahydrate 147,400 mg, sodium chloride 49,800 mg, iron gluconate 10,900 mg, manganese sulfate monohydrate 3120 mg, zinc sulfate heptahydrate 4670 mg, copper sulfate pentahydrate 620 mg, potassium iodide 160 mg, cobalt chloride hexahydrate 80 mg, ammonium molybdate 60 mg, sodium selenite 20 mg.

and 3 h after injection as six fish per point, the serum was separated immediately and frozen until further analysis was required.

2.4. Biochemical parameters analyses

Experimental feeds and whole-body samples were dried at 105 °C until constant weight and then used for the determination of crude protein and crude lipid (Horwitz, 2010). Crude protein contents of feeds, whole-body and muscle samples were analyzed using a semi-automatic Kjeldahl system (FOSS, Sweden). Crude lipid contents of feeds, whole-body samples were determined by the chloroform/methanol (2:1, vol/vol) method (Folch et al., 1957).

Serum and hepatic triglyceride (Catalog number: A110-1-1, GPO-PAP method, 510 nm, detection limit: 0.3 to 11.4 mmol/L) and cholesterol (Catalog number: A111-1-1, COD-PAP method, 510 nm, detection limit: 0 to 10.34 mmol/L) concentrations were analyzed using commercial kits (Jiancheng Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Serum glucose (Catalog number: BC2505, GOD-POD method, 505 nm, detection limit: 1.4 to 28 mmol/mL) and insulin levels (Catalog number: HB407-QT, detection limit: 2.5 to 80 nmol/mL) of farming trials and glucose tolerance test were determined by using a glucose assay kit (Beijing Solarbio Science&Technology Co., Ltd., Beijing, China) and an Ultra Sensitive Fish Insulin ELISA Kit (Shanghai Hengyuan Biotech Co., Shanghai, China), respectively. Serum alanine aminotransferase (ALT) (Catalog number: C009-2-1, Reitman-Frankel method, 510 nm, detection limit: 0 to 72.3 U/L) and aspartate aminotransferase (AST) (Catalog number: C010-2-1, Reitman-Frankel method, 510 nm, detection limit: 0 to 72.3 U/L) activities were analyzed using commercial kits (Jiancheng Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Hepatic pyruvate, lactate and glycogen contents, and phosphofructokinase (PFK) activity were measured using commercial kits (Catalog number: A081-1-1, A019-2-1, A043-1-1, and A128-1-1, respectively. Jiancheng Biotech Co., Ltd., Nanjing, China). Briefly, Pyruvate interacts with 2,4-dinitrophenylhydrazine to produce pyruvate-2,4-dinitrophenylhydrazone, which is cherry red in alkaline solution (505 nm). Using NAD^+ as the hydrogen acceptor, lactate dehydrogenase catalyzes the dehydrogenation of lactate to produce pyruvate, which converts NAD^+ to NADH and produces a purple colorimetric product, the OD of which is linearly related to the lactate content at 530 nm. Glycogen can be dehydrated in the presence of concentrated sulfuric acid to form glyoxal derivatives, which in turn interact with anthrone to form blue compounds that are quantified colorimetrically with standard glucose solutions treated with the same method. PFK catalyzes the formation of fructose-1,6-bisphosphate and ADP from fructose-6-phosphate and ATP. Pyruvate kinase and lactate dehydrogenase further catalyze the oxidation of NADH to generate NAD^+ in turn, and the rate of NADH decrease is measured at 340 nm to react PFK activity.

2.5. Hepatic AcCoA concentration measurements

Determination of hepatic AcCoA concentration was conducted according to the existing method (Li et al., 2020). To minimize the possibility of baseline drift, an isocratic mobile phase at constant room temperature (25 °C) was used in this study. The mobile phase used consisted of 100 mmol/L monosodium phosphate and 75 mmol/L sodium acetate. The pH was adjusted to 4.6 by using concentrated phosphoric acid. Acetonitrile (ACN) was added to the prepared mobile phase at the ratio of 6 (ACN) to 94 (mobile phase) (vol/vol). The freshly prepared mobile phase was filtered and degassed before high-performance liquid chromatography (HPLC) analysis. The wavelength for UV detection was set to 259 nm. The column was kept at room temperature with a flow rate of 0.5 mL/min and an injection volume of 30 μL. Under these conditions, acetyl CoA eluted after 9.0 min.

2.6. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from the liver of Nile tilapia using Trizol reagent (Megen, China), and 1% denaturing agarose gel electrophoresis was used to verify the integrity of total RNA. The quality and quantity of total RNA were assessed by NanoDrop 2000 Spectrophotometer (Thermo, United States). The RNA with a 260/280 ratio of 1.9 to 2.0 was used to synthesize cDNA using HiScript III RT SuperMix for qPCR reagent Kit (R323-01, Vazyme, China) according to the manufacturer's instructions. The details of primer sequences used in the present study are provided in Table 2. The qRT-PCR reaction was performed in a 20-μL mixture containing 8 μL

Table 2
Primer nucleotide sequences of the genes used for qRT-PCR.

Gene		Primer sequences (5′ – 3′)	Accession no.
Lipid metabolism			
fasn	F	TCATCCAGCAGTTCACTGGCATT	GU433188
	R	TAGCTGAAGAGGAGGGTGCAAGA	
acly	F	AAAAGCTTTGATGAGCTTGGGG	XM_003442027
	R	TACAGTGGGAGGAGGCAACTCTT	
srebp1	F	TGCAGCAGAGAGACTGTATCCGA	XM_005471970
	R	ACTGCCCTGAATGTGTTCAGACA	
pparg	F	TGGACTACACAAACATGCACAGC	KF918712
	R	CACGGGACTATCTGAGTACTGTGGA	
hsl	F	AGTTCACTCCAGCCATTCGG	XM_005463937.4
	R	TGGCTGCTACCCCTATTCCT	
atgl	F	GACACATGCTGCAAAGCACT	XM_003440346.5
	R	ACCAGGACGTTTTCTCCGTC	
cpt1a	F	TTTCCAGGCCTCCTTACCCA	XM_013268638.3
	R	TTGTACTGCTCATTGTCCAGCAGA	
pparα	F	CTGATAAAGCTTCGGGCTTCCA	KF871430
	R	CGCTCACACTTATCATACTCCAGCT	
Glucose metabolism			
glut2	F	CATTGGCATTCTAATCAGCCAGGT	XM_003442884.5
	R	TTGTAATATTGCTGGCGCTCCA	
glut4	F	GCAGGAGGAAAGCCATGCTTATA	XM_003458705.4
	R	ATCATTTCAAAGGAGCGGCAGA	
gk	F	GACATGAGGACATTGACAAGGGAA	XM_003451020.2
	R	CTTGATGGCGTCTCTGAGTAAACC	
pehe1a1	F	AATCCACAGCCAGTCACT	XM_013264731.3
	R	GCAGAGTTCATCCAGACAA	
pdk2	F	GCAGAGTTCATCCAGACAA	XM_003448725.5
	R	GACCTGTAGTGCTTATCTGAT	
pdk4	F	AATCCACAGCCAGTCACT	XM_003457260.5
	R	GCAGAGTTCATCCAGACAA	
gs	F	CCTCACTCTGCGCTGTTATTC	XM_013276796.3
	R	CAGCGGCATGCCTTCAGTTT	
fbpase	F	ACCGGACAATAGCGGAAAATACA	XM_003449650.4
	R	TGGCGAATATTGTTCCTATGGAGA	
pepck	F	TGGAAGAACAAACCTTGGCG	XM_003448375
	R	TGGGTCAATAATGGGACACTGTCT	
g6pase	F	AGACCTTATTGGTGGGTTCACGA	XM_003448671.4
	R	CTGAAGGACTTCCTGGTCCAGTTT	
TCA cycle			
cs	F	AGCACCACAGTTTACCAG	XM_003438897
	R	AGTGTTGACAAACCCAGA	
idh	F	ACGCATCGCTGAGTACGCCTT	XM_003437590.5
	R	AGACCGTCTGACATCCGCATGA	
cicb	F	TCCATGGGGTGAGGGAGATT	XM_003452972.5
	R	ACATAGAAGCGGATGGCCTG	
Protein metabolism			
gls2	F	AGCTGGAAAGTCAACGACCT	XM_025902818.1
	R	GCTGCGACAAACGACACAAA	
bckdha	F	ACGCCATCTCTACCCCTACA	XM_003459112.5
	R	ACACGGATGGACAGCATACC	
gdh1	F	GGTTCCAGGACAAGACCTTCGT	XM_003457465.3
	R	TCTCCGACTCCGACGCACTT	
glns	F	AAGCTGGTGCTGTGTGAAGT	NM_001279668.1
	R	TCGACTACGTCCCTGCCATA	
asns	F	CAGAAGACTGACAGCGTGGTGA	XM_019355582.1
	R	GGTGTTGGAGCCTTGTGGAAGT	
Inflammation			
nfkb	F	CGACCACTACCTACACGCTC	XM_019363515.2
	R	GATGTCGTTTGAGGCATCGC	
tnfa	F	CAGAAGCACTAAAGGCGAAGAACA	NM_001279533
	R	TTCTAGATGGATGGCTGCCTTG	
il1b	F	GAGCACAGAATTCCAGGATGAAAG	JF957370
	R	TGAACTGAGGTGGTCCAGCTGT	
il8	F	CTGTGAAGGCATGGGTGTGGAG	NM_001279704.1
	R	TCGCAGTGGGAGTTGGGAAGAA	
tgfb	F	AAGAGGAGGAGGAATACTTTGCCA	XM_003459454
	R	GAAGCTCATTGAGATGACTTTGGG	
il10	F	CAGCAGCAGGAGCATCAGCATT	KP645180.1
	R	CACAGGAGGACGGTCTGAGAAGT	
ef1α	F	ATCAAGAAGATCGGCTACAACCCT	KJ123689.1

Table 2 (*continued*)

Gene		Primer sequences (5′ – 3′)	Accession no.
	R	ATCCCTTGAACCAGCTCATCTTGT	
β-actin	F	AGCCTTCCTTCCTTGGTATGGAAT	KJ126772.1
	R	TGTTGGCGTACAGGTCCTTACG	

fasn = fatty acid synthase; *acly* = ATP-citrate lyase; *srebp1* = sterol regulatory element-binding protein 1; *ppara/g* = peroxisome proliferator-activated receptor α/g; *hsl* = hormone-sensitive lipase; *atgl* = adipose triglyceride lipase; *cpt1a* = carnitine palmitoyltransferase 1a; *glut2/4* = glucose transporter 2/4; *gk* = glucokinase; *pdhe1a1* = pyruvate dehydrogenase E1 component subunit alpha; *pdk2/4* = pyruvate dehydrogenase kinase isozyme 2/4; *gs* = glycogen synthase; *fbpase* = fructose 1 = 6-bisphosphatase; *pepck* = phosphoenolpyruvate carboxykinase; *g6pase* = glucose 6-phosphatase; cs = citrate synthase; *idh* = isocitrate dehydrogenase; *cicb* = citrate carrier; *gls2* = glutaminase 2; *bckdha* = branched chain keto acid dehydrogenase E1 subunit alpha; *gdh1* = glutamate dehydrogenase 1; *glns* = glutamine synthetase; *asns* = asparagine synthetase; *nfκb* = nuclear factor kappa B; *tnfa* = tumor necrosis factor alpha; *il1b* = interleukin 1 beta; *il8* = interleukin 8; *tgfb* = transforming growth factor beta; *il10* = interleukin 10; *ef1α* = elongation factor 1α; F = forward primer; R = reverse primer.

diluted cDNA (with nuclease-free water), 10 μL 2× ChamQ Universal SYBR qPCR Master Mix (Q711-03, Vazyme, China) and 2 μL forward and reverse each gene-specific primer. The reaction conditions were as follows: 95 °C, 30 s for pre-deformation; 95 °C, 10 s followed by 60 °C, 30 s, 40 cycles for amplification reaction; and then, 95 °C, 15 s, 60 °C, 60 s, 95 °C, 15 s for melt curve. The melting curves of amplified products were generated to ensure the specificity of assays at the end of each qRT-PCR. The qRT-PCR efficiency was between 98% and 102% and the correlation coefficient was over 0.97 for each gene. Elongation factor 1 alpha (*ef1α*) and β-actin were used as the reference genes. Three replicate qRT-PCR analyses were performed per group. The relative expression levels of the target genes were calculated following the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001).

2.7. Western blotting

The liver and muscle homogenates after lysis (RIPA lysis buffer, Beyotime Biotechnology, China) were used for Western blot (WB) analysis. Briefly, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the proteins (P2012, New Cell & Molecular Biotech Co., Ltd, Jiangsu, China), and then electrophoresis was transferred to the nitrocellulose membrane. Membranes were incubated for 20 min in a quick blocking buffer (Beyotime Biotechnology, China). Then, membranes were washed briefly in a mixture of tris-buffered saline and polysorbate 20, and incubated overnight at 4 °C with antibodies (Table 3). Afterward, the blots were washed to remove excessive primary antibody binding and finally were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. The WB images were obtained using Odyssey CLx Imager (Licor, USA).

2.8. Calculations and statistical analysis

Weight gain ratio, condition factor, feed conversion ratio, carcass ratio, viscerosomatic index, hepatosomatic index and survival rate were calculated using the following formulas:

Weight gain ratio (%) = 100 × (final mean body weight − initial mean body weight)/initial mean body weight

Condition factor (g/cm^3) = final body weight/body length^3

Feed conversion ratio = dry feed intake (g)/(final body weight − initial body weight)

Carcass ratio (%) = 100 × carcass weight/body weight

Hepatosomatic index (%) = 100 × liver weight/body weight

Viscerosomatic index (%) = 100 × viscera weight/body weight

Mesenteric fat index (%) = 100 × mesenteric fat weight/body weight

Survival rate (%) = 100 × final fish number/initial fish number

Before conducting the analysis of sample differences, we have checked the normal distribution using D'Agostino & Pearson normality test, and the criterion for passing the normality test is a *P*-value > 0.05. The samples passed the normal distribution check and then one-way ANOVA and independent *t*-tests were conducted. Tukey's multiple comparisons were performed in the case of a significant overall difference between the experimental group and the control ($P < 0.05$). Independent-samples *t* test was performed to evaluate the significant differences among measured variables between treatments. Values are means ± SEM (one culture tank as one treatment, $n = 3$ for all indexes). Difference from group C: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Independent-samples *t* test). All data were analyzed using the GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Dietary sodium citrate had no effects on growth performance, but induced crude protein and hepatic glycogen deposition

At the end of the 8-week feeding trial, there were no significant differences in final body weight, weight gain ratio, feed intake, survival rate, condition factor, viscerosomatic index and carcass ratio of Nile tilapia among treatments (Table 4). Dietary supplementation of sodium citrate decreased the hepatosomatic index (HSI), and the 2% sodium citrate group had significantly lower HSI value than the control group (Table 4). Additionally, the 4% sodium citrate group significantly increased the mesenteric fat index, while there was no significant difference between the 2% sodium citrate group and the control group (Table 4). Compared to the control group, the supplementation of 4% sodium citrate also significantly increased crude protein content, serum triglyceride and liver glycogen content of tilapia (Table 4). To further investigate the mechanism of altered metabolic homeostasis by sodium citrate, only the 4% sodium citrate group and the control group were selected for the following assays.

Table 3
Antibodies used for Western blotting assay.

Antibodies	Source	Identifier
G6pc	Abclone	A16234
Acly	Abcam	ab40793
Srebp1	Proteintech	14088-1-AP
Cpt1a	Proteintech	15184-1-AP
p-Pdhe1a (Ser293)	Abways	CY7247
Pdk2	Abways	CY7193
t-Acc	Abways	CY5575
p-Acc	Abways	CY6008
t-Ampk	Abways	CY5326
p-Ampk (Thr172)	Cell Signaling Technology	#2535S
S6	Cell Signaling Technology	#2217S
p-S6 (Ser235/236)	Cell Signaling Technology	#4856S
t-mTor	Cell Signaling Technology	#2972S
p-mTor	Cell Signaling Technology	#2971S
p-NF-κB p65 (Ser536)	Affibiotech	AF2006
α-Tubulin	HuaBio	M1501-1

G6pc = glucose-6-phosphatase catalytic subunit; Acly = ATP-citrate lyase; Srebp1 = sterol regulatory element-binding protein 1; Cpt1a = carnitine palmitoyltransferase 1a; p-Pdhe1a = phosphor-pyruvate dehydrogenase E1 component subunit alpha; Pdk2 = pyruvate dehydrogenase kinase isozyme 2; t/p-Acc = total/phosphor-acetyl-CoA carboxylase 1; t/p-Ampk = total/phosphor-AMP-activated protein kinase; t/p-S6 = total/phosphor-ribosomal protein s6; t/p-mTor = total/phosphor-mammalian target of rapamycin; p-NF-κB p65 (Ser536) = phosphor-nuclear factor kappa B RelA.

3.2. Dietary sodium citrate activated glycolysis and gluconeogenesis while caused insulin resistance

The 4% sodium citrate-treated fish showed remarkably higher levels of serum glucose (Fig. 1A), however, serum insulin level was also significantly elevated by 4% sodium citrate treatment compared with control group (Fig. 1B). Besides, hepatic pyruvate contents of the 4% sodium citrate-treated fish were significantly higher than those of control (Fig. 1C), while 4% sodium citrate treatment reduced hepatic lactate content (Fig. 1D). Nevertheless, 4% sodium citrate supplementation markedly enhanced hepatic PFK activity compared to the control group (Fig. 1E). The glucose tolerance test results showed that the serum glucose in the 4% sodium citrate group was higher than that in the control group after glucose injection at all time points (Fig. 1F). The glucose tolerance test results also showed that serum insulin in the 4% sodium citrate group was significantly higher than that in the control group at all time points after glucose injection (Fig. 1G).

The gene and protein expression of glucose metabolism showed significant differences between control and 4% sodium citrate groups (Fig. 1H, I and J). The expression of the genes involved in glucose transport, including glucose transporter 2 (*glut2*) and 4 (*glut4*), were not changed between groups (Fig. 1I). Moreover, the expressions of the glycolysis-related genes, including glucokinase (*gk*) (+7.5 times), pyruvate dehydrogenase E1 component subunit alpha (*pdhe1a1*) (+1.6 times), pyruvate dehydrogenase kinase 2 (*pdk2*) (+6 times) and *pdk4* (+1.8 times), were remarkably increased in the 4% sodium citrate group (Fig. 1H). Accordingly, the 4% sodium citrate group had lower protein expression levels of p-Pdhe1a and Pdk2, which are key enzymes that drive the conversion of pyruvate to AcCoA, than those in the control group (Fig. 1J). Meanwhile, compared with the control group, the 4% sodium citrate group had higher expressions of glycogen synthase (*gs*) (+6 times) and glucose 6-phosphatase (*g6pase*) (+2.5 times), which are glycogen synthesis- and gluconeogenesis-related genes, respectively, whereas the expressions of fructose 1,6-bisphosphatase (*fbpase*) and phosphoenolpyruvate carboxykinase (*pepck*) were not changed (Fig. 1H). The protein expression level of G6pc was also significantly increased in the 4% sodium citrate group (Fig. 1J). In addition, 4% sodium citrate treatment significantly increased the expressions of TCA cycle-related genes, including citrate synthase (*cs*) (+1.5 times) and isocitrate dehydrogenase (*idh*) (+1.5 times), while the citrate carrier subunit b (*cicb*) was not affected (Fig. 1I). These results suggest that the ingestion of 4% sodium citrate led to systemic insulin resistance in Nile tilapia, although both hepatic glycolysis and gluconeogenesis were enhanced.

3.3. Dietary sodium citrate triggered lipid accumulation through inhibition of lipid catabolism

Sodium citrate treatment significantly altered the pattern of hepatic lipid metabolism of tilapia (Fig. 2). The concentrations of triglyceride in the liver and cholesterol in serum were significantly

Table 4
Effects of sodium citrate on growth performance, feed utilization and organ indices in Nile tilapia ($n = 3$).

Experiment duration	Control	2% Sodium citrate	4% Sodium citrate
Final body weight, g	29.31 ± 0.72	27.73 ± 0.23	28.23 ± 0.76
Weight gain ratio, %	944.4 ± 25.7	891.7 ± 8.7	904.7 ± 24.4
Condition factor, %	3.693 ± 0.098	3.595 ± 0.160	3.488 ± 0.100
Viscerosomatic index, %	12.39 ± 0.26	11.59 ± 0.52	12.23 ± 0.08
Hepatosomatic index, %	3.22 ± 0.03^{a}	2.78 ± 0.15^{b}	2.96 ± 0.01^{ab}
Mesenteric fat index, %	0.830 ± 0.001^{b}	0.734 ± 0.037^{b}	1.122 ± 0.049^{a}
Feed intake, g/per fish	24.75 ± 0.73	22.42 ± 0.020	23.42 ± 0.46
Carcass ratio, %	53.57 ± 2.11	55.91 ± 0.49	51.34 ± 1.7
Feed conversion ratio	0.931 ± 0.030	0.849 ± 0.010	0.885 ± 0.030
Survival rate, %	85.56 ± 2.94	92.22 ± 1.11	85.56 ± 1.11
Crude protein, %, wet	42.27 ± 1.74^{b}	44.72 ± 1.11^{ab}	48.75 ± 0.089^{a}
Crude lipid, %, wet	12.84 ± 0.13	12.13 ± 0.45	14.10 ± 0.70
Serum triglyceride, mmol/L	2.75 ± 0.24^{b}	3.47 ± 0.30^{ab}	4.26 ± 0.12^{a}
Liver glycogen, mg/g, wet	45.03 ± 1.5^{b}	47.72 ± 2.6^{ab}	54.07 ± 2.0^{a}

Values in the same row with different superscript letters indicate significant differences ($P < 0.05$).

increased in the 4% sodium citrate-treated fish (Fig. 2A and B), while there was no significant difference in hepatic cholesterol contents between groups (Fig. 2C). Hepatic AcCoA level, a substrate for DNL, in the 4% sodium citrate group was significantly higher than that of control (Fig. 2D). Interestingly, the expressions of the genes involved in lipogenesis, including sterol regulatory element-binding protein 1 (*srebp1*) (−0.5 times), peroxisome proliferator-activated receptor gamma (*pparg*), fatty acid synthase (*fasn*) (−5 times), *acly* (−5 times), and fatty acid β-oxidation, including carnitine palmitoyltransferase Iα (*cpt1a*) (−0.5 times), were notably decreased in the liver of 4% sodium citrate group (Fig. 2E and F), whereas the expressions of *ppara* and the genes involved in lipolysis, including adipose triglyceride lipase (*atgl*) and hormone-sensitive lipase (*hsl*), were not changed between treatments (Fig. 2F). Correspondingly, the 4% sodium citrate group had significantly lower protein expression levels of Acly and Cpt1a, while the total-acetyl-CoA carboxylase/phosphor-acetyl-CoA carboxylase (t-Acc/p-Acc) and mSrebp1 level was not affected by 4% sodium citrate treatment (Fig. 2G). These results suggest that the 4% dietary sodium citrate probably induced hepatic lipid accumulation by the inhibition of lipid oxidative catabolism.

3.4. Dietary sodium citrate improved protein deposition in muscle

Sodium citrate treatment also significantly affected the protein metabolism of Nile tilapia (Fig. 3). Supplementation of 4% sodium citrate prominently increased crude protein content in the muscle compared with the control group (Fig. 3A). The expressions of the genes related to muscle proteolytic metabolism in Nile tilapia, such as glutamate dehydrogenase 1 (*gdh1*), was down-regulated after sodium citrate ingestion, while the glutaminase (*gls2*) and branched-chain alpha-keto acid dehydrogenase (*bckdha*) expressions were not affected (Fig. 3B). The *gdh1* gene expression, which promotes glutamate catabolism into the TCA cycle, was significantly decreased compared to the control group (Fig. 3B). Meanwhile, the expression of glutamine synthetase (*glns*), a protein synthesis-related gene, showed an approximately 4-fold increase in 4% sodium citrate group compared with the control group. The expression of asparagine synthetase (*asns*), another protein synthesis-related gene, decreased by nearly half after 4% sodium citrate treatment (Fig. 3B). Protein expressions of t-mTor and t-S6 in the muscle of 4% sodium citrate group were significantly higher than those of the control, although the protein expressions of p-mTor and p-S6 showed no difference between two groups (Fig. 3C). Accordingly, the protein expression of t-AMPK and p-AMPK were significantly decreased by approximately 50% in the muscle of the 4% sodium citrate-treated fish compared with the control (Fig. 3C). These results suggest that the 4% sodium citrate treatment could inhibit protein catabolism and lead to protein deposition by activating the mTOR signaling pathway in muscle.

3.5. Dietary sodium citrate changed liver inflammatory status

The effect of dietary supplementation of 4% sodium citrate on liver health was also examined (Fig. 4). Serum AST and ALT activities were significantly reduced by 4% sodium citrate treatment compared to the control group (Fig. 4A and B). The histological results showed that the dietary supplementation of sodium citrate resulted in more pronounced vacuolization in the liver cells, suggesting more lipid deposition in the liver, than the control group (Fig. 4C). In addition, no significant signs of tissue damage or inflammation were observed (Fig. 4C). The expression of anti-inflammatory-related gene, transforming growth factor beta (*tgfb*) did not differ significantly between the two groups, while the expression of interleukin 10 (*il10*) in the 4% sodium citrate group was significantly down-regulated (Fig. 4D). On the other hand, the expressions of liver pro-inflammatory-related genes, such as nuclear factor kappa B (*nfκb*), tumor necrosis factor alpha (*tnfa*) and *il8*, were significantly lower in the 4% sodium citrate group than in the control group (Fig. 4E). However, there was a near twofold elevation in *il1b* mRNA expression in the 4% sodium citrate group compared to the control (Fig. 4E). Given the divergence in pro-inflammatory factor expressions, we further examined the protein expression of p65, an important downstream signaling element of the NFκB pathway, and found that the 4% sodium citrate treatment significantly activated the phosphorylation of p65 in the liver (Fig. 4F). These results indicate that although dietary supplementation with 4% sodium citrate caused a mild inflammatory response in the liver of Nile tilapia, no significant liver injury was found.

4. Discussion

4.1. Sodium citrate enhanced liver fat and muscle protein deposition by inhibiting catabolism

In previous studies, it was found that organic acid salts have growth-promoting and metabolism-improving effects in fish (Li et al., 2022), but the growth-promoting effects of sodium citrate in mammals and fish have rarely been reported. Some studies on the application of sodium citrate in yellowfin seabream and goldfish have shown the growth-promoting effect (Azari et al., 2021; Sotoudeh et al., 2020), but Romano et al. (2016) indicated that the dietary sodium citrate cause a growth-inhibiting effect and resulted

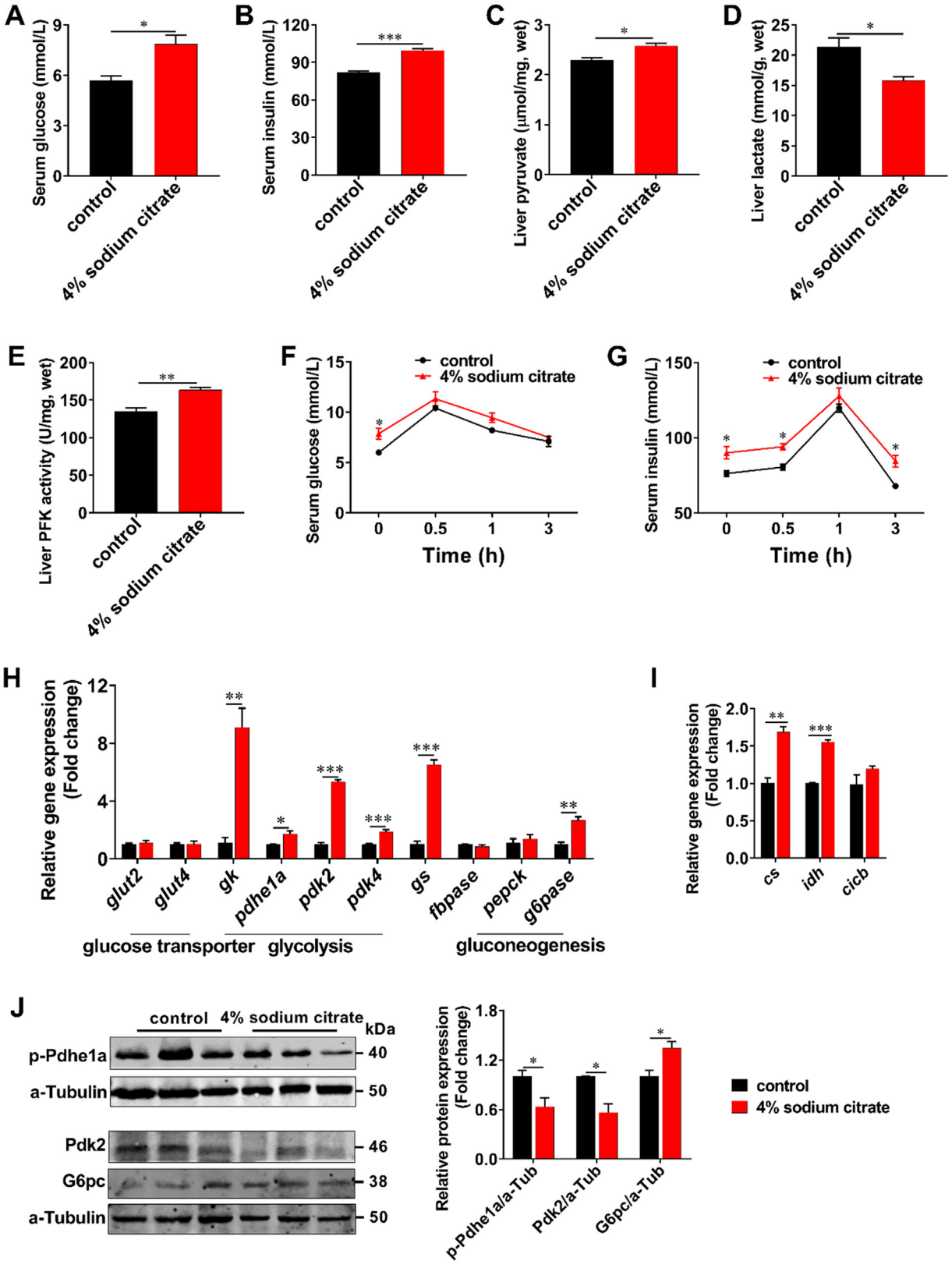

Fig. 1. Effects of sodium citrate on glucose metabolism in the liver of Nile tilapia. (A) Serum glucose, $n = 3$; (B) serum insulin, $n = 3$; (C) liver pyruvate, $n = 3$; (D) liver lactate, $n = 3$; (E) liver phosphofructokinase (PFK) activity; (F and G) Serum glucose and insulin of glucose tolerance test, $n = 3$; (H) mRNA expression of genes related to glucose metabolism, $n = 3$; (I) mRNA expression of genes related to TCA cycle, $n = 3$; (J) expression of proteins related to glucose metabolism, $n = 3$. Values are means ± SEM. Difference from group control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Independent-samples t test). *glut2* = glucose transporter protein 2; *glut4* = glucose transporter protein 4; *gk* = glucokinase; *pdhe1a1* = pyruvate dehydrogenase E1 component subunit alpha 1; *pdk2* = pyruvate dehydrogenase kinase isozyme 2; *pdk4* = pyruvate dehydrogenase kinase isozyme 4; *gs* = glycogen synthase; *fbpase* = fructose-1,6-bisphosphatase; *pepck* = phosphoenolpyruvate carboxykinase; *g6pase* = glucose-6-phosphatase; *cs* = citrate synthase; *idh* = isocitrate dehydrogenase; *cicb* = citrate carrier; p-Pdhe1a = phosphor-pyruvate dehydrogenase E1 component subunit alpha; Pdk2 = pyruvate dehydrogenase kinase isozyme 2; G6pc = glucose-6-phosphatase catalytic subunit.

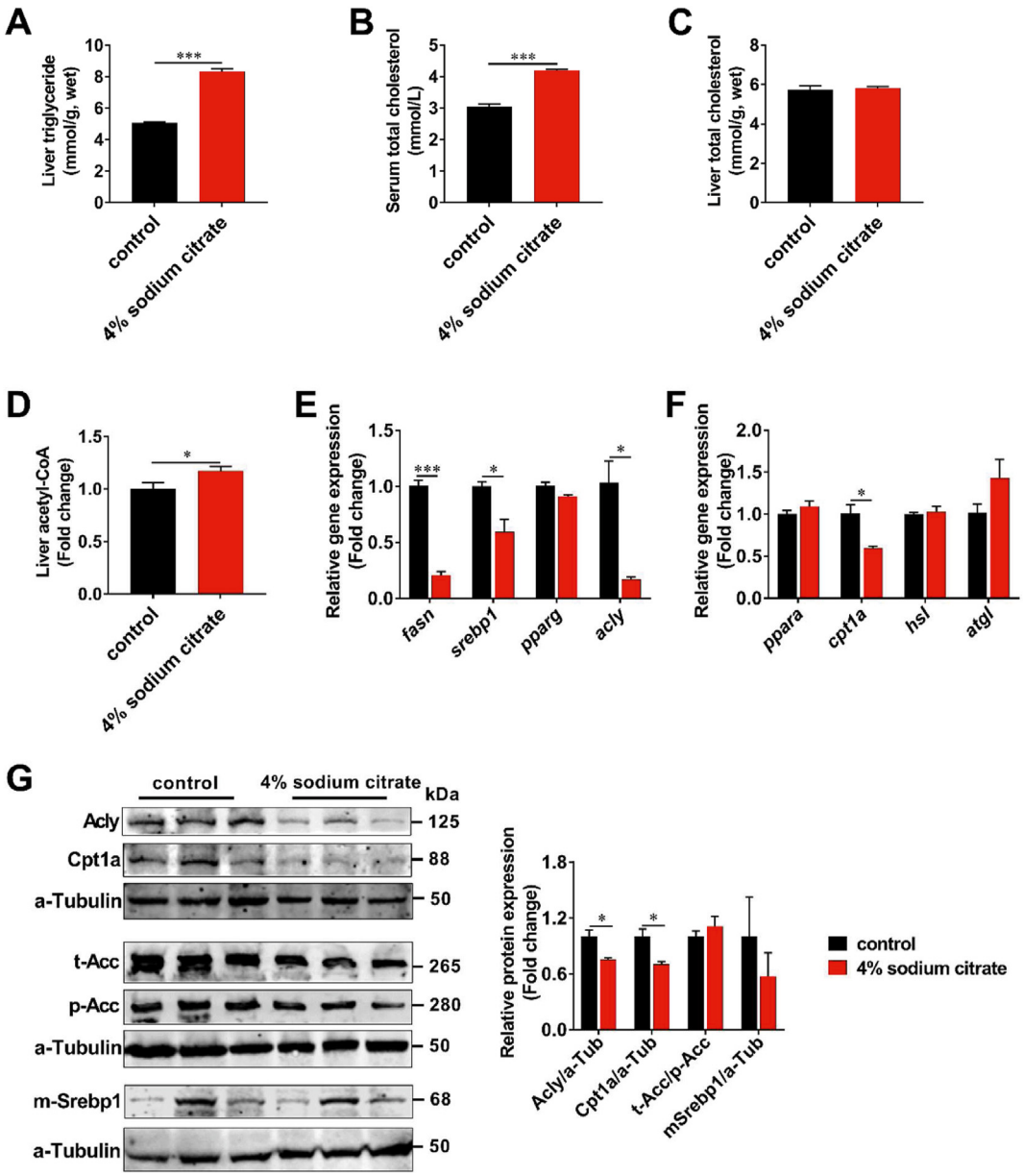

Fig. 2. Effects of sodium citrate treatment on lipid metabolism in the liver of Nile tilapia. (A) Liver triglyceride, $n = 3$; (B) serum cholesterol, $n = 3$; (C) liver cholesterol, $n = 3$; (D) liver acetyl-CoA, $n = 3$; (E and F) mRNA expression of genes related to lipid metabolism, $n = 3$; (G) expression of proteins related to lipid metabolism, $n = 3$. Values are means ± SEM. Difference from group control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Independent-samples *t* test). *fasn* = fatty acid synthase; m-Srebp1/*srebp1* = (mature) sterol regulatory element-binding protein 1; *ppara*/*pparg* = peroxisome proliferator activated receptor a/g; Acly/*acly* = ATP-citrate lyase; *hsl* = hormone-sensitive lipase; *atgl* = adipose triglyceride lipase; Cpt1a/*cpt1a* = carnitine palmitoyltransferase 1a; t/p-Acc = total/phosphor-acetyl-CoA carboxylase 1.

in liver damage in tilapia. The results of present study revealed no significant differences in growth performance and feed utilization of Nile tilapia between control group and sodium citrate groups. Romano et al. (2016) and the present study suggest that supplementing sodium citrate to Nile tilapia did not act as a growth promoter and the growth-promoting effect of sodium citrate in farmed fish is highly species-specific. In addition, previous studies on citrate have focused on the deposition of metal elements and trace elements in fish, and there is a lack of research on nutrient metabolism (Azari et al., 2021; Khajepour and Hosseini, 2012a, 2012b; Romano et al., 2016; Sarker et al., 2007; Sotoudeh et al., 2020). Therefore, it is important to investigate how sodium citrate affects nutrient metabolism in fish to assess whether sodium citrate can be used as a potential feed additive for aquaculture.

Liver plays an important role in the removal of citrate (Li et al., 2016) by breaking down it into AcCoA as the substrate for lipid synthesis (Mosaoa et al., 2021). In the present study, we found that 4% sodium citrate treatment significantly increased the AcCoA and triglyceride contents in the liver of Nile tilapia. Further study found that the *cpt1a* gene and Cpt1a protein expression, the enzyme that normally exerts flux and controls mitochondrial fatty acid β-oxidation (Li et al., 2020), were significantly decreased when lipid was accumulated. Malonyl-CoA inhibits the oxidative decomposition of fatty acids through its allosteric inhibition of Cpt1 (Saggerson, 2008). Although malonyl-CoA levels were not examined in the present experiment, we found no decrease in protein expression of t/p-Acc, which catalyzes the generation of malonyl coenzyme A from

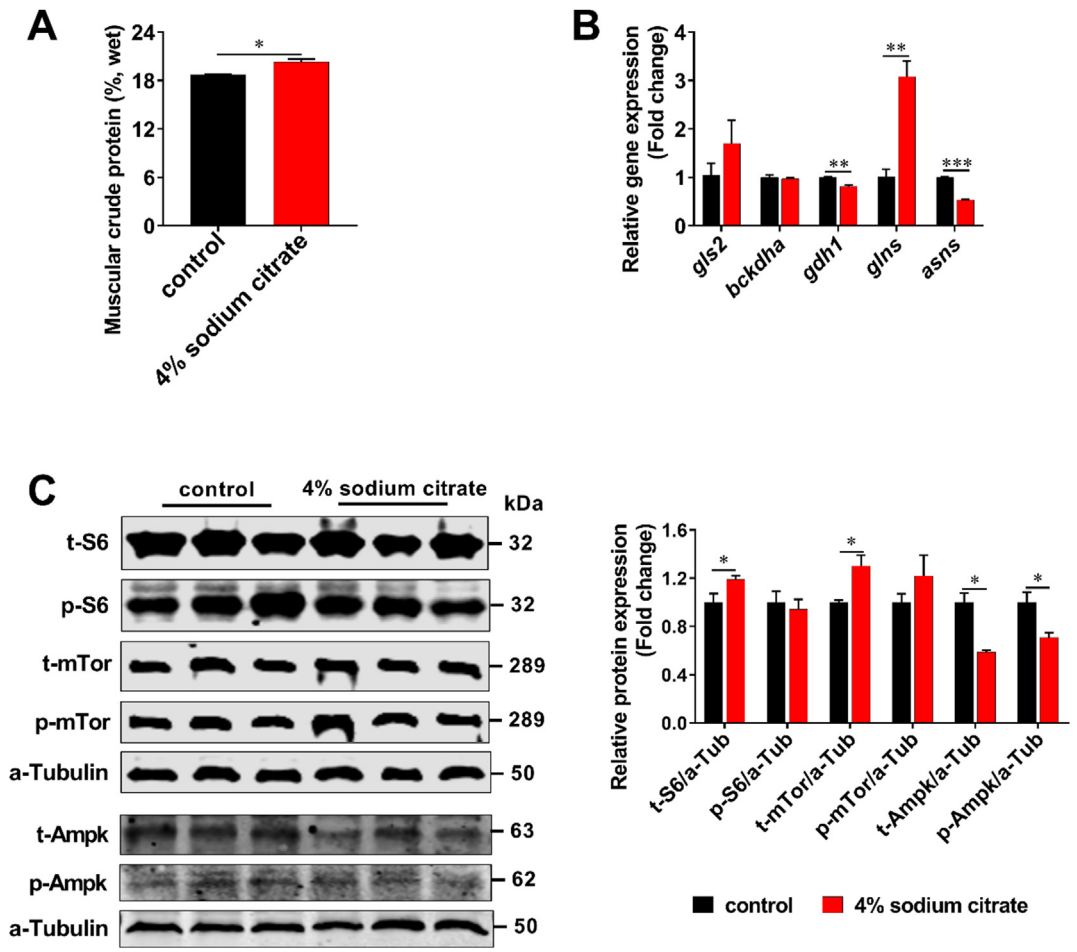

Fig. 3. Effects of sodium citrate treatment on the protein deposition in the muscle of Nile tilapia. (A) Crude protein in the muscle, $n = 3$; (B and C) expression of genes ($n = 3$) and proteins related to protein metabolism ($n = 3$). Values are means ± SEM. Difference from group control: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Independent-samples t test). *gls2* = glutaminase 2; *bckdha* = branched chain keto acid dehydrogenase E1 subunit alpha; *gdh1* = glutamate dehydrogenase 1; *glns* = glutamine synthetase; *asns* = asparagine synthetase; t/p-S6 = total/phosphor-ribosomal protein s6; t/p-mTor = total/phosphor-mammalian target of rapamycin; t/p-Ampk = total/phosphor-AMP-activated protein kinase.

AcCoA, in the liver of Nile tilapia after 4% sodium citrate treatment. These results indicate that 4% sodium citrate treatment could reduce mitochondrial fatty acid β-oxidation in the liver of Nile tilapia. Meanwhile, we also found decreased expression of the lipogenesis-related genes such as *srebp1, acly, pparg* and *fasn* in the 4% sodium citrate group, along with the decreased protein expression of Acly and mSrebp1. Numerous studies have shown that the reduced expression of triglyceride synthesis-related genes may be caused by a feedback mechanism of lipid over-accumulation in the liver (Kim et al., 2004; Kreeft et al., 2005; Yan et al., 2015). Therefore, these results illustrate that the sodium citrate-induced lipid accumulation in the liver of Nile tilapia was mainly caused by the inhibition of lipid catabolism rather than the upregulation of lipogenesis (Fig. 5).

In the present study, whole-body and muscle crude protein contents of Nile tilapia in the 4% sodium citrate group were significantly increased. Dietary sodium citrate supplementation was found to improve the metabolic status and extend lifespan in *Drosophila* fed high-fat diet. These effects depend on the activated AMPK and inhibited mTOR signaling pathways, suggesting a direct or indirect regulatory relationship between citrate and both pathways (Fan et al., 2021), which has not yet been confirmed in fish. Studies in fish demonstrated that activation of mTor and its downstream target protein S6 has been shown to promote protein deposition (Li et al., 2020). In the present experiment, we found that 4% sodium citrate treatment significantly down-regulated the expression of proteolytic-related gene *gdh1* and up-regulated the expression of protein synthesis gene *glns*. As expected, we also observed the Nile tilapia fed 4% sodium citrate has significantly higher protein expressions of t-mTor and t-S6 and significantly lower protein expressions of t-Ampk and p-Ampk. The above results suggest that the sodium citrate treatment could change the overall muscle protein metabolism by inhibiting catabolism and promoting deposition (Fig. 5).

It has been reported that sodium citrate treatment led to a significant decrease in whole-body crude lipid content (approximately 0.4%) and whole-body crude protein content (approximately 5%) of hybrid tilapia (Romano et al., 2016). In contrast with this, we found an increasing trend in whole-body crude lipid content, and significantly higher whole-body and muscle crude protein content in Nile tilapia treatment with 4% sodium citrate. We hypothesize that this difference could be due to the high percentage of soybean meal (54.87%) in the feed of hybrid tilapia (Romano et al., 2016), because dietary soybean meal above 22.5% has been reported to cause liver damage and reduced growth in hybrid tilapia (Lin and Luo, 2011). This suggests that the effects of dietary sodium citrate on fish physiological process could largely depend on the dietary components.

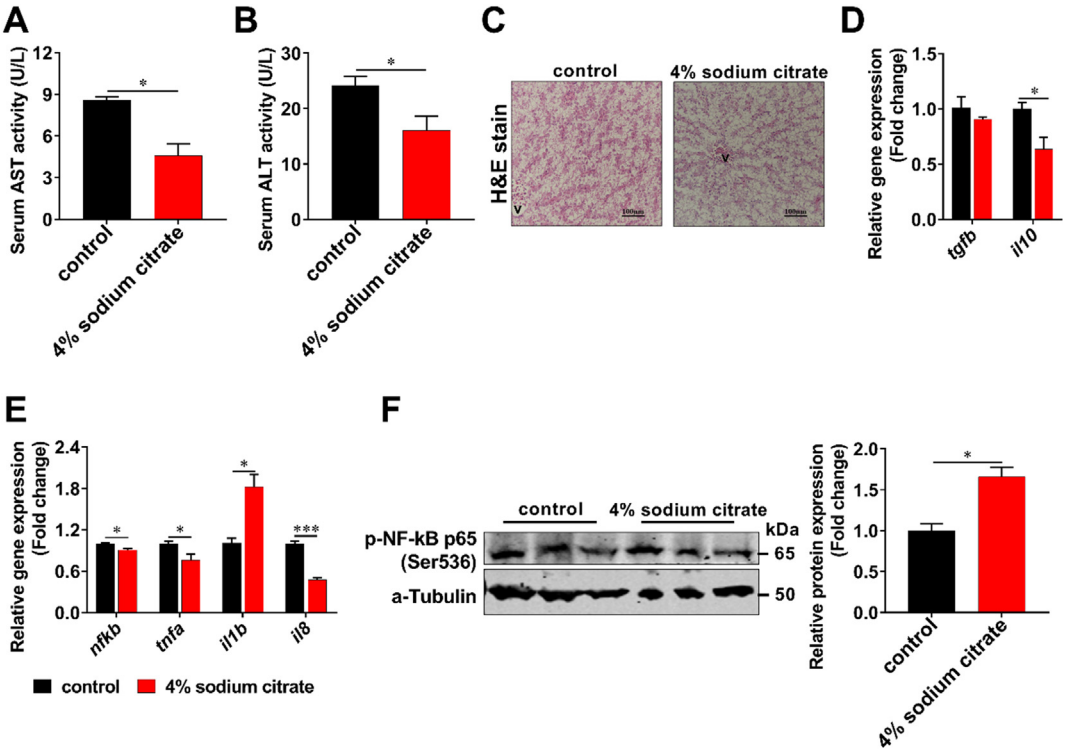

Fig. 4. Effects of sodium citrate treatment on the liver health of Nile tilapia. (A) Serum AST activity, $n = 3$; (B) serum ALT activity, $n = 3$; (C) H&E staining of the liver; (D and E) expression of genes related to inflammation, $n = 3$; (F) expression of p-NF-κB p65 (Ser536) protein, $n = 3$. Values are means ± SEM. Difference from group control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Independent-samples *t* test). AST = aspartate aminotransferase; ALT = alanine aminotransferase; *nfκb* = nuclear factor kappa B; *tnfa* = tumor necrosis factor alpha; *il1b* = interleukin 1 beta; *il8* = interleukin 8; *tgfb* = transforming growth factor beta; *il10* = interleukin 10.

4.2. Citrate induced hepatic gluconeogenesis and insulin resistance but enhanced pyruvate oxidation for energy supply

Intracellular citrate is an important regulator of energy production, as citrate inhibits and/or induces important strategic enzymes located at the entrance and/or exit of glycolysis, TCA cycle, gluconeogenesis and fatty acid synthesis (Iacobazzi and Infantino, 2014). In the present study, the serum glucose level was significantly increased in Nile tilapia after 4% sodium citrate treatment, while no significant changes were found in the mRNA expression of glucose transport-related genes (*glut2* and *glut4*). This indicates that 4% sodium citrate treatment did not affect glucose uptake in the liver of Nile tilapia. We next observed that the 4% sodium citrate-treated group of Nile tilapia showed an approximately 7-fold increment in liver *gk* mRNA expression, as well as a significant enhancement in PFK activity, which is considered to be the rate-limiting enzyme of glycolysis and is critical in determining glycolytic flux (Mor et al., 2011). In addition, glucose tolerance experiments revealed that serum glucose and insulin levels were higher in the 4% sodium citrate group of Nile tilapia than in the control group after glucose injection. These results suggest that sodium citrate treatment in basal metabolic state led to glucose intolerance and insulin resistance in Nile tilapia. On the other hand, citrate is a potent allosteric activator of Fbpase, an enzyme that converts fructose-1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis (Wang and Dong, 2019). Although there was no significant change in mRNA expression of liver *fbpase* and *pepck* of Nile tilapia in this experiment, we found a nearly 2.5-fold increase in *g6pase* mRNA expression, a key enzyme that controls the gluconeogenic flux (Wang and Dong, 2019), after 4% sodium citrate treatment. Accordingly, there was also a significant increase in protein expression of G6pc in 4% sodium citrate group. Meanwhile,

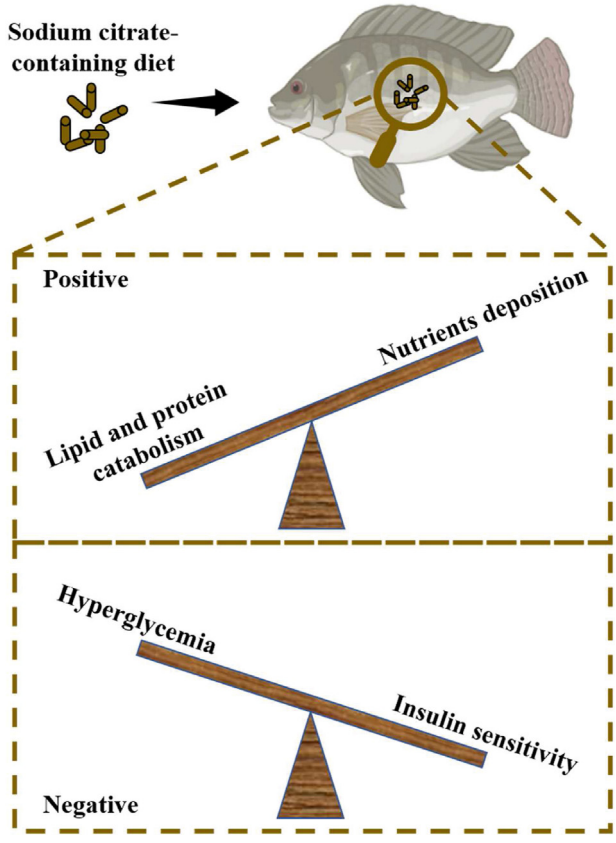

Fig. 5. Sodium citrate could exhibit a double-edged effect in Nile tilapia, with the positive aspect in promoting nutrient deposition and the negative aspect in causing hyperglycemia and insulin resistance.

liver pyruvate was significantly accumulated, as well as glycogen content and *gs* mRNA expression (approximately 6-fold increase), while lactate content was notably decreased by 4% sodium citrate treatment. These results suggest that 4% sodium citrate treatment prominently enhanced gluconeogenesis and hepatic glucose synthesize in the liver of Nile tilapia. In the present study, the protein expression of p-Pdhe1a and Pdk2, which inactivates Pdh activity, were significantly downregulated. In addition, the rate-limiting enzymes of the TCA cycle *cs* and *idh* mRNA expression were significantly increased. These data collectively suggest that the supplementation of 4% sodium citrate to Nile tilapia activated Pdh complex and enhanced the flux of hepatic pyruvate into the TCA cycle after oxidation to AcCoA, suggesting the enhanced TCA cycle.

4.3. Citrate treatment caused inflammation in the liver

The development of inflammation has been regarded as one of the main causes of systemic insulin resistance and severe lipid accumulation in the liver (Cao et al., 2020). Although the reduced serum AST and ALT activities in 4% sodium citrate group indicate that the liver was not significantly damaged during the feeding trial. 4% sodium citrate treatment resulted in a significant decrease in the expression of the pro-inflammatory-related genes *tnfa* and *il8* in the liver of Nile tilapia, while elevated gene expression of *il1b*. Recently, a close relationship between citrate and inflammation has been reported (Branco et al., 2021; Liu et al., 2022; Liu et al., 2022). Under normal conditions, dietary supplementation of citrate leads to polarization of mouse liver macrophages toward the M1 type and promotes inflammation (Branco et al., 2021). On the other hand, blocking the citrate shuttle can alleviate inflammation by reshaping cellular metabolic patterns (Liu et al., 2022) and blocking inflammatory signaling pathways (Liu et al., 2022). Meanwhile, the significant activation of hepatic p65 phosphorylation in the sodium citrate-treated group reinforced the conviction that 4% sodium citrate treatment could induce liver inflammation in Nile tilapia. However, compared to the control group, no significant tissue damage or cellular infiltration was found in the liver sections of the 4% sodium citrate group. These results imply that 4% dietary sodium citrate supplementation could induce inflammation signals in the liver of Nile tilapia, but did not lead to liver injury.

5. Conclusion

The results of this study showed that sodium citrate (4%) promoted lipid and protein deposition in Nile tilapia by inhibiting their catabolism but had no effect on growth performance. Sodium citrate treatment was able to enhance both hepatic glycolysis and the entry of pyruvate into the TCA cycle for catabolic energy supply. Although no significant liver injury was found in the sodium citrate-fed fish, dietary sodium citrate could induce hepatic gluconeogenesis, systemic insulin resistance and activate inflammatory signaling pathways. The present study suggests that at least in tilapia, dietary sodium citrate has a double-edged effect in fish, with the positive aspect in promoting nutrient deposition and the negative aspect in causing hyperglycemia and insulin resistance (Fig. 5). Our study brings novel information in understanding the roles of citrate in nutrient metabolism in fish and is helpful for the precise application of citrate in aquafeed formulation.

Author contributions

Zhen-Yu Du, **Yuan Luo**, and **Jun-Xian Wang:** conceived the study and designed the experiments. **Jun-Xian Wang:** carried out the experiments, analyzed the data, and wrote the manuscript. **Fang Qiao**, **Li-Qiao Chen** and **Mei-Ling Zhang:** contributed reagents/materials/analysis tools. **Zhen-Yu Du**, **Yuan Luo**, and **Jun-Xian Wang:** wrote and revised the manuscript. All authors read and approved the final manuscript.

Availability of data and material

The datasets and materials used in this study are available from the corresponding author on reasonable request.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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