Journal of Trace Elements in Medicine and Biology 68 (2021) 126851

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Dietary copper supplementation enhances lipolysis in Rex rabbits

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ARTICLE INFO

Keywords:
Rex rabbit
Copper
Lipid metabolism
Hormone
PPAR

ABSTRACT

Background: Copper is an important regulator of lipid metabolism in mammals, as a cofactor of many enzymes and is involved in the lipolysis. Copper deficiency has been considered as a significant factor in human diseases related to abnormal lipid metabolism, while adding copper to the diet seems to be the simplest and most effective way to prevent copper deficiency.

Aims: The aim of this study was to investigate the effects of dietary copper level on lipid metabolism in Rex Rabbits.

Methods: A total of 120 90-d-old Rex Rabbits were randomly allotted into three treatments, with 40 replicates (20 males, 20 females) in each treatment (1 rabbit per replicate). The diets included 1) control (8.4 mg/kg), normal-copper diet (39.1 mg/kg), 3) high-copper diet (67.5 mg/kg). The trial including a one-week adaptation period and a five-week experimental period.

Result: The results showed that copper (39.1 mg/kg) diet increased average daily feed intake (ADFI) ($P < 0.05$, N = 34), and tended to increase the final body weight (FBW) ($P = 0.0556$, N = 34). Moreover, dietary copper addition (39.1 and 67.5 mg/kg) significantly increased the foreleg and hindleg weight ($P < 0.05$, N = 8), and decreased the weight of Perirenal fat and the concentration of triglycerides (TG) in the liver ($P < 0.05$, N = 8). The concentration of triglycerides (TG), epinephrine (EPI), and glucagon (GC) in serum were obviously higher than that in control group ($P < 0.05$, N = 8), and the concentration of insulin (INS), and very low-density lipoprotein (VLDL) in serum were significantly decreased ($P < 0.05$, N = 8). The copper group (39.1 mg/kg) showed up-regulated gene expression levels of carnitine palmitoyl transferases (CPT-1 and CPT-2) and peroxisome proliferator-activated receptor (PPAR-α) in liver ($P < 0.05$, N = 8) and down-regulated gene expression levels of fatty acid synthase (FAS) and Acetyl-CoA carboxylase (ACC) ($P < 0.05$, N = 8). In skeletal muscle, CPT-1, CPT-2, PPAR-α, fatty acid transport protein (FATP), fatty acid-binding protein (FABP) and lipoprotein lipase (LPL) levels were significantly up-regulated by copper treatment ($P < 0.05$, N = 8). Rex Rabbits receiving copper addition had higher CPT-1, CPT-2, PPAR-a and hormone-sensitive lipase (HSL) mRNA levels in adipose tissue ($P < 0.05$, N = 8).

Conclusion: Copper diets promoted skeletal muscle growth and reduced fat accumulation by enhancing fatty acid oxidation, at the same time, dietary copper inhibited De novo lipogenesis in the liver. PPAR-α signaling in liver, skeletal muscle and adipose tissues were involved in the regulation of lipid metabolism by copper.

1. Introduction

Fat and fatty acids are the most commonly stored and circulating forms of energy in mammals [1]. Generally speaking, the liver, adipose tissue and skeletal muscle play a more important role in fatty acid metabolism compared with other tissues [2]. The relative importance of these tissues in lipid metabolism depends on species differences: in poultry, the liver is the main site of *De novo* lipogenesis [3]; in ruminants, on the contrary, the liver produces only a small amount of *De novo* lipogenesis compared with adipose tissue [4]; in other mammals, both the liver and adipose tissue play an important role in *De novo* lipogenesis.

A variety of regulatory factors play a regulatory role in the utilization of fatty acids. Fatty acid synthase (FAS) and Acetyl-CoA carboxylase (ACC) are rate-limiting enzymes in the process of fatty acid synthesis, and carnitine palmitoyl transferases (CPT-1 and CPT-2) are rate-limiting

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https://doi.org/10.1016/j.jtemb.2021.126851
Received 15 May 2021; Received in revised form 30 July 2021; Accepted 24 August 2021
Available online 26 August 2021
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enzyme for the oxidation of long-chain fatty acids [5]. Hormone-sensitive lipase (HSL) enzymes is a multifunctional enzyme that hydrolyzes lipids in various tissues [6,7]. The lipoprotein lipase (LPL) is synthesized from adipose tissue and secreted into the blood, which hydrolyzes triglycerides in very low-density lipoprotein (VLDL) and chylous particles to enhance lipolysis. In skeletal muscle, the fatty acid transport protein (FATP) and the fatty acid-binding protein (FABP) promote the cellular binding, uptake, and transport of fatty acids. Peroxisome proliferators-activated receptors (PPARs) is a ligand-activated receptor in the nuclear transcription factors family, three subtypes (PPAR-α, PPAR-β, PPAR-γ) of it have been found in different species, which control various cellular metabolic processes and belong to ligand-induced nuclear receptors [8]. PPAR-α can enhance fatty acid catabolism (β and ω oxidation pathways) [9] and widely found in the liver, adipose tissue and skeletal muscle [10]. PPAR-γ regulates adipogenic differentiation of adipocytes in vitro and fat deposition in vivo [11], and the main sources of PPAR-γ are fat, macrophages and mammary tissue [12]. PPAR-γ can regulate the expression of genes related to lipid metabolism in adipose tissue such as LPL and FABP [13]. In addition, hormone (e.g., insulin, glucagon, and epinephrine) [14,15] and nutritional state (e.g., copper and energy) [16,17] also regulate lipid metabolism to a certain extent.

Copper (Cu) is an indispensable trace element for mammals [18], and plays an important role in the regulating of lipid metabolism [16] and whose improper regulation contributes to obesity and diseases (e.g., diabetes, cancer, and cardiovascular diseases) [19–21]. However, it is noting that humans are rarely diagnosed with copper deficiency [22]. The addition of copper to the diet is often ignored, which happens to be an important way to supplement copper [23]. In rats, Dietary copper deficiency can regulate the level of plasma cholesterol and lipoprotein [24]. Changes in lipid metabolism were also observed in patients and mice with genetic defects in copper metabolism [25,26]. Indeed, dietary copper can alter fat storage [27]. Sui et al. [2] reported that dietary copper supplementation significantly decreased the perirenal fat yield in growth Rex rabbits. However, studies in pigs have not yielded similar results [28]. Rabbits has been widely used as animal model in biomedical researches because of the closely phylogenetic relatedness to human being, short vital cycle and other advantages [29]. In particular, rabbit resembles human closely with respect to lipoprotein metabolism, therefore, rabbit is considered to be the preferred animal model for the study of human lipid metabolism [29,30]. In general, studies have shown the important role of copper in the regulating of lipid metabolism, but these results need to be verified through a holistic study on the regulatory mechanism of copper on lipid metabolism.

Abnormal lipid metabolism and diseases related to abnormal lipid metabolism are becoming global health problems in the middle-aged and elderly. Cardiovascular disease (CVD) caused by hyperlipidemia is one of the leading causes of death in the United States [31]. In fact, hyperlipidemia and obesity in children and adolescents has been increasing in the past few decades [32]. At the same time, recent study has shown that childhood obesity is associated with a long-term increase in cardiovascular disease [33]. In view of the important regulatory role of copper on lipid metabolism in *vivo* and *vitro*. The present study explored the effect of dietary copper supplementation on the lipid deposition, skeletal muscle growth and serum hormone of Rex rabbits. At the same time, we also studied the effects of dietary copper on signaling pathway (PPARs) related to lipid metabolism in the liver, skeletal muscle, and adipose tissue of Rex rabbits.

2. Materials and methods

2.1. Animal protocol and dietary treatments

This study was carried out in the Rabbit Nutrition Laboratory of Shandong Agricultural University (Tai'an, China). All protocols of this study were approved by the local experimental animal care committee and the institutional ethics committee of the Nutrition and Clinical Nutrition Department (Shandong Agricultural University).

120 90-day-old healthy Rex rabbits (60 males, 60 females) with an average body weight of 1,947.07 g were divided into three treatments, with 40 replicates (20 males, 20 females) in each treatment (1 rabbit per replicate). Copper was added as copper sulfate pentahydrate (CAS: 7758−99-8; Shanghai aladdin Biochemical Technology Co., Ltd., Shanghai, People's Republic of China). The treatments were a basal diet (control: measured Cu content 8.4 mg/kg) or a basal diet supplemented with 30 or 60 mg/kg Cu (measured Cu content 39.1 and 67.5 mg/kg, respectively). The rabbits were housed in single cages and fed twice a day, at 8:00 and 16:00. The rabbits could drink water freely. Before the experiment, the experimental environment was thoroughly cleaned and disinfected and fumigated with potassium permanganate and formaldehyde. Natural lighting and ventilation were maintained throughout the experimental period. The cages were sterilized once a week. The trial lasted for six weeks (including a one-week adaptation period and a five-week experimental period). The composition and chemical analysis of the basal diet are shown in Table 1.

2.2. Sample collection and preparation

At the end of the trial, 8 rabbits (half male and half female) per group were electrically stunned (70 V, pulsed direct current, 50 Hz for 5 s), and 10 mL of blood sample was collected by cardiac puncture applying serum separation tube (BD-Pharmingen, USA). The blood samples were centrifuged at 3000 r/min for 10 min, ant the upper serum was transferred to 1.5 mL centrifuge tubes and stored at -80℃ until analysis. The rabbits were killed by cervical dislocation, and the liver, foreleg, hindleg, shoulder fat, perirenal fat and perigastric fat were weighed. According to the effects of copper on lipid accumulation and serum hormone, the copper dose of 30 mg/kg (measured copper content 39.1 mg/kg) were selected for the subsequent experiments. 1−2 g liver, Perirenal fat and hindleg muscle were placed in a frozen tube (Thermo Fisher, Carlsbad, Ca, USA), cooled with liquid nitrogen and preserved at −80 °C for gene expression analysis. About 5 g shoulder fat, Perirenal fat, Perigastric fat were placed in a frozen tube, cooled with liquid nitrogen and preserved at −80 °C for tissue mineral contents analysis.

2.3. Sample analysis

2.3.1. Growth performance

The body weight of rabbits was recorded at the beginning and end of

Table 1
Composition and nutrient levels of the basal diet (air-dry basis) fed to Rex rabbits.

Ingredients	%	Chemical analysis[b]	%
Corn	10.50	DE (MJ/kg)	10.37
Soybean meal	6.00	DM	89.16
Corn germ meal	20.00	CP	16.87
Wheat bran	18.00	Ash	6.83
Husk powder	11.00	EE	4.39
Sunflower meal	12.00	CF	15.79
Alfalfa	6.00	Ca	0.70
Soya bean stem meal	12.00	P	0.54
Artemisia apiacea flour	3.00	Lys	0.53
Premix[a]	1.50	Met	0.89
Total	100.00		

[a] The premix provided the following per kilogram of diet: vitamin A, 10,000 IU; vitamin D4, 100 IU; vitamin E, 60 mg; vitamin K3, 2 mg; vitamin B1, 5 mg; vitamin B2, 10 mg, vitamin B11, 2.5 mg; vitamin B12, 0.01 mg; choline chloride, 600 mg; iron (as ferrous sulfate), 50 mg; zinc, 50 mg; selenium, 4 mg; iodine, 0.6 mg; manganese, 4 mg; $CaHPO_4$, 1600 mg; NaCl, 4800 mg; lysine, 1000 mg; methionine, 2000 mg; stone powder, 1600 mg.

[b] Digestive energy is theoretically calculated, and other nutritional indicators are measured values.

the experimental period. The feed intake of each rabbit was recorded daily. The average daily feed intake (ADFI), average daily weight gain (ADG), and feed/gain ratio (F/G) were calculated.

ADG = {final body weight – initial body weight} / 35

ADFI = total feed intake per rabbit during the whole experimental period / 35

F/G = ADFI/ADG

2.3.2. Serum sample

The concentrations of serum triglyceride (TG), total cholesterol (TCHO), urea nitrogen (UREA), high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL) (kits supplied by College of Animal Science and Technology, Shandong Agricultural University, Tai'an, People's Republic of China) was measured by an automatic biochemical analyzer (Hitachi 7020, Hitachi High Technologies, Inc., Ibaraki, Japan). The content of very low-density lipoprotein (VLDL), glucagon (GC), insulin (INS) and epinephrine (EPI) in serum were determined using Shanghai Enzyme-linked Biotechnology kits (Shanghai Enzyme-Linked Biotechnology Co., LTD, Shanghai, People's Republic of China).

2.3.3. Tissue mineral contents

The Cu content in samples were measured by atomic absorption spectrophotometry (Analytik Jena novAA 400 P, Jena, Germany) and all results are expressed in fresh weight.

2.3.4. Liver triglyceride

Extraction of TG from liver was used Methanol chloroform method. Liver sample (0.1 g) was transferred into centrifuge tube while adding mill bead (1 mm) (Servicebio, Wuhan, Hubei, People's Republic of China) and 0.3 mL methanol (Tianjin Cartatton Industry Co., LTD., Tianjin, People's Republic of China) and then transferred the centrifuge tube to the KZ-III-FP high-speed and low-temperature tissue grinder (Servicebio, Wuhan, Hubei, People's Republic of China) 4℃ for 120 s. And then 0.6 mL chloroform was added to the centrifuge tube, and fully oscillating mixing. The homogenate was extracted for 24 h, and the lower organic solvents were carefully extracted and incubated at 37℃ for 20 min, and then centrifuged at 6000 r/min for 10 min. Finally, the concentration of TG was determined according to the triglyceride determination kit (Shanghai Enzyme-Linked Biotechnology Co., LTD, Shanghai, People's Republic of China).

2.3.5. RNA isolation and analysis

Total RNA was extracted by the Trizol (Thermo Fisher, Carlsbad, Ca, USA) method. The RNA concentration was measured on a DU 640 nucleic acid spectrophotometer (Beckman Coulter, Inc., 250 S. Kraemer Boulevard Brea, Ca, USA). The quality of extracted RNA was detected by agarose gel electrophoresis. Reverse transcription reactions (20 μL) contained 1000 ng of total RNA, 4 μL 5 ×Evo M-MLVRT Master Mix (supplied by the Accurate Biotechnology Co., Ltd., Hunan, People's Republic of China). Real-time PCR analysis was carried out with an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster, CA, USA). Each RT reaction served as a template in a 20 μL PCR containing 0.2 mol/L of each primer and SYBR Green master mix (Takara, Dalian, People's Republic of China). Real-time PCRs were performed at 95℃ for 10 s of pre-denaturation, followed by 40 cycles of denaturation at 95 ℃ for 5 s and annealing and extension at 60℃ for 40 s. A standard curve was plotted to calculate the efficiency of real-time PCR primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the normalization gene, and the results of the relative mRNA quantification was verified using β-actin levels [2,34], and mRNA expression was analyzed using the $2^{-\Delta\Delta CT}$ method [35]. The primer sequences are shown in Table 2.

Table 2
Primer sequences of related genes.

Genes [a]	GenBank accession number	Primer sequences (5′ - 3′)	Product size/bp
PPAR-α	XM_002723354	F: 5′-AGGCCCTCTTCAGAACCTGT-3′ R: 5′-GTGGCTTTCTGTTCCCAGAG-3′	122
PPAR-γ	NM_001082148.1	F: 5′-GGAGCAGAGCAAAGAAGTCG-3′ R:5′-CTCACAAAGCCAGGGATGTT-3′	111
FATP	XM_002722970	F: 5′-GGCCTACCTCTCTGGTGATG-3′ R:5′-TCAGTGGTGGACACGTTCTC-3′	111
FABP	XM_002716060	F: 5′-AGCTGGTGGACAGCAAGAAT-3′ R:5′-TCAGGGTGATGATGTCTCCA-3′	129
CPT1	XM_002724092.2	F: 5′-ATTCTCACCGCTTTGGGAGG-3′ R:5′-ACGGGGTTTTCTAGGAGCAC-3′	196
CPT2	XM_008265231.1	F: 5′-ATGACCGTTTCTGCCATCC-3′ R:5′-AAGGTGTTGGTGTCGCTTCT-3′	101
FAS	KF201292.1	F: 5′-ACCACGTCCAAGGAGAGCA-3′ R:5′-AGTTCTGCACCGAGTTGAGC-3′	112
ACC	XM_002719077.2	F: 5′-GTGGTCTTCGTGTGAACTGG-3′ R:5′-TTCTTCTGCTGCCTTTAGCC-3′	122
HSL	XM_008249691.2	F: 5′-CCAGGCTAAACTCGCATCCA-3′ R:5′-ATTTGGCTCTCTGGACTGGC-3′	119
LPL	NM_001177330.1	F: 5′-TTCAACCACAGCAGCAAGAC-3′ R:5′-TAACAGCCAGTCCACCACAA-3′	141
GAPDH	NM_001082253.1	F: 5′-CACCAGGGCTGCTTTTAACTCT-3′ R:5′-CTTCCCGTTCTCAGCCTTGACC-3′	163
β-actin	XM_002722894.3	F: 5′-CGCAGAAACGAGACGAGATT -3′ R:5′-GCAGAACTTTGGGGACTTTG -3′	123

[a] PPARα = peroxisome proliferators-activated receptor α; PPARγ = peroxisome proliferators-activated receptor γ; FATP = fatty acid transport protein; FABP = fatty acid binding protein; CPT1 = carnitine palmitoyltransferase 1; CPT2 = carnitine palmitoyltransferase 2; FAS = fatty acid synthase; ACC = Acetyl-CoA carboxylase; HSL = Hormone-sensitive lipase; LPL = lipoprotein lipase; GAPDH = glyceraldehyde phosphate dehydrogenase.

2.4. Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was performed on more than two groups of data, and then Dunnett's multiple comparisons or Tukey's HSD were carried out. *T*-Test was performed on the two groups of data. All statistical analyses were carried out using JMP Pro software (SAS Institute, Cary, NC). Before the analysis of ANOVA or the t-test, the variance homogeneity of the data was analyzed. For growth performance, n = 34; for tissue and organ weight analysis, n = 8; for blood biochemistry analysis, n = 8; and for mRNA levels analysis, n = 8. The difference at $P < 0.05$ was considered to be significant, and *P*-value between 0.05 and 0.1 were considered as a trend.

3. Results

3.1. Effects of dietary copper levels on the performance of Rex rabbits

The effects of dietary Cu supplementation on the growth performance of Rex rabbits are shown in Table 3. Average daily feed intake (ADFI) were greatly influenced with the supplementation of copper ($P < 0.05$). During the whole trial period, there was no significant difference in average daily gain (ADG) and the feed weight ratio (F/G) among the treatment groups ($P > 0.05$), but the final body weight (FBW) tended to increase with the increase of dietary copper ($0.05 < P < 0.1$).

3.2. Effect of dietary copper levels on liver growth, skeletal muscle growth and lipid accumulation of Rex rabbits

The effects of dietary Cu supplementation on liver growth, skeletal muscle growth and lipid accumulation of Rex rabbits are shown in Table 4. The dietary addition of copper significantly decreased the ratio and yield of perirenal fat compared to the control group ($P < 0.05$) and the foreleg and hindleg yield was significantly increased ($P < 0.05$), but it had no significant effect on the rate of foreleg and hindleg. Dietary copper treatment demonstrated no significant effect on the weight or ratio of liver, shoulder fat and perigastric fat.

3.3. Effect of dietary copper levels on the serum biochemistry in Rex rabbits

Compared with the control group, the Cu level did not significantly affect the serum levels of UREA, TCHO, HDL, LDL ($P > 0.05$, Table 5). However, copper treatment significantly increased the TG content in the serum ($P < 0.05$, Fig. 1A). In contrast, copper treatment significantly decreased the VLDL content in the serum ($P < 0.05$, Fig. 1A).

Compared with the control group, copper treatment significantly increased the concentration of EPI and GC in the serum ($P < 0.05$, Fig. 1B; C). The INS content in the group supplemented with copper were lower than that in the control group ($P < 0.05$, Fig. 1C).

Copper treatment significantly decreased the concentration of TG in the liver compared with the control group ($P < 0.05$, Fig. 2).

3.4. Effect of dietary copper levels on adipose tissue concentrations of copper in Rex rabbits

The effects of dietary Cu addition on adipose tissue Cu contents in Rex rabbits are shown in Table 6. Dietary copper treatment demonstrated no significant effect on adipose tissue concentrations of copper.

Table 3
Effects of the level of dietary copper supplementation on growth performance of Rex rabbits.

Items	Level of dietary copper supplement (mg/kg)			
	0	30	60	*P*-value
IBW(g)	1926.94 ± 32.06	1985.29 ± 28.49	1928.97 ± 39.11	0.3801
FBW(g)	2674.71^{b} ± 34.48	2787.65^{a} ± 37.08	2712.50^{ab} ± 27.72	0.0556
ADG(g/d)	21.36 ± 0.49	22.92 ± 0.49	22.39 ± 0.66	0.1327
ADFI(g/d)	164.95^{b} ± 0.38	166.26^{a} ± 0.28	166.13^{a} ± 0.32	0.0102
F/G	7.89 ± 0.23	7.36 ± 0.16	7.62 ± 0.21	0.1930

IBW = initial body weight; FBW = final body weight; ADG = average daily weight gain; ADFI = average daily feed intake; F/G = feed/gain.
^{a,b}Mean values in a row sharing no common superscript are significantly different ($P < 0.05$).

Table 4
Effect of dietary copper levels on liver growth, skeletal muscle growth and lipid accumulation in Rex rabbits.

Items	Dietary copper supplemental level (mg/kg)			
	0	30	60	*P*-value
Live weight, g	2640.00 ± 19.64	2677.86 ± 20.50	2682.86 ± 17.72	0.256
Liver yield, g	67.89 ± 2.34	71.22 ± 2.70	73.49 ± 2.70	0.3389
Liver yield/BW, %	2.57 ± 0.08	2.66 ± 0.09	2.74 ± 0.10	0.4425
Foreleg yield, g	151.82^{b}±3.57	165.44^{a}±3.95	165.49^{a}±2.23	0.0129
Hindleg yield, g	336.24^{a}±2.97	348.89^{b}±4.24	350.34^{b}±2.12	0.0116
Foreleg yield/BW, %	5.76 ± 0.17	6.18 ± 0.18	6.17 ± 0.11	0.1154
Hindleg yield/BW, %	12.74 ± 0.14	13.03 ± 0.17	13.06 ± 0.11	0.2378
Shoulder fat yield, g	6.73 ± 0.86	4.95 ± 0.53	5.13 ± 0.88	0.2266
Perirenal fat yield, g	19.89 ± 1.39	13.53 ± 1.00	15.77 ± 0.97	0.0032
Perigastric fat yield, g	5.23 ± 0.65	7.40 ± 1.39	7.78 ± 0.49	0.1418
Shoulder fat yield/BW, %	0.25 ± 0.03	0.19 ± 0.02	0.19 ± 0.03	0.1960
Perirenal fat yield/BW, %	0.75^{a}±0.05	0.50^{b}±0.04	0.59^{b}±0.04	0.0024
Perigastric fat yield/BW, %	0.20 ± 0.03	0.28 ± 0.05	0.29 ± 0.02	0.1740

The organ index was a percentage to BW and expressed as %.
^{a,b}Mean values in a row sharing no common superscript are significantly different ($P < 0.05$).

Table 5
Effect of dietary copper levels on the serum parameters of Rex rabbits.

Items	Dietary copper supplemental level (mg/kg)			
	0	30	60	*P*-value
UREA (mmol/L)	7.42 ± 0.41	7.35 ± 0.29	7.54 ± 0.21	0.9096
TCHO (mmol/L)	1.47 ± 0.11	1.56 ± 0.19	1.15 ± 0.11	0.1328
HDL (g/L)	0.08 ± 0.01	0.09 ± 0.02	0.06 ± 0.01	0.2776
LDL (g/L)	0.24 ± 0.05	0.28 ± 0.06	0.16 ± 0.03	0.3112

TCHO = total cholesterol; HDL = high-density lipoprotein cholesterol; LDL = low-density lipoprotein cholesterol.
^{a,b}Mean values in a row sharing no common superscript are significantly different ($P < 0.05$).

3.5. Effect of dietary copper levels on the enzyme mRNA levels of Rex rabbits

Effect of dietary copper treatment on relative gene expression in lipid metabolism in the liver, skeletal muscle, and adipose tissue were shown in Fig. 3. Through the determination of the expression of genes related to lipid metabolism, the results showed that the addition of copper to the diet significantly increased gene expression of *CPT-1*, *CPT-2* and *PPAR-α*, and decreased gene expression of *FAS* and *ACC* in the liver ($P < 0.05$, Fig. 3A). Gene expression of *CPT-1, CPT-2, FATP*, *FABP*, *PPAR-α* and *LPL* in the skeletal muscle was significantly upregulated by copper treatment ($P < 0.05$, Fig. 3B). Compared with the control group, copper treatment significantly increased the mRNA levels of *CPT-1, CPT-2, PPAR-α* and *HSL* in adipose tissue($P < 0.05$, Fig. 3C). However, there were no significant influence on the mRNA levels of *FAS*, *ACC*, *PPAR-γ* and *LPL*.

4. Discussion

4.1. Effects of dietary copper level on the performance, tissue development, and lipid deposition of Rex rabbits

Copper is widely used as a growth promoter in livestock and poultry

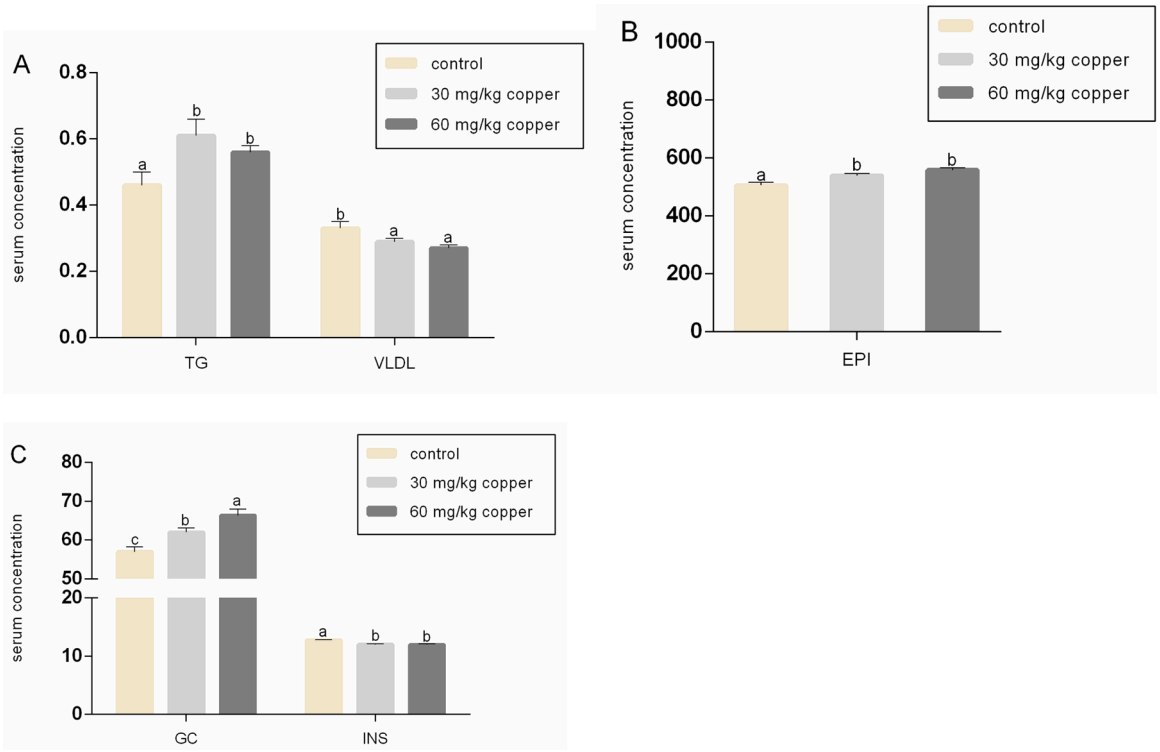

Fig. 1. Effect of dietary copper levels on serum concentrations of TG (mmol/L) (Fig. 1A), VLDL (mmol/L) (Fig. 1A), EPI (pg/mL) (Fig. 1B), GC (mU/L) (Fig. 1C) and INS (mU/L) (Fig. 1C) in Rex rabbits.
Values were obtained from duplicates of each sample; values are means ± SEM (n = 8).
[a–b] means with different letters differ significantly (P < 0.05) as shown by ANOVA.
(TG, triglycerides; VLDL, very low-density lipoprotein; GC, glucagon; INS, insulin; EPI, epinephrine).

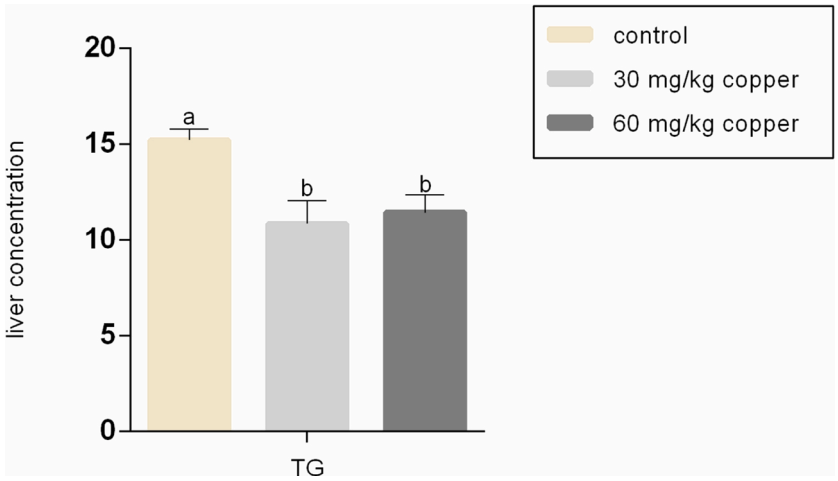

Fig. 2. Effect of dietary copper levels on liver concentration of TG (mmol/kg) in Rex rabbits.
Values were obtained from duplicates of each sample; values are means ± SEM (n = 8).
[a–b] means with different letters differ significantly (P < 0.05) as shown by ANOVA. (TG, triglycerides).

production [36]. In line with previous reports in weanling pigs [37], Rex rabbits [18] and young male mink (*Mustela vison*) [38], diet supplemented with copper increased the average daily feed intake (ADFI) (Table 3). However, in our experiment, the increased ADFI had no significant effect on the average daily gain (ADG), and the final body weight (FBW) tended to increase (Table 3), this might be because the Rex rabbits were at the end of the growing period, when Rex rabbits have slower growth. Meanwhile, our results showed that, copper addition decreased Perirenal fat weight, and increased foreleg and hindleg weight (Table 4). Interestingly, copper treatment had no significant effect on the foreleg and hindleg rate, it may be due to the tendency of the increased final body weight (FBW). Sui et al. [2] reported that dietary copper addition significantly decreased the weight of liver, which was inconsistent with our results. Therefore, we analyzed the concentration of TG in the liver and found that copper treatment significantly reduced the concentration of TG in liver (Fig. 2). These results indicating the changed redistribution of energy that reduced lipid deposition and promoted lipolysis. Meanwhile, we measured the copper content in adipose tissue (Table 6), and dietary copper addition had no significantly effects on the copper content in adipose tissue, which were

Table 6
Effect of dietary copper levels on adipose tissue concentrations of copper in Rex rabbits.

Items	Dietary copper supplemental level (mg/kg)			*P*-value
	0	30	60	
Shoulder fat (mg/kg)	0.95 ± 0.07	1.01 ± 0.05	1.08 ± 0.08	0.3762
Perirenal fat (mg/kg)	0.92 ± 0.08	1.06 ± 0.06	0.95 ± 0.05	0.3122
Perigastric fat (mg/kg)	0.99 ± 0.08	0.98 ± 0.06	1.01 ± 0.07	0.9721

[a,b]Mean values in a row sharing no common superscript are significantly different ($P < 0.05$).

consistent with previous studies [39].

4.2. Effects of dietary copper level on the serum parameters of Rex rabbits

Consistent with previous studies on rabbits [40], the present study showed that dietary copper addition had no significant effect on serum total cholesterol (TCHO), urea (UREA), high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) in Rex rabbits. Triglycerides (TG) is important components of lipids in serum [41,42]. Generally speaking, it is considered that the concentration of triglyceride in serum may reflect the status of lipid metabolism in animals [43]. In poultry, studies have shown that low-energy diets reduce lipid deposition accompanied by an increase in serum triglyceride content [34]. In our study, we found that the serum TG was significantly increased in the group supplemented with copper compared with the control group (Fig. 1A), at the same time, diets supplemented with copper significantly decreased the weight of Perirenal fat (Table 4), reflecting that copper promoted lipolysis and reduced fat deposition. The main function of very low-density lipoprotein (VLDL) is to transport endogenous triglycerides synthesized in the liver into the blood [44]. Compared with the control group, copper supplementation significantly decreased the content of VLDL in serum (Fig. 1A). The decrease of serum VLDL content may be due to the decrease of the substrate in the liver, that is, the decrease of newly synthesized lipids in the liver, this result is also related to the decreased transcription levels of *FAS* and *ACC* in the liver (Fig. 3A).

4.3. Effects of dietary copper level on the serum hormones of Rex rabbits

The regulation of hormones on lipid metabolism (e.g., glucagon, insulin, and epinephrine) has been reported [15,45,46]. The regulation of fatty acid synthase (FAS) by insulin (INS) and glucagon (GC) has been described in liver and adipocytes [47], insulin availability activates the enzyme whereas glucagon and inhibit its activity (via cyclic adenosine monophosphate (cAMP)-dependent phosphorylation) [1]. In Sprague-Dawley rats, dietary copper deficiency can lead to liver steatosis and insulin resistance [48], at the same time, copper supplementation can lead to an increase in insulin sensitivity [49]. Our result showed that dietary copper decreased serum insulin levels significantly (Fig. 1C), and the decrease of insulin level may be related to the increase of insulin sensitivity caused by dietary copper supplementation. Meanwhile, the level of glucagon in serum increased significantly in our result (Fig. 1C). Previous studies have shown that elevated epinephrine levels stimulate the hormone-sensitive lipase (HSL) through β_2-epinephrine, which leads to the breakdown of TG in adipose tissue [50]. In line with our results (Fig. 1B), Lin et al. [51] reported that diet supplemented with Cu significantly increased the concentration of epinephrine in plasma. In a word, our results showed that dietary copper supplementation increased the contents of hormones that promote lipolysis in serum and decreased the insulin content in serum which main function is to promote the synthesis of glycogen, fat and protein. Which explained the

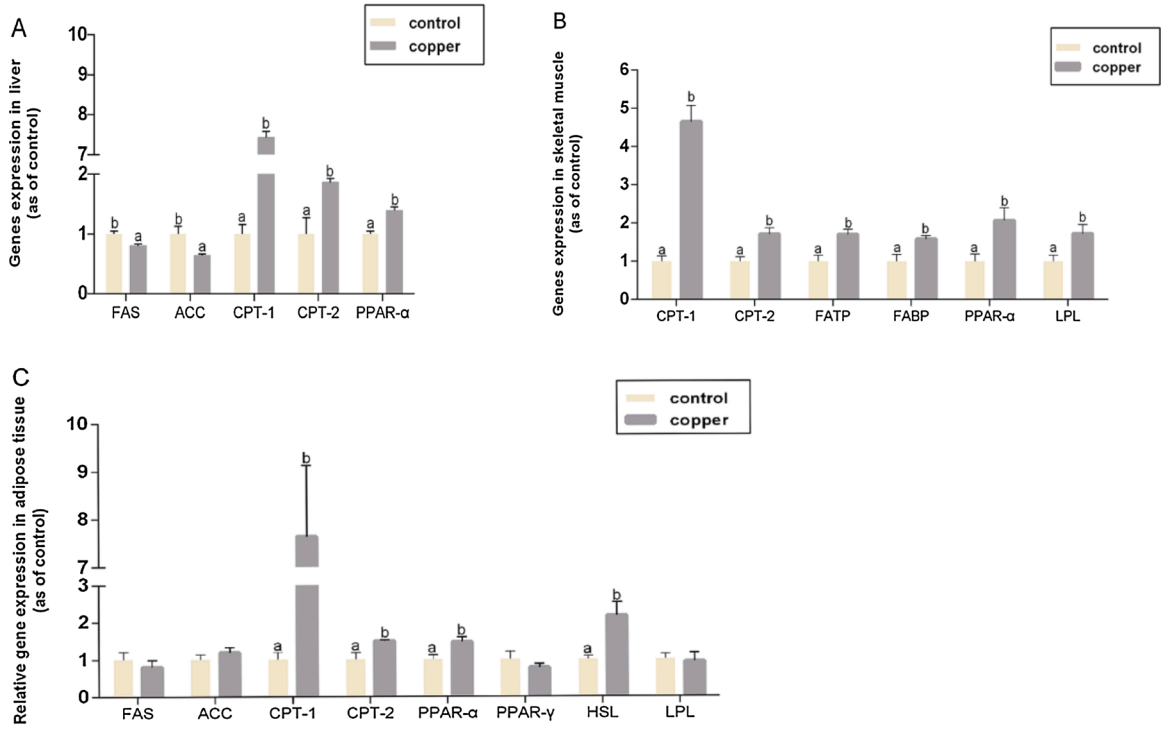

Fig. 3. Effect of dietary copper treatment (30 mg/kg) on relative gene expression in lipid metabolism in the liver (Fig. 3A), skeletal muscle (Fig. 3B), and adipose tissue (Fig. 3C).
Values were obtained from duplicates of each sample; values are means ± SEM (n = 8).
[a–b] means with different letters differ significantly (P < 0.05) as shown by ANOVA.
(FAS, fatty acid synthase; ACC, Acetyl-CoA carboxylase; CPT-1, carnitine palmitoyltransferase-1; CPT-2, carnitine palmitoyltransferase-2; PPAR-α, Peroxisome proliferator-activated receptor-α; FATP, fatty acid transport protein; FABP, fatty acid-binding protein; LPL, lipoprotein lipase; HSL, hormone-sensitive lipase; PPAR-γ, Peroxisome proliferator-activated receptor-γ).

decrease in the weight of perirenal fat and the increase in the weight of the skeletal muscle in our results (Table 4).

4.4. Effects of dietary copper level on enzyme mRNA levels of Rex rabbits

The liver is the hub of fatty acid synthesis and lipid circulation through lipoprotein, and plays a key role in lipid metabolism [1]. Our results showed that copper treatment decreased hepatic *FAS* and *ACC* gene expression and increased hepatic *CPT-1, CPT-2* and *PPAR-α*gene expression (Fig. 3A), which is consistent with previous studies in grass carp [52]. Decreased *FAS* and *ACC* mRNA levels indicated a decreased in *De novo* lipogenesis in the liver. Carnitine palmitoyl transferases (CPT) has two isozyme forms: CPT-1 and CPT-2, Located on the mitochondrial outer membrane and the inner side of the inner membrane of the mitochondria, respectively [53]. CPT-1 converts acyl-CoA into coenzyme A and fatty acyl carnitine in the cytoplasm, and then under the action of CPT-2, fatty acyl carnitine into carnitine and acyl-CoA. Finally, acyl-CoA enters the mitochondrial matrix for fatty acid β oxidation [54]. In present study, dietary copper addition can up-regulate the gene expression of CPT-1 and CPT-2 to enhance fatty acid oxidation in the liver. We believe that the increase in *CPT-1* and *CPT-2* gene expression may be due to the increase in energy required for detoxification of reactive oxygen species caused by high copper [55]. *PPAR-α* acts as key messengers responsible for lipid metabolism and *PPAR-α* is responsible for transforming nutritional, metabolic and drug stimuli into changes in gene expression, especially those related to lipid metabolism (e.g., FAS, ACC, and FATP) [1]. Studies have shown that mRNA level of *CPT-1* was positively correlated with the mRNA level of *PPAR-α* [56], and a similar correlation was obtained in this study, which was consistent with previous studies on grass carp [52]. In conclusion, we believed that the decrease concentration of TG (Fig. 2) in liver was due to the decreased fatty acid *De novo* synthesis caused by the decrease of *FAS* and *ACC* transcription levels and the enhanced fatty acid oxidation caused by the increase of *CPT-1* and *CPT-2* transcription levels.

Cardiac and skeletal muscle are the main sites of lipid oxidative metabolism [57]. FATP and FABP have been shown to have the role of uptake and transport fatty acid in many species [58,59]. In line with previous results in the *Synechogobius hasta* [60], our results shows that the mRNA levels of *FATP* and *FABP* were significantly increased in skeletal muscle with copper treatment (Fig. 3B), it may be due to the increase of TG in serum, suggesting that the ability of muscle cells to absorb fatty acids may be enhanced. Lipoprotein lipase (LPL) is a rate-limiting enzyme for the degradation of triglycerides to glycerol and free fatty acids. It is considered that the increase of LPL activity in skeletal muscle is beneficial to the utilization of energy [61], the present study showed that copper increased *LPL* gene expression in skeletal muscle (Fig. 3B), indicated that copper can enhance the hydrolysis of TG in skeletal muscle cells. At the same time, dietary copper addition can promote the oxidation of fatty acid by upregulate the mRNA levels of *CPT-1* and *CPT-2* (Fig. 3B), suggesting copper enhance the energy supply and promote the growth of skeletal muscle. In addition, the present study showed that the increased expression level of *PPAR-α* in skeletal muscle (Fig. 3B), suggesting that *PPAR-α* was involved in lipid metabolism in skeletal muscle.

HSL is a multifunctional enzyme that participates in fatty acid metabolism and hydrolyzes extracellular and intracellular triglycerides in various tissues, especially adipose tissue [7]. Our study showed that dietary copper addition increased the mRNA level of *HSL* in adipose tissue (Fig. 3C), which was consistent with the research of Chen et al. [60] in the intestinal, which may be the reason for the decrease of perirenal fat weight (Table 4). In addition, the increased expression of *HSL* in adipose tissue may also be due to the decrease concentration of INS in serum, Lan et al [6] reported that HSL is strictly controlled by insulin regulation through the central and peripheral systems, and HSL is the total rate-limiting enzyme of lipolysis, while insulin is the inhibitor of HSL. At the same time, copper enhanced fatty acid oxidation and lipolysis by upregulate the mRNA levels of *CPT-1* and *CPT-2* (Fig. 3C). Consistent with our results in skeletal and liver (Fig. 3A, B), Copper treatment upregulated the expression level of *PPAR-α* in adipose tissue (Fig. 3C). Therefore, we believed that the regulation of copper on lipid metabolism in liver, skeletal muscle and adipose tissue may be through the activation of PPAR-α signaling pathway. Although, both the liver and adipose tissue play an important role in *De novo* lipogenesis in mammals. Interestingly, copper treatment had no significant effect on the mRNA levels of *FAS* and *ACC* in adipose tissue (Fig. 3C), which were inconsistent with the results in the liver. It is possible that the liver plays a more important role in *De novo* lipogenesis than adipose tissue in Rex rabbits. To sum up, our results suggest that copper promotes the oxidation of fatty acids and accelerates the lipolysis process in adipose tissue.

In summary, our research indicated that copper promoted skeletal muscle growth and reduced lipid deposition. However, it is strange that copper addition did not increase the amount of copper in adipose tissue. These results suggested that copper is not a direct regulator of lipolysis. At the same time, we detected a decrease in the content of INS and an increase in the content of GC and EPI in serum. Therefore, we believed the regulatory effect of copper on lipolysis may be realized by regulating the content of hormones in serum. The decreased content of insulin in serum by copper seems to be related to the increased of insulin sensitivity. Consistent with previous studies, we found the activation of PPAR-α signaling in the liver, skeletal muscle, and adipose tissue. Which may be the reason for the increase fatty acids β-oxidation caused by the increase of mRNA levels of *CPT-1* and *CPT-2* in liver, skeletal muscle and adipose tissue. Meanwhile, the increased mRNA levels of *LPL*、*FATP*、*FABP* in skeletal muscle and HSL in adipose tissue indicated the increased uptake of fatty acids by skeletal muscle and the enhanced lipid decomposition in adipose tissue. In addition, it is important that we found that copper addition reduced the *De novo* lipogenesis in the liver.

5. Conclusion

The overall results of this study suggest that dietary copper supplementation has a positive effect on the regulation of lipid metabolism.

Therefore, from the point of view of lipid metabolism, copper can be considered as a potential positive regulator of lipid metabolism, especially the regulatory of copper on insulin, could be have significant influence in future drug exploitation and the treatment of abnormal lipid metabolism diseases. Various outstanding questions regarding the mechanistic signaling pathways of the regulating of copper on insulin, which involve many key regulators and organs of metabolism of the body, need to be identified, and various molecules in these pathways may also be targets for treatment. Currently, extensive studies have reported the regulatory effect of copper on insulin. However, more efforts should be made to clarify the molecular mechanism of the regulatory of copper on insulin. Meanwhile, the toxic effect of copper should also be fully considered.

CRediT authorship contribution statement

Fan Li: Conceptualization, Validation, Writing - original draft, Formal analysis, Investigation, Writing - original draft & Journal Preproof editing, Software, **Xiaojing Wu:** Conceptualization, Methodology, **Hongli Liu:** Conceptualization, Resources, **Bin Zhang:** Validation, **Lei Liu:** Methodology, **Fuchang Li:** Project administration, Funding acquisition

Compliance with ethical standards

All the experimental procedures were carried out in accordance with the local Experimental Animal Care Committee and approved by the Ethics Committee of the Department of Nutrition and Clinical Nutrition of Shandong Agricultural University.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgements

This work was supported by the Natural Science Foundation of Shandong Province (ZR2018MC025), the Natural Science Foundation of China (31972594), the Modern Agri-industry Technology Research System (CARS-43-B-1), and funds from the Shandong "Double Tops" Program.

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