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Transcriptome Analysis Identified the Regulation of Dietary Zinc on Hepatic Lipid Metabolism in Rats

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Abstract: The objective of the study was to investigate the regulation of dietary Zinc (Zn) on transcription of key enzymes involved into hepatic lipid metabolism. Rats were fed a Zinc-Adequate diet (ZA, 46.39 mg Zn/kg basis diet), a Zinc-Deficient diet (ZD, 3.20 mg Zn/kg basis diet) and a Zinc-Overdose diet (ZO, 234.39 mg Zn/kg basis diet) for 5 weeks. Pair-Fed group (PF) rats were fed zinc-adequate diet with food intake of ZD group. Zinc concentrations of liver and femur and activity of Alkaline Phosphatase (ALP) in ZD group were significantly decreased compared with PF group whereas those in ZO group were markedly increased compared with ZA group. Content of oleic acid was markedly decreased in ZD but the contents of palmitic acid and oleic acid were significantly increased in ZO. Concentrations of hepatic Free Fatty Acid (FFA) and Triglyceride (TG) were markedly raised in ZD group whereas these in ZO group was reduced. Of 9753 genes analyzed by cDNA microarray, 13 genes related to hepatic lipid metabolism showed altered expression levels in livers of ZD and ZO rats, respectively with. The changes of four genes were confirmed by relative quantitative real-time RT-PCR. Compared with PF group, mRNA level of Acetyl-CoA Carboxylase (ACC) was significantly increased but Stearoyl-CoA Desaturase-1 (SCD-1) was reduced in ZD group. Relative to ZA group, mRNA levels of Fatty Acid Synthase (FAS) and ACC was obviously decreased in ZO group but these of Carnitine Palmiroyl Transferase-1 (CPT-1) and SCD-1 were significantly elevated. These results indicated that dietary zinc might affect hepatic fatty acid synthesis, β-oxidation and desaturation through regulation on FAS, ACC, CPT-1 and SCD-1 at the transcriptional level.

Key words: Zinc, liver, gene expression, cDNA microarry, fatty acid metabolism, desaturation

INTRODUCTION

Todd *et al*. (1934) found that zinc was an essential trace element required for animals and human being and played comprehensive roles in the physiological and biochemical metabolisms. Zinc is involved in the processes of genetic stability and gene expression in a variety of ways including the structure of chromatin, the replication of DNA and transcript of RNA through the activity of transcription factors and RNA and DNA polymerase as well as DNA repair and programmed cell death (Falchuk, 1998; Dreosti, 2001).

Zinc deficiency has been shown to accentuate the symptoms of Essential Fatty Acid (EFA) deficiency, suggesting a role of zinc in fatty acid metabolism (Eder *et al*., 1996). Previous studies reported major changes in lipid metabolism in zinc-deficient rats (Bettger *et al*., 1979; Shen *et al*., 2007; Nakashima, 2011). Besides alterations in lipoprotein formation, fatty acid composition of lipids in different organs was changed by zinc deficiency (Wu *et al*., 1998). It has been demonstrated that zinc deficiency could increased the activities of some lipogenic enzymes and decreased the activities of enzymes needed for fatty acid degradation (Eder and Kirchgessner, 1995a, b). However, the underlying mechanisms for these pronounced changes in hepatic lipid metabolism were still unknown.

The study employed cDNA microarrays and real-time RT-PCR for assessing changes in the transcriptome as global screening methods and analysis of key functions genes related to hepatic lipid metabolism to explore the action of dietary zinc on lipid metabolism process thus supplied theoretical evidence for future research on the role of zinc in biological metabolism and its mechanism.

MATERIALS AND METHODS

Animal and diet: Zinc deficiency in rats was achieved as described earlier (Sun *et al*., 2005). In brief, male Sprague-Dawley rats (n = 100) with a mean body weight of 90±5 g (Shanghai SLAC Laboratory Animal Co. Ltd., China) were housed in acid-washed, stainless steel cages

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within a barrier system (22-25°C, 40-60% relative humidity, 12 h light/darkness cycle). Food and deionized water were provided ad libitum. After three-day adaptation period with nutritionally complete control diet, animals were randomly divided into four groups of 25 rats each. All rats were fed a purified diet (AIN 93 G) with casein low in zinc as the protein source. The first group was fed a Zinc-Deficient diet (ZD, 3.20 mg Zn/kg basis diet), the second group was pair-fed zinc-adequate diet with food intake of ZD group (PF), the third group was fed Zinc-Adequate diet (ZA, 46.39 mg Zn/kg basis diet) and the last group was fed zinc-overdose diet (ZO, 234.39 mg Zn/kg basis diet). After 5 weeks, rats of each group were anesthetized with urethane. Then blood was collected from intraperitoneal vein and centrifuged for 30 min after collection. The resected tissues were immediately flash-frozen in liquid nitrogen and stored at -80°C.

Zinc concentration and activity of ALP: The concentrations of Zn in liver, femur and serum were determined by Flame Atomic Absorption Spectroscopy (FAAS) according to standard procedures (AA-650, Shimadzu Corp., Kyoto, Japan). The activity of ALP in plasma was assessed according to kit protocol (Ningbo Cicheng biochemical kit company).

Heptic fatty acids composition and concentrations of FFA and TG: About 0.3 g liver lipids were extracted in chloroform, methanol (2:1). Tissues were homogenized and the solution was incubated in 37°C water bath for 30 min, centrifuged at 3000 rpm for 5 min, recovered the bottom phase using insert pipette through upper phase and washed with 0.73% sodium chloride (Folch *et al*., 1957). After vortex and centrifuged, the bottom phase was evaporated to dryness under nitrogen. The lipids were methylated with 3.0 mL 14% BF_3-methanol/aether. The mixture was incubated in 60°C water bath for 30 min, cooled in ice bath, added 0.3 mL 0.73% sodium chloride and 1 mL hexane and vortexed well. The upper phase was evaporated under nitrogen and added 0.1 mL hexane to dissolve. About 1.0 mL fatty acid esters were separated on capillary column using gas chromatography (Angilent Technologies 6890N). The concentrations of hepatic FFA and TG were assessed according to kit protocol (Ningbo Cicheng biochemical kit company).

cDNA microarray: cDNA microarray analysis was performed by using the Rat Expression Array V1.2 (Subsidiary of Shanghai biochip Co., Ltd.) containing 9753 genes there of 7116 Unigene and 1758 homologous genes.

RNA isolation and probe preparation: Total RNA from rat liver was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Then, pooled RNA (n = 5) was isolated with the reagent (QIAGEN Oligotex mRNA purification kits) according to the manufacturer's protocol. RNA quality was ensured by spectrophotometric analysis (OD260/OD280) and agarose gel electrophoresis. For labeling of cDNA probe, nucleic acid labeled by CyDye (Cy3 or Cy5) was incorporated into cDNA strands with reverse transcriptase during the synthesis of the first stand of cDNA. The reaction system containing total RNA, oligo-dT and Rnase-free H_2O was incubated at 70°C for 10 min. In succession, the mixture consisting of 4 μL first-strand buffer, 2.0 μL of 0.1 M Dithio Threi Tol (DTT), 1.0 μL of unlabeled dNTP mixture, 1.0 mL 1 of mM Cy3/Cy5 dCTP, 1.0 μL of RNase inhibitor and 200 U of Superscript 2 were added to the reaction system and incubated at 42°C for 2 h followed by 70°C for 5 min. Labeled probes were purified (QIAquick PCR purification kit) and qualified (Eisen *et al*., 1998; Schena *et al*., 1998).

cDNA array hybridization and image analysis: Labeled cDNA probes were hybridized to a microarray containing 9753 genes. After hybridization for 18 h at 42°C, glass slides were washed four times at 55°C with a wash solution (1X SSC/0.2% Sodium Dodecyl Sulfate (SDS) twice for 10 min each then 0.1X SSC/0.2% SDS twice for 10 min each) and twice at room temperature with 0.1X SSC for 5 min. After a final 2 min wash with double distilled water (ddH2O), slides were centrifugated at 1500 rpm for 5 min and then exposed to fluorescence imaging screen. The image was obtained under dry conditions (Affymetrix 428 Array Scanner; Santa Clara, CA) and analyzed using Imagene 5.0 Software. Hybridization signal intensity of each gene was estimated by fluorescence intensity and transformed into number. The data were normalized according to the supplier's instruction. The ratio value of each gene was obtained through the division of the Cy3 value by the Cy5 value. Finally, the average of three parallel ratios in each dietary treatment was calculated. If Ratio values = 1.600-fold, genes were considered up-regulated and if Ratio values = 0.625-fold, genes were considered down-regulated in liver of ZD rats relative to PF rats, ZO-ZA.

Relative quantification of specific mRNA by real-time RT-PCR: RNA was from above experiment. Single-strand cDNA was synthesized using first-strand synthesis system for RT-PCR (promega molecular system) according to the manufacturer's protocol. The abundances of FAS, ACC, CPT-1, SCD-1 and β-actin mRNAs were assessed by a real-time reverse transcription polymerase chain reaction (RT-PCR) (iCycler iQ™ Real time PCR detector system, Bio-Rad). Primers of FAS, ACC, CPT-1, SCD-1 and β-actin were designed according to the corresponding sequences of the rat genes obtained from GenBank (Table 1).

Table 1: Sequences and parameters of primers

Gene	Product (bp)	Annealing temp (°C)	Primer sequences (5'-3')
FAS	336	55	GCCAAGCACATTCGGGAGT
			GGCGAAGCCGTAGTTAGTTT
ACC	466	58	CCATCAATCCTCGGCACAT
			TCCACAAACCAGCGTCTCA
CPT-1	726	55	ATCATCACTGGTGTGTTCCC
			GAGTTCCTCACGGTCTAATG
SCD-1	395	61	CACTGAACGAGAGGGTTGGTTGT
			CTCTGGATTGGGCTACAGGGACA
β-actin	524	60	AGGCACCAGGGTGTGATGGT
			TTAATGTCACGCACGATTTCC

For online detection during real-time PCR analysis, the fluorescent dye SYBRGreen from faststart SYBRGreen[2] (Takara, Japan) was used. The housekeeping gene β-actin was used as an internal standard for the determination of targeted cDNA levels. The amount of target cDNA in each sample was established by determining a fractional PCR threshold Cycle number (C_T). Melting curves and native gel electrophoresis were carried out to characterize the final products. The data were analyzed using Eq. $2^{-\Delta\Delta C}{}_T$ where $2^{-\Delta\Delta C}{}_T = (C_{T, Target} - C_{T, Actin})_{zinc\text{-}treatment} - (C_{T, Target} C_{T, Actin})_{control}$ (Livak and Schmittgen, 2001). PF group was used as control group for ZD group, ZA as control group for ZO group.

Statistical analysis: SSPS 13.0 statistical package was used for all statistical analysis. Values were expressed as the mean±SEM. Data were analyzed by a one-way ANOVA. Significant differences were accepted at $P<0.05$.

RESULTS AND DISCUSSION

Zinc status *in vivo* and activity of ALP: ZD rats exhibited typical symptoms such as lethargy and lower activity, poor appetite, sparse and coarse hair, alopecia, dermal lesions and leanness whereas ZO rats maintained good phenotype and ingestive behavior similar to ZA rats. the changes in zinc status *in vivo* and activity of ALP were showed in Table 2. Compared with PF group, zinc levels of liver and femur and activity of ALP in ZD group were reduced by 21.51%, 25.60% and 22.98 ($p<0.05$), respectively. Compared with ZA group, zinc levels of liver and femur and activity of ALP in ZO group were increased by 19.22 17.71 and 11.16% ($p<0.05$), respectively.

Concentrations of FFA and TG and fatty acid composition in liver: ZD rats had significantly greater triglyceride concentrations relative to liver wet weight than PF rats whereas that in ZO rats was lower, compared with ZA rats. The concentration of FFA showed the similar alteration (Table 3).

Table 2: Effects of dietary Zn on zinc concentrations of serum and tissues and activity of ALP

Items	ZD	PF	ZA	ZO
Liver (μg/g)	23.57±0.43^{c}	30.03±0.50^{b}	31.42±0.43^{b}	37.46±0.46^{a}
Femur (μg/g)	104.56±2.94^{d}	140.54±1.86^{c}	174.86±2.07^{b}	205.83±1.56^{a}
Serum (μg/g)	88.32±1.50	95.14±1.38	94.96±1.21	96.12±1.49
ALP (IU/L)	134.34±4.64^{c}	174.42±3.99^{b}	193.84±3.89^{ab}	215.48±4.58^{a}

Values presented as Mean±SEM in the same row with different letter superscripts mean significant difference ($p<0.05$), same or no letter superscripts mean no difference ($p>0.05$), n = 5

Four fatty acids composition were analyzed which related with SCD-1 activity in liver. The content of oleic acid in ZD rats was reduced relative to PF rats but the contents of palmitic acid and Oleic acid in ZO rats were significantly increased, compared with ZA rats.

Identification of gene encoding proteins involved in hepatic fatty acid metabolism with altered expression: To monitor changes in gene expression of liver due to zinc deficiency and zinc overdose, the gene expression profiling was analyzed using cDNA arrays. Regulated genes were clustered according to known function. mRNA levels of 67 genes were found to be altered by zinc deficiency and 76 genes were changed by zinc overdose (unlisted). There of 13 genes relative to fatty acid metabolism were significantly altered (Table 4) including FAS, ACC, CPT-1 and SCD-1.

Confirmation of changes in mRNA level for selected genes: The effect of zinc on gene expression levels of FAS, ACC-1, CPT-1 and SCD-1 was shown in Table 5. The mRNA levels of ACC in ZD group and CPT-1 and SCD-1 in ZO group were increased by 1.99, 2.58 and 4.61 folds, respectively but the expression level of SCD-1 in ZD rats and FAS and ACC in ZO rats were decreased by 0.38, 0.47 and 0.43 folds, respectively. The expression levels of FAS and CPT-1 were not significantly changed in ZD rats.

The main goal of cDNA microarray analysis was to identify hepatic transcript alterations that might provide a clue for understanding the changes in fatty acid metabolism responding to dietary zinc deficiency (Eder and Kirchgessner, 1994a,b, 1995a, b) and zinc overdose in rats. Liver of rodent is not only one of the most important metabolism tissues but also the regulation centre of lipid metabolism. Thus, rat liver was employed to research the fatty acid metabolism. A total of 9753 transcripts were inspected by cDNA microarray technology. There of 13 genes involved in fatty acid metabolism were obviously altered.

FAS and CPT-1 play key roles in the de novo lipogenesis and beta-oxidation and ACC, catalyzing the formation of malonyl-CoA from acetyl-CoA is important in the regulation of fatty acid metabolism. Although, zinc is not the essential component for the active domains of

Table 3: Fatty acids composition and concentrations of FFA and TG in liver

Items	ZD	PF	ZA	ZO
Palmitic acid (%)	17.53±1.12^{c}	17.12±1.04^{c}	20.31±0.72^{b}	22.58±1.22^{a}
Palmitoleic acid (%)	0.26±0.017^{a}	0.27±0.014^{a}	0.19±0.014^{b}	0.17±0.020^{b}
Stearic acid (%)	13.12±1.20	14.51±1.14	14.64±1.11	15.32±0.96
Oleic acid (%)	7.05±0.73^{c}	9.75±0.71^{b}	10.36±0.51^{b}	12.32±0.84^{a}
FFA (μg/mg)	36.11±2.89^{a}	24.15±2.30^{c}	30.39±2.10^{b}	22.23±1.81^{c}
TG (μmol/g)	6.49±0.32^{a}	5.38±0.24^{b}	5.60±0.27^{b}	4.55±0.38^{c}

a-c values presented as Mean±SEM in the same row with different letter superscripts mean significant difference ($p<0.05$), same or no letter superscripts mean no difference ($p>0.05$), n = 5

Table 4: Changes in mRNA levels in ZA and ZO rat livers detected by cDNA microarray

Accession No.	ZD	ZO	Encoded protein
NM_022252	1.646	0.558	Acetyl-CoA transferase
M23737	1.829	0.593	Acetyl-CoA carboxylase
L07736	0.430	1.504	Carnitine palmiroyl transferase-1
NM_057107	0.623	1.609	Fatty acid ligase
NM_024381	1.972	0.325	Glycerokinase (TG synthesis)
NM_024162	0.314	1.687	Fatty acid binding protein 3
BC003782	0.209	1.609	Phospholipid transfer protein
U13253	2.016	0.599	Lipid-binding protein
U68550	1.873	0.515	Phospholipase D
NM_017332	0.548	1.768	Fatty acid synthase
NM_013013	0.613	2.224	Prosaposin
NM_053445	0.462	1.687	Fatty acid Δ5 desaturase
NM_139192	0.327	1.971	Stearoyl-CoA desaturase-1

If ratio values = 1.600-fold, genes were considered up-regulated and if ratio values = 0.625-fold, genes were considered down-regulated in liver of ZD rats relative to PF rats, ZO-ZA, n = 3

Table 5: Changes in mRNA levels of key enzymes associated with hepatic fatty acid metabolism verified by real-time RT-PCR

Gene	ZD	ZO
FAS	1.24±0.044	0.47±0.021
ACC	1.99±0.081	0.43±0.056
CPT-1	0.78±0.042	2.58±0.12
SCD1	0.38±0.039	4.61±0.29

If ratio values = 1.600-fold, genes were considered up-regulated, and if ratio values = 0.625-fold, genes were considered down-regulated in liver of ZD rats relative to PF rats, ZO-ZA, n = 3

these enzymes, the transcript levels of the enzymes was altered by dietary zinc. The current study found that the transcription levels of ACC was increased by zinc deficiency but these of FAS and ACC were decreased by zinc overdose whereas transcription level of CPT-1 put out inverse change as observed previously in the same animal model (Tom *et al*., 2005). Meanwhile, the concentrations of TG and FFA displayed increased levels in zinc deficient rats, consisting with previous result in force-fed zinc-deficient rats (Yousef *et al*., 2002). Together with the results, it demonstrated that zinc might depress fatty acid synthesis and promote the beta-oxidation. Similarly (Tom *et al*., 2005) postulated a complex regulatory network of zinc-dependent alterations in hepatic lipid metabolism, zinc deficiency inhibited fatty acid beta-oxidation but induced the fatty acid synthesis and triglyceride synthesis.

Many of the symptoms of zinc deficiency resemble those of an EFA deficiency and zinc deficiency aggravated the symptoms of EFA deficiency (Eder and Kirchgessner, 1993). A change in tissue fatty acid composition depending on zinc status has been reported for a variety of tissues with major differences in concentrations of oleic and linoleic acid (Clejan *et al*., 1982; Cunnane, 1988). Zinc was postulated to directly affect desaturation processes (Ayala and Brenner, 1983; Eder and Kirchgessner, 1994). We observed a substantially reduced transcription level of SCD-1 and content of oleic acid in zinc-deficiency rats but opposite changes in zinc-overdose rats, indicating that zinc mainly affect stearic acid desaturation (Kudo *et al*., 1990; Eder and Kirchgessner, 1996).

CONCLUSION

The study provided the evidence for the regulation of dietary zinc on hepatic fatty acid metabolism in rats. In particularly, gene groups such as FAS, ACC, CPT-1 and SCD-1 showed major alterations in transcription level, demonstrating that dietary zinc might reduce fatty acid synthesis but increased the beta-oxidation and desaturation.

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