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Original Article

The chronic effects of 5-aminolevulinic acid plus sodium ferrous citrate on pre-diabetic wistar rats

Zhenhua Xu[1], Fangfang Zhou[1,2], Hongyan Chen[1], Takeshi Hara[3], Motowo Nakajima[3], Chengde Zheng[1], Tohru Tanaka[3], Daru Lu[1]

[1]*State Key Laboratory of Genetic Engineering, School of Life Sciences and Zhongshan Hospital, Fudan University, Shanghai 200438, China;* [2]*Shanghai Jiao Tong University School of Medicine, Shanghai, China;* [3]*SBI Pharmaceuticals Co., Ltd., Tokyo, Japan*

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Abstract: 5-aminolevulinic acid (5-ALA) is produced by mitochondria and is expected to improve many health issues. In this study, we investigated the effects and mechanisms of 5-ALA plus sodium ferrous citrate (5-ALA/SFC) on obese rats. Wistar rats on a high-fat diet were orally administered 5-ALA/SFC at different dosages daily for 6 months. We found that 5-ALA/SFC effectively reduced plasma glucose levels and insulin resistance. Interestingly, although 5-ALA/SFC improved appetite, decreases in body weight and visceral fat were observed. The promotion of appetite may depend on the regulation of two appetite factors, amylin and peptide YY (PYY). Furthermore, adiponectin, inflammatory factor monocyte chemotactic protein-1 (MCP-1), and tumor necrosis factor α (TNF-α) might participate in the glucose and lipid metabolism. More importantly, 5-ALA/SFC was likely to regulate the expression of *COXIV, UCP1, UCP2*, *Glut2,* and *SERBP-1c* in related tissues to maintain the homeostasis of the mitochondrial metabolism, reduce fat accumulation in the liver, decrease visceral fat accumulation, and further promote glucose and fat metabolism. Our data indicated 5-ALA/SFC may ameliorate obesity and prevent the emergence of pre-diabetes states.

Keywords: 5-aminolevulinic acid, sodium ferrous citrate, mitochondrial metabolism, pre-diabetes

Introduction

Diabetes mellitus and obesity are increasing rapidly worldwide. In 2014, about 422 million people were diagnosed with diabetes mellitus, and the morbidity has risen from 4.75% in 1980 to 8.5% among adults [1]. Pre-diabetes is defined as impaired fasting glucose (IFG) and glucose tolerance (IGT), elevated glycated hemoglobin (HbA1c), according to the American Diabetes Association (ADA). The metabolism of glucose, lipids and protein is abnormal and a higher amount of HbA1c causes poor control of blood glucose levels in pre-diabetes [2]. Several studies have shown that patients with pre-diabetes may develop diabetic retinopathy [3] or peripheral neuropathy [4, 5]. In view of such a serious situation, it is important in pre-diabetes to prevent diabetes-related complications with early care.

5-ALA, a natural amino acid synthesized in the mitochondria, is the precursor of both chlorophyll and heme, which relate to the origin of life. It is widely distributed in both animals and plants and can be found in many common foods such as green vegetables, fruits, and fish [6]. It has been used in agriculture, as a food additive, and in cosmetics, and also in the medical field such as photodynamic diagnosis and sonodynamic therapy for chaos or tumors [7-10]. What's more, 5-ALA can improve other health problems such as anemia, dyssomnia, and metabolic syndrome [6, 11-13].

Visceral adiposity is usually observed in obesity and type 2 diabetic patients. The increase of adipose tissue leads to a decrease in insulin sensitivity by adipocytokines, such as adiponectin, leptin and other inflammatory factors, all of which are associated with insulin function [14-16]. Recent studies have shown that 5-ALA/SFC could reduce adiposity and improve glucose tolerance by enhancing mitochondrial function [17]. It could also lower the plasma glucose and HbA1c levels in obese rats [18]. Also,

Table 1. Composition of high-fat diet

Ingredients	Ratio (g/100 g)
Breeding feed	53.8
Lard	18.9
Cholesterol	1.3
Bile salt	1.3
Sucrose	11.2
Casein	8.7
Premix	1.8
Maltodextrin	3
Total	100

two large-scale intervention studies about pre-diabetes patients taking 5-ALA/SFC suggested that 5-ALA/SFC could effectively improve glucose tolerance [11, 19]. These findings explain the beneficial effects of 5-ALA on metabolism abnormality to a certain extent. However, the effect and mechanism of 5-ALA on obesity and pre-diabetic conditions has not been elucidated. In this study, we designed an experiment to feed a high-fat diet to Wistar rats with 5-ALA/SFC for 6 months to explore the diet's effects on pre-diabetic conditions and the mechanisms of 5-ALA/SFC on energy metabolism.

Materials and methods

Animals

Male Wistar rats 4-5 weeks old and with a body weight 150-180 g were obtained from the SLAC Laboratory Animal, Shanghai, China. The animals were maintained in groups of two or three in the Laboratory Animal Center of the Second Military Medical University, Shanghai, China and fed a standard pellet diet and water ad libitum. Before the experiment, the rats were accustomed for one week to the laboratory environment at a limited temperature range (20 ± 2°C) and a 12 h light/12 h dark cycle. The protocol of this study was approved by the institutional ethical committee of Fudan University.

Grouping and animal treatments

The Wistar rats were randomly divided into the five groups listed below.

Group 1: Normal Diet rats administered distilled water orally daily for 6 months (ND group). Group 2: High fat diet (**Table 1**) rats administered distilled water orally daily for 6 months (HFD group). Group 3: High fat diet rats simultaneously administered 5-ALA/SFC (50 mg/kg and 7.9 mg/kg each) in an aqueous solution orally daily for 6 months (ALA50 group). Group 4: High fat diet rats simultaneously administered 5-ALA/SFC (100 mg/kg and 15.7 mg/kg each) in an aqueous solution orally daily for 6 months (ALA100 group). Group 5: High fat diet rats simultaneously administered 5-ALA/SFC (300 mg/kg and 47.1 mg/kg each) in an aqueous solution orally daily for 6 months (ALA300 group).

Each group had at least nine rats. The 5-ALA (5-Aminolevulinic acid hydrochloride; lot number: HCL-KK08-04-1-1; purity: 99.8%) was obtained from the Cosmo Oil Co., Ltd (Tokyo, Japan). The SFC was obtained from the Komatsuya Corporation (Osaka, Japan). And all the treatments were performed using a gavage needle every afternoon from 6 to 30 weeks of age. The average weights, food intake and plasma glucose levels of the rats were recorded every one or two weeks.

Plasma and tissue preparation and biochemical assays

After 6 months of administration, the rats were fasted overnight (6:00 pm to 8:00 am of next day) and sacrificed by cervical dislocation after being anaesthetized with ether. Blood samples were collected from the orbital vein. The plasma was collected from the blood samples after it was centrifuged at 1000 rpm for 10 min at 4°C and then stored at -20°C until analyzed. Tissues samples from the liver, neck brown fat, epididymal and retroperitoneal fats were collected, weighed, and immediately frozen in liquid nitrogen before storage at -80°C. Plasma concentrations of the biochemical indexes were measured using a kit (serum glucose, free fatty acid, and total cholesterol) from the Jiancheng Bioengineering Institute, Nanjing, China and liquid chip detection kits (Milliplex: RMHMAG-84K-06 for insulin, C-peptide, amylin, MCP-1, gastric inhibitory polypeptide (GIP) and PYY; RADPCMAG-81K-01 for adiponectin, RECYTMAG-65K-07 for interleukine-1 β (IL-1β), leptin, TNF-α and vascular endothelial growth factor (VEGF)) from Merck Millipore, United States, respectively. The serum used for the 2 h postprandial blood glucose (PBG) were collected two hours later after the fasting (6:00 pm to 8:00 am of next day) rats were administrated of 2.5 g/kg glucose one week (Not anesthetized, blood collection from orbital venous

Table 2. Primers used for q-PCR

Gene	Primer sequence (5'-3')
LPK	(Forward, F) 5'-CTCCCCACTCAGCTACAGACC-3'
	(Reverse, R) 5'-CCCTTCACAATTTCCACCTCC-3'
Glut2	(Forward, F) 5'-TGAAGGATCTGCTCACATAGTCA-3'
	(Reverse, R) 5'–CCAACATGGCTTTGATCCTT-3'
SERBP-1c	(Forward, F) 5'-CCGTTTCTTCGTGGATGG-3'
	(Reverse, R) 5'-CACAGAATAGTCGGGTCACCT-3'
Cpt1a	(Forward, F) 5'-AAGAAGTTCATCCGGTTCAAGA-3'
	(Reverse, R) 5'-GCATGCATGGATGAAATCAC-3'
UCP2	(Forward, F) 5'-GTTCTACACCAAGGGCTCAGA-3'
	(Reverse, R) 5'-GACCTTTACCACATCTGTAGGTTG-3'
UCP1	(Forward, F) 5'-TCAGGGCTGATTCCTTTTGG-3'
	(Reverse, R) 5'-GCGGACTTTGGCGGTGT-3'
Scd1	(Forward, F) 5'-ATCCCCTCCTCCAAGGTCTA-3'
	(Reverse, R) 5'-CCGAGCCTTGTAAGTCCTGT-3'
GCK	(Forward, F) 5'-GCCTCAGGAGTCAGGAACAT-3'
	(Reverse, R) 5'-AACTCTGCCAGGATCTGCTC-3'
COXI	(Forward, F) 5'-GGAGGCTTCGGGAACTGA-3'
	(Reverse, R) 5'-GCTTCTACTATGGAGGATGCTAAA-3'
COXIV	(Forward, F) 5'-CACGTCAAGCTGCTGTCTG-3'
	(Reverse, R) 5'-CGTTAAACTGGATGCGGTACA-3'
β-actin	(Forward, F) 5'-AAGGCCAACCGTGAAAAGAT-3'
	(Reverse, R) 5'-ACCAGAGGCATACAGGGACA-3'

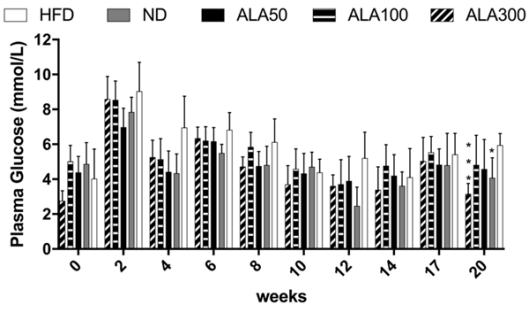

Figure 1. Change in weekly average plasma glucose. Data represent the mean ± SD. Statistical analyses were performed using the One-way Analysis of Variance between each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the HFD control group.

plexus) before the rats were sacrificed (anesthetized). The serum used for plasma fasting plasma glucose (FPG), free fatty acid (FFA), total cholesterol, insulin, C-peptide, amylin, MCP-1, GIP, and the other measurements was collected during the sacrificing process (with 14 hours of fasting). The serum used for ordinary FPG we measured every two weeks after 14 hours of fasting (not anesthetized).

Histology

The liver tissues of each group was embedded in 4% paraformaldehyde up to 48 h, then they were dehydrated, processed for paraffin embedding and sectioned. 5-7 um thick paraffin sections were stained with hematoxylin and eosin (HE). The samples were assessed and photographed under a microscope.

Quantitative PCR

Total mRNA was extracted from the liver and visceral fats using Trizol (Invitrogen, Carlsbad, CA) and was reverse transcripted to cDNA using the RT reagent Kit (TOYOBO, Japan), and Quantitative PCR (q-PCR) was performed using a 7900HT (ABI, Foster City, CA). Primers used for q-PCR by SYBR Premix (TOYOBO, Japan) are shown in **Table 2**. PCR amplification was performed in a total reaction of 10 μL, and the amplification conditions were 95°C 3 min, 45 cycles of a run with 95°C 15 s and 60°C 30 s, followed by dissociation on the ABI7900 Real-Time PCR System (ABI, US). The relative quantities of target mRNA expression levels were measured by relative quantification and the results for each sample were normalized to β-actin.

Statistical analyses

All statistical analyses were performed using SPSS 17.0. The data were expressed as the means ± SD. Statistical differences between groups were evaluated using the One-way Analysis of Variance. *P*-values < 0.05 were considered statistically significant.

Results

The Influence of 5-ALA/SFC on the glucose and lipid profiles

Hyperglycemia and hyperlipidemia widely occur in obesity and pre-diabetic conditions. As shown in **Figure 1** and **Table 3**, during a high fat diet for 6 months, the FPG and PBG levels of the HFD group increased significantly, compared with ND group. Nevertheless, with the administration of 5-ALA/SFC, these biochemical factors were significantly improved in the ALA300 group. Also, the plasma cholesterol

Table 3. Plasma biochemistry of glucose and lipid related metabolism

Parameters	ND (n = 11)	HFD (n = 11)	ALA50 (n = 10)	ALA100 (n = 10)	ALA300 (n = 9)
FPG (mM)	4.1 ± 1.2*	6.1 ± 0.6	4.6 ± 1.7	4.8 ± 1.7	3.1 ± 0.6***
PBG (mM)	7.4 ± 0.3*	8.0 ± 0.5	7.9 ± 0.7	7.7 ± 0.7	7.3 ± 0.5**
FFA (μmol/dL)	80.6 ± 5.8	92.5 ± 13.2	82.9 ± 6.1	95.1 ± 11.4$^{\#}$	79.0 ± 14.2
Cholesterol (mg/dL)	79.5 ± 13.5	82.1 ± 12.8	73.6 ± 9.0	57.8 ± 12.9$^{***,\#\#}$	79.3 ± 16.0
Insulin (ng/mL)	3.4 ± 1.0	4.3 ± 1.1	2.3 ± 0.7**	4.3 ± 0.6	3.5 ± 1.6
HOMA-IR index	15.2 ± 6.9***	28.0 ± 6.5	9.4 ± 4.7***	20.1 ± 5.1*	12.0 ± 5.1***
C-peptide (ng/mL)	3.8 ± 0.4	3.9 ± 0.8	3.8 ± 0.7	4.3 ± 1.3	3.1 ± 0.6
Adiponectin (ng/mL)	9.8 ± 1.8***	19.5 ± 3.6	15.6± 2.1$^{**,\#\#\#}$	15.4 ± 2.5$^{**,\#\#\#}$	16.4 ±1.8$^{**,\#\#\#}$
Leptin (ng/mL)	35.5 ± 8.7**	90.8 ± 42.6	56.4 ± 16.4	79.7 ± 42.2	50.9 ± 16.1

FPG: Fasting plasma glucose; PBG: 2 h postprandial blood glucose; FFA: Free fatty acid; Adip: Adiponectin. HOMA-IR: Homeostasis model assessment of insulin resistance. Values are means ± SD. Statistical analyses were performed using the One-way Analysis of Variance between each groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the HFD control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the ND control group.

levels were decreased significantly in the ALA100 group.

In view of the influence on glucose and lipid, we considered that related hormones might participate in the regulation. The plasma level of adiponectin was remarkably increased in the HFD group and the plasma level of the ALA/SFC treated groups were significantly decreased ($P < 0.01$, $P < 0.001$, respectively; **Table 3**). The plasma level of insulin was visibly reduced in the ALA50 group, but the other dosage groups were not affected ($P < 0.001$; **Table 3**). The homeostasis model assessment of insulin resistance (HOMA-IR) index was lower in the ND group and the 5-ALA/SFC treated groups compared with the HFD group ($P < 0.05$, $P < 0.001$, respectively; **Table 3**). The HFD group and ALA/SFC treated groups presented a higher leptin level compared with the ND group. The plasma level of C-peptide was not affected by the 5-ALA/SFC.

The influence of 5-ALA/SFC on body weight and food intake

During the 6 month period, we also followed up on the rats' body weight and food intake. The body weight of the ALA50 and ALA300 groups dropped about 7.5% and 25% respectively, compared with the HFD group ($P < 0.01$, $P < 0.001$, respectively; **Figure 2A**; **Table 4**). The average body weight of the rats in the ALA300 group was even significantly lower than the average body weight of the ND rats ($P < 0.01$, **Figure 2A**; **Table 4**). The food intake per gram of weight of the 5ALA/SFC treated rats was increased compared with the HFD rats ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; **Figure 2B**; **Table 4**), but the rats in ND group ate more food than the other groups because the number of calories in the ND's food was lower ($P < 0.001$, **Figure 2B**; **Table 4**).

5-ALA/SFC prevents visceral adipose deposition

To indicate the fat deposition of viscera, we measured the fat content of two major tissues of viscera: epididymal and retroperitonea. The ratio of epididymal fat to the whole body weight in the HFD group as well as in the 5-ALA/SFC treated group was notably higher than the ND group ($P < 0.001$; **Figure 3**). However, compared with the HFD group, only the ALA300 group had a slightly reduced weight ratio of epididymal fat. The weight ratio of retroperitoneal fat was substantially declined in the ALA/SFC 50 group and in the ALA/SFC 100 group ($P < 0.001$, $P < 0.01$, respectively; **Figure 3**).

To gather more information, we also performed assays to observe the liver histology. HE staining showed no necrosis or hyperplasia in the livers of the ND rats, but in the HFD rat livers, there was fat accumulation and some necrosis and cell infiltration, and even the local hepatic cord was abnormal. In the 5-ALA/SFC of the different dosage treated rats, the liver still had a small amount of fat and some tissue damage, but in the maximum drug dosage ALA300 treated rats, the liver structure was dense and had less fat accumulation and was just the same as the ND rats (**Figure 4**).

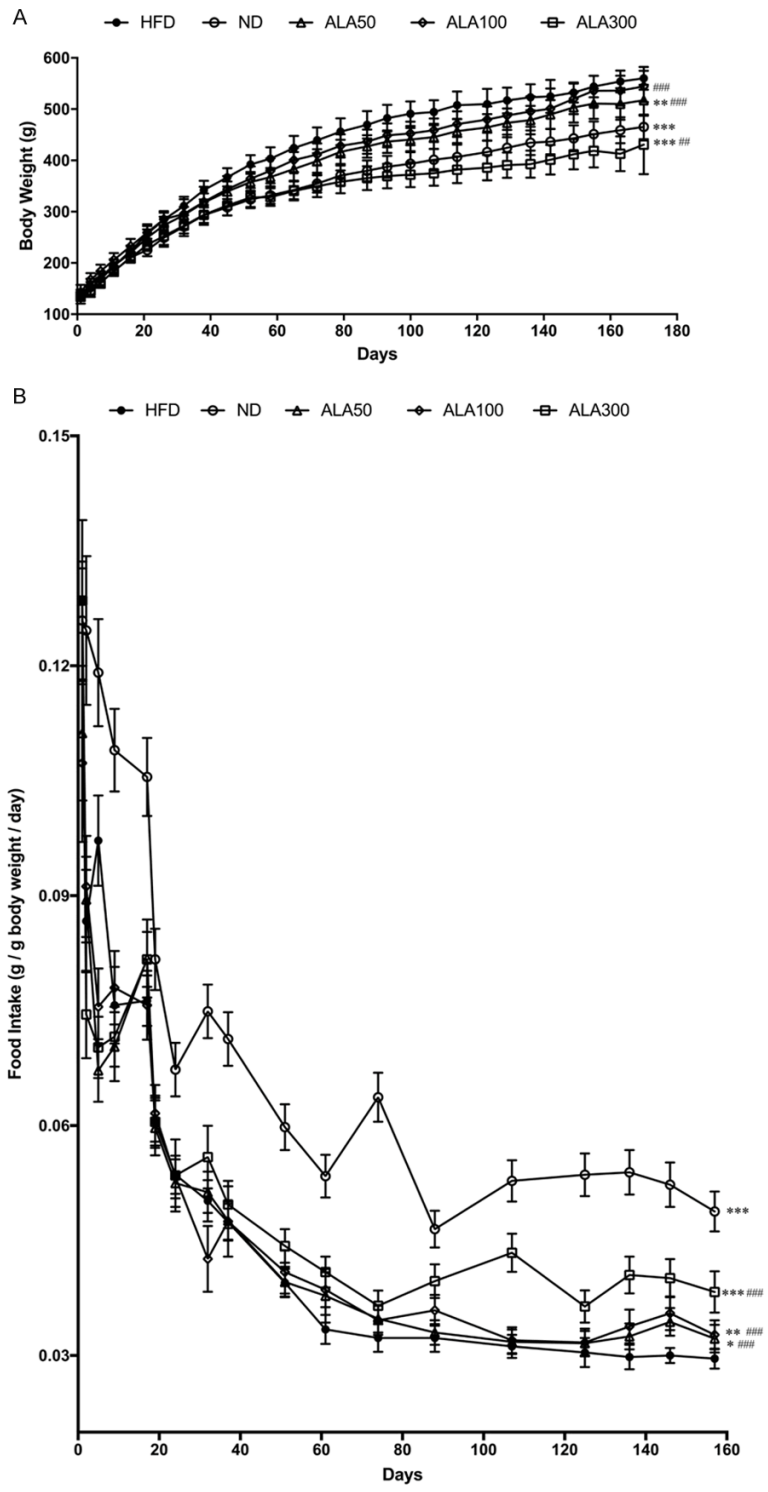

Figure 2. Change in weekly average body weight and food intake. (A) Body weight and (B) food intake were measured during the 25 weeks with different diets and treatment with 5-ALA/SFC (mean ± SD). Data represent the mean ± SD. Statistical analyses were performed using the One-way Analysis of Variance between each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the HFD control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the ND control group. (ND, n = 11; HFD, n = 11; ALA50, n = 10; ALA100, n = 10; ALA300, n = 9).

5-ALA/SFC influences the levels of inflammatory and appetite related factors

Several studies have shown that some inflammatory factors including IL-1β, TNF-α, VEGF, and MCP-1 play important roles in the development of diabetes [20-23]. As shown in **Table 5**, the levels of inflammatory factors such as IL-1β, and VEGF did not change much in any of the groups whether they were treated with ALA/SFC or not, but the concentration of MCP-1 in ALA300 was significantly lower than it was in the HFD rats and the TNF-α in the ALA300 group was remarkably reduced compared with the ND group ($P < 0.01$, $P < 0.05$, respectively; **Table 5**).

As 5-ALA/SFC improved the appetite of rats as shown in **Figure 2B**, the plasma levels of the three appetite related factors amylin, GIP, and PYY was examined. The plasma amylin level of HFD was much higher than it was in the ND group ($P < 0.001$, **Table 5**), while that of the 5-ALA/SFC treated group was visibly reduced, especially in the ALA100 and ALA300 group ($P < 0.001$, **Table 5**). The concentrations of GIP and PYY in the rats treated with ALA300 showed increases to varying degrees.

The effect of 5-ALA/SFC on the expression of glucose metabolism and lipid metabolism related genes in the liver

To evaluate how the ALA/SFC improves the metabolisms of glucose and lipids, we employed q-PCR to analyze the expressions of relative genes.

Table 4. Body weight and average food intake of the rats with 25 weeks of different diet and treated with 5-ALA/SFC

Parameters	ND (n = 11)	HFD (n = 11)	ALA50 (n = 10)	ALA100 (n = 10)	ALA300 (n = 9)
Body weight (g)	465.5 ± 22.4***	559.7 ± 16.3	517.2 ± 16.4$^{**,\#\#\#}$	544.5 ± 17.5$^{\#\#\#}$	416.4 ± 15.7$^{***,\#\#}$
Food intake (g/g body weight/day)	0.0489 ± 0.0027***	0.0296 ± 0.0014	0.0322 ± 0.0019$^{*,\#\#\#}$	0.0327 ± 0.0020$^{**,\#\#\#}$	0.0383 ± 0.0029$^{***,\#\#\#}$

Values are the means ± SD. Statistical analyses were performed using the One-way Analysis of Variance between each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the HFD control group; $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 vs. the ND control group.

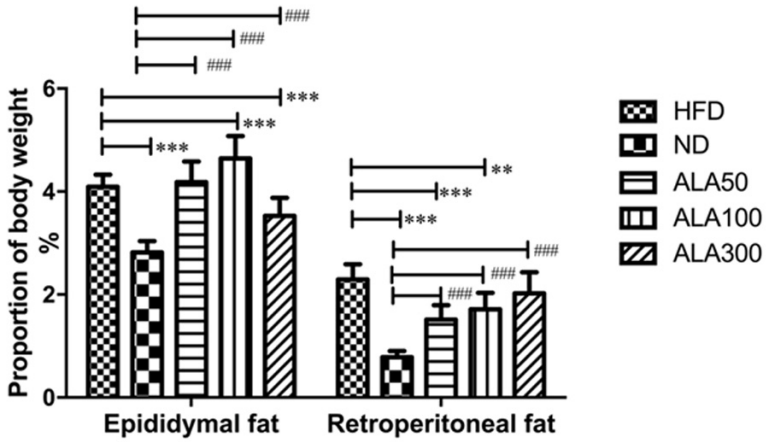

Figure 3. The proportions of epididymal and retroperitoneal fat in body weight. The proportions of epididymal and retroperitoneal fat in body weight in the different treatment group rats were measured (organs weight/body weight). Data represent means ± SD. Statistical analyses were performed using the One-way Analysis of Variance between each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the HFD control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the ND control group. (ND, n = 11; HFD, n = 11; ALA50, n = 10; ALA100, n = 10; ALA300, n = 9).

We found that in the livers, the gene expressions of *COX*IV and *SREBP-1c* of 5-ALA/SFC treated rats were significantly higher than those of the HFD rats ($P < 0.05$, $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively; **Figure 5**), but the expressions of *Glut2* and *LPK* of the 5-ALA/SFC treated rats were significantly lower compared with those of the HFD and ND rats (**Figure 5**). Moreover, the expression of *GCK* in the HFD and 5-ALA/SFC treated groups was much higher than it was in the ND group, but there was no difference between the HFD and 5-ALA/SFC treated groups (**Figure 5**). Furthermore, the expression of *Cpt1a*, one of the fatty acid metabolism related genes in the liver, was significantly reduced in the ALA50 group. More importantly, the expression of *UCP2* in liver, and *UCP1* in the brown and white fat of the 5-ALA/SFC treated rats increased significantly more than the expression in the HFD and ND group rats (**Figures 5** and **6**). At the same time, *COX*I and *Scd1* showed little difference among the groups (**Figure 5**).

Discussion

In the present study, the effects of the different doses of 5-ALA/SFC on obesity and pre-diabetic conditions were investigated. We demonstrated that the administration of 5-ALA/SFC increased rats' appetites but reduced their plasma glucose levels, insulin resistance, body weight, and visceral adiposity. Finally, we found that 5-ALA/SFC effectively ameliorates obesity and prevents the development of pre-diabetes. We speculated that these results might be mediated by the regulation of metabolism-related hormones and genes, appetite-related factors, and inflammation-related hormones.

In studies, the effects of 5-ALA/SFC on the appetite were inconsistent. We for the first time found that 5-ALA/SFC significantly increases the appetite by regulating the plasma levels of three appetite related hormones: amylin, GIP, and PYY. In vivo, amylin mediates satiation and reduces food intake via the brain by slowing down gastric emptying and inhibiting digestive secretion [24, 25]. In our study, the plasma level of amylin in 5-ALA/SFC treated rats was significantly reduced, which could cause an increased appetite. GIP can induce postprandial insulin secretion, regulate gastrointestinal motility and food intake, and then promote adipogenesis by stimulating lipoprotein lipase activity in adipocytes [26]. Meanwhile, as shown in our results, there was no statistical difference in the plasma GIP levels among the groups. PYY exerts its action by inhibiting gastric motility and increasing water and electrolyte absorption in the colon [27], thus suppressing pancreatic secretion, reducing appetite and slowing the gastric emptying [28], PYY can also increase

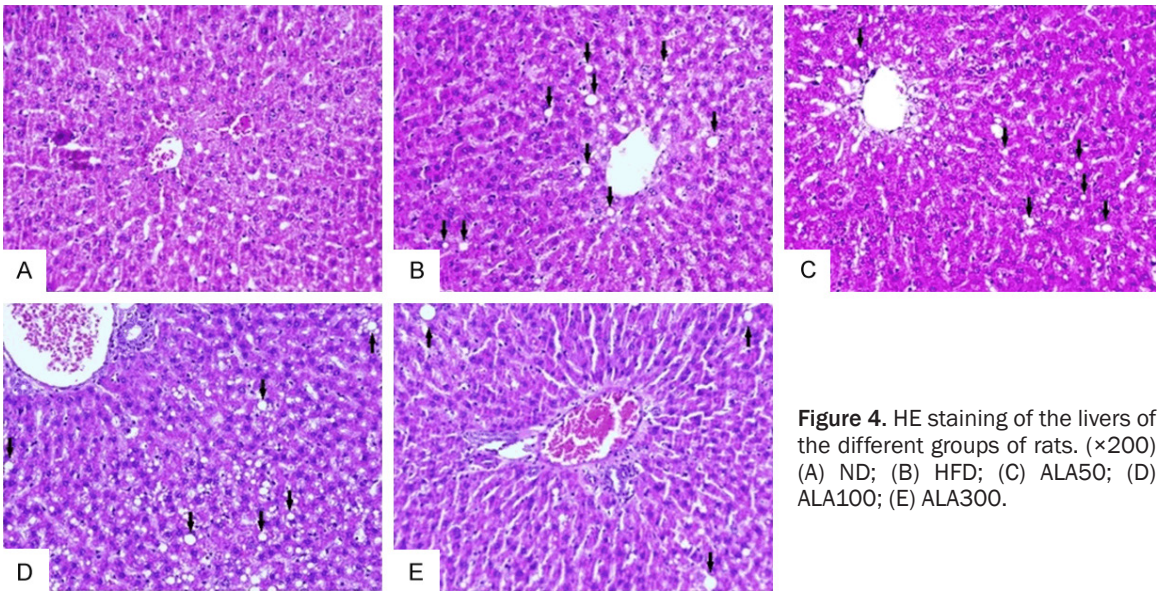

Figure 4. HE staining of the livers of the different groups of rats. (×200) (A) ND; (B) HFD; (C) ALA50; (D) ALA100; (E) ALA300.

Table 5. Plasma biochemistry of inflammatory and appetite related hormones

Parameters	ND (n = 11)	HFD (n = 11)	ALA50 (n = 10)	ALA100 (n = 10)	ALA300 (n = 9)
MCP-1 (ng/mL)	3.0 ± 0.6	3.5 ± 0.7	3.5 ± 0.8	3.4 ± 0.6	2.8 ± 0.4**
IL-1β (pg/mL)	40.4 ± 21.5	74.2 ± 51.7	41.9 ± 31.1	75.3 ± 70	71.6 ± 43.9
TNF-α (pg/mL)	3.0 ± 0.9	4.5 ± 4.4	2.5 ± 0.9	3.0 ± 2.0	1.4 ± 0.9#
VEGF (pg/mL)	26.7 ± 9.1*	43.7 ± 23.3	32.6 ± 15.8	32.9 ± 13.0	37.1 ± 14.7
Amylin (pg/mL)	527.54 ± 10.5***	730.8 ± 77.6	522.68***,①	0***,###,②	0***,###,③
GIP (pg/mL)	95.5 ± 33.5	81.8 ± 17.1	78.9 ± 53.5	74.6 ± 13.7	240.8 ± 249.0
PYY (pg/mL)	275.2 ± 81.2	294.3 ± 44.0	298.4 ± 49.3	286.5 ± 89.8	362.5 ± 67.7*,##

MCP-1: monocyte chemotactic protein 1; IL-1β: interleukine-1 β; TNF-α: tumor necrosis factor α; VEGF: vascular endothelial growth factor; Amylin: Islet Amyloid Polypeptide, IAPP; GIP: gastric inhibitory peptide or glucose-dependent insulinotropic peptide; PYY: peptide YY. Values are the means ± SD. Statistical analyses were performed using the One-way Analysis of Variance between each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the HFD control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the ND control group. ①Only one rat of ALA50 group rats tested positive for amylin, the rest of the rats tested negative for amylin. ②, ③None of the ALA100 or the ALA300 group rats tested positive for amylin.

the efficiency of digestion and nutrient absorption. Unexpectedly, we found that the plasma levels of this hormone in the ALA300 group was significantly increased, which could cause a decrease in appetite. The trends of these appetite-related factors were inconsistent, so we speculate the influence of amylin may be stronger than PYY. The underlying specific mechanism still needs further investigation.

Also, previous studies indicated that the mechanism of 5-ALA/SFC improves the metabolism of lipid and glucose through the induction of heme oxygenase-1 (HO-1) or mitochondrial OXPHOS complexes III, IV, and V [17, 18]. We further investigated the molecular mechanisms of these effects through other related genes. First, mitochondrial activity increased in the liver, white fat and brown adipose tissue as the expression of *COX*IV, *UCP2* and *UCP1* significantly increased. *COX*IV is a component of the mitochondrial complex. Our study showed that 5-ALA/SFC indeed contributed to the improvement of COX activity, which is consistent with a previous study [29]. *UCP2* can promote fatty acid oxidation [30] and control the production of reactive oxygen species to protect and resist oxidative stress [31]. In the present study, the expression of *UCP2* significantly increased in the liver, suggesting that the reduction of fat deposition observed in the liver might be caused by higher mitochondrial activity, especially by *UCP2* activation. *UCP1* is restricted to brown adipose tissue. Since white adipose tissue can transform into brown adipose tissue, *UCP1* could be produced in the transformation

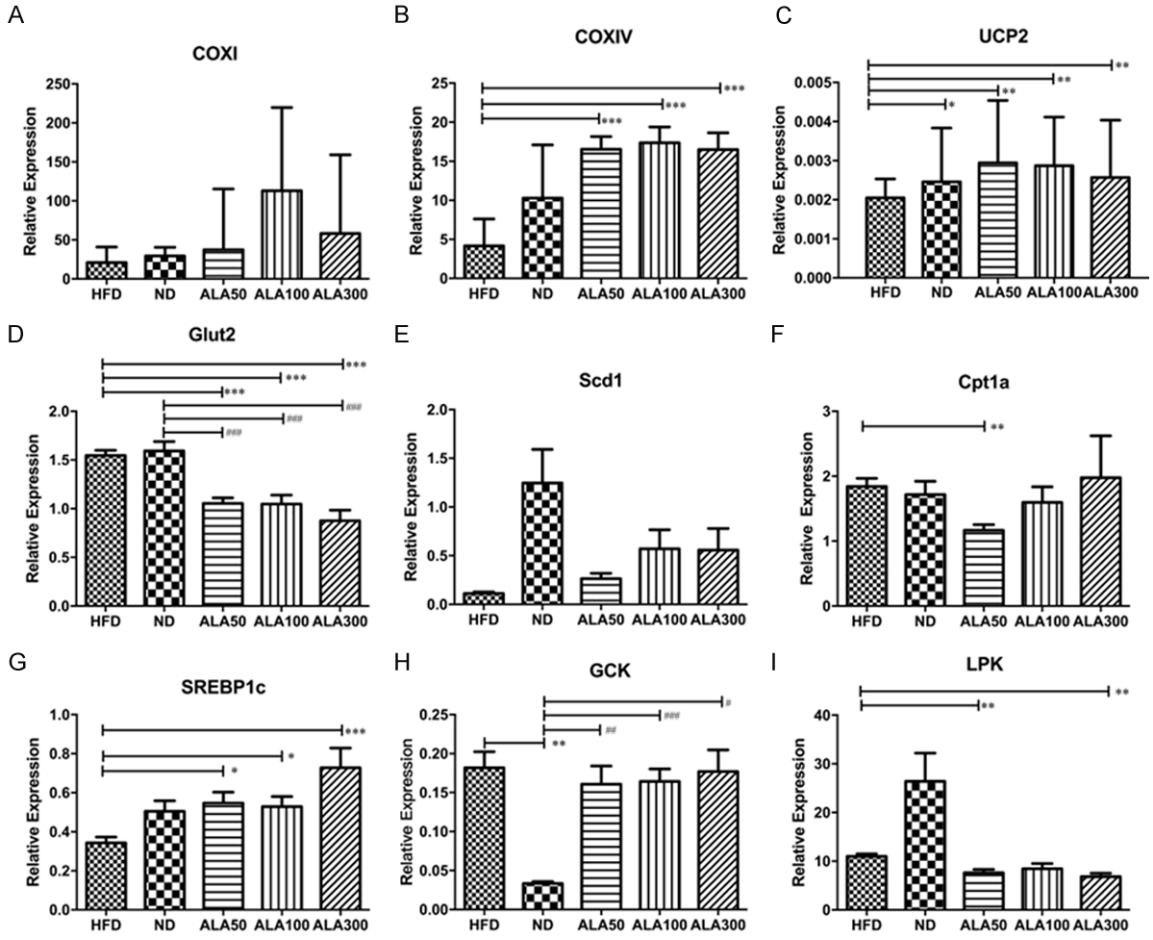

Figure 5. The expressions of the related genes in the liver. Data represent the mean ± SD. Statistical analyses were performed using the One-way Analysis of Variance between each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the HFD control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the ND control group. (ND, n = 11; HFD, n = 11; ALA50, n = 10; ALA100, n = 10; ALA300, n = 9).

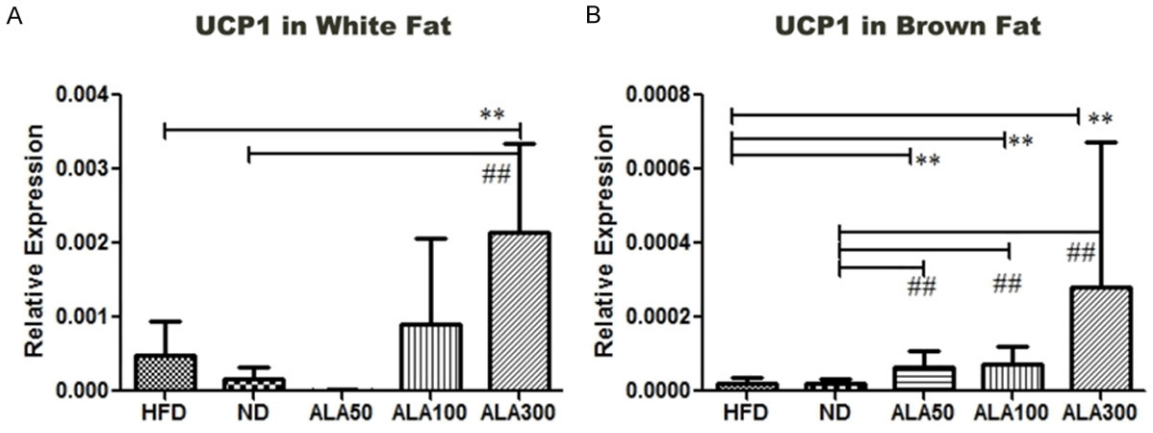

Figure 6. The expression of UCP1 in related fat. A. The expression of UCP1 in white fat. B. The expression of UCP1 in brown fat. Data represent the mean ± SD. Statistical analyses were performed using the One-way Analysis of Variance between each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the HFD control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the ND control group. (ND, n=11; HFD, n = 11; ALA50, n = 10; ALA100, n = 10; ALA300, n = 9).

state of white adipose tissue. *UCP1* could mediate heat generation in brown fat, uncoupling the respiratory chain. Even in the case of a low rate of ATP production, it can still mediate fast substrate oxidation [32, 33]. As shown in **Figure 6**, the expression of *UCP1* was higher in white adipose tissue and brown adipose tissue in the 5-ALA/SFC treated rats, especially in the ALA300 group. Therefore, the retroperitoneal fat was lower in the 5-ALA/SFC treated group compared with the HFD group. Secondly, as an important facilitative glucose transporter in the liver, *Glut2* controls the glucose across the membranes [34]. A previous study showed that reducing the expression of *Glut2* might improve dyslipidemia and hepatic steatosis by decreasing fructose into hepatic cells [35]. In our results, the expression of *Glut2* in the 5-ALA/SFC treated groups was significantly lower compared with the expression in the HFD and ND rats, suggesting that 5-ALA/SFC might decrease the expression of *Glut2* and then result in less glucose being transported into the hepatic cells as a precursor of lipogenesis. It also to a certain degree explains the reason why 5-ALA/SFC reduces fat deposition in liver. As a lipogenic transcription factor, *SREBP-1c* mainly controls the lipogenic process through the activation of fatty acid and triglyceride synthesis related genes [36]. The transcription of *SREBP-1c* is mainly regulated by liver X-activated receptors (LXR), insulin, and glucagon. Also, the unsaturated fatty acids can reduce the mRNA expression of *SREBP-1c* through LXR [37]. In our results, the expression of *SREBP-1c* was significantly lower in the HFD than it was in the 5-ALA/SFC treated groups. This implies that a high-fat diet might inhibit the transcription of *SREBP-1c,* and that 5-ALA/SFC might restore the expression of *SREBP-1c* to maintain the metabolic balance. These indicated that 5-ALA/SFC might improve the pathoglycemia and dyslipidemia, reduce fat deposition, and body weight by enhancing mitochondrial activity and regulating the lipid and glucose metabolism related genes.

In the present study, we found that 5-ALA/SFC could improve insulin resistance. We also investigated other glucose and lipid related hormones besides insulin. HOMAIR is used for assessing insulin resistance based on fasting plasma glucose and insulin concentration levels [38]. It showed that the HOMA-IR index was remarkably decreased in the 5-ALA/SFC treated groups and in the ND group compared with the HFD group, indicating that 5-ALA/SFC could effectively improve insulin resistance. C-peptide secretes with insulin from pancreatic β cells but has a longer half-life than insulin. The plasma level of C-peptide reflects the status of pancreatic β cells. Due to the dysfunction of the β cells, the concentration of C-peptide might decrease in the later stages of type 2 diabetes [39, 40]. In our results, there was no difference between each group. It indicated that the 5-ALA/SFC might have no influence on pancreatic β cells. Adiponectin is secreted from adipocytes and plays an important role in the regulation of glucose and lipid metabolism [41]. Both type 1 and type 2 diabetes patients with nephropathy were found to have higher levels of adiponectin [42]. The plasma level of adiponectin in the 5-ALA/SFC treated groups was significantly lower than it was in the HFD group, suggesting that 5-ALA/SFC might influence the secretion of adiponectin to prevent the development of diabetic complications. These results demonstrated that 5-ALA/SFC might play a role in preventing metabolism disorders through above hormones.

Previous studies have shown that inflammatory factors might influence the development of diabetes. Hyperglycemia tends to result in metabolic stress and inflammatory response. We explored the effects of 5-ALA/SFC on the inflammatory related hormones for the first time. MCP-1 is a monocyte chemotactic factor, which was first found in the media of human myelomonocytic cell lines. It has been found that MCP-1 is associated with insulin resistance and significantly increased in the obesity and type 2 diabetic conditions [23, 43-46]. In addition, as shown in our results, the concentration of MCP-1 in the ALA300 group was significantly lower than it was in the HFD group. Moreover, TNF-α plays an important role in the catabolism of adipocytes, and it is increased in type 2 diabetic conditions [21, 23]. The plasma level of TNF-α was significantly reduced in ALA300 group compared with ND group. These results mean that 5-ALA/SFC might prevent obesity and the development of diabetes through regulating insulin resistance and catabolism.

Body weight was significantly lower in the ALA-300 group compared with the ND group, suggesting that 5-ALA/SFC might exist some toxic effects. Moreover, *LPK* is a key enzyme in glu-

cose metabolism and catalyzes the last step of glycolysis. It has been shown that the level of *LPK*, which is decreased in diabetic rats, directly influences the utilization of glucose in the liver [47, 48]. We found the baseline of *LKP* in HFD group was lower compared with the ND group. However, giving 5-ALA/SFC further inhibited its expression. It is necessary to examine the effects of long-term treatment with 5-ALA/SFC in rats with a normal diet in future studies.

In summary, 5-ALA/SFC effectively promotes appetite, while ameliorating obesity and preventing the development of pre-diabetes. It suggests these effects achieved their functions by promoting metabolism and enhancing mitochondrial activity. However, the deeper molecular mechanism underlying the improvement of appetite and the promotion of metabolism needs further investigation.

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Disclosure of conflict of interest

None.

Abbreviations

5-ALA, 5-aminolevulinic acid; SFC, sodium ferrous citrate; HFD, high fat diet; ND, Normal Diet; FPG, fasting plasma glucose; PBG, 2 h postprandial blood glucose; IL-1β, Interleukine-1 β; TNF α, Tumor Necrosis Factor α; VEGF, vascular endothelial growth factor; COXI, Cytochrome C oxidase subunit I; COXIV, Cytochrome C oxidase subunit IV; UCP1, uncoupling protein 1; UCP2, uncoupling protein 2; GIP, gastric inhibitory polypeptide; PYY, peptide YY; HE, hematoxylin and eosin; q-PCR, Quantitative PCR; LPK, liver pyruvate kinase; glut2, Glucose transporter 2; SREBF1, Sterol regulatory element-binding transcription factor 1; Cpt1a, carnitine palmitoyltransferase 1a; Scd1, stearoyl-CoA desaturase-1; GCK, glucokinase; MCP-1, monocyte chemotactic protein 1.

Address correspondence to: Daru Lu, State Key Laboratory of Genetic Engineering, School of Life Sciences and Zhongshan Hospital, Fudan University, 2005 Songhu Road, Shanghai 200438, China. E-mail: darulu@163.com

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