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Original Research Article

Paternal Zn-deficiency abolishes metabolic effects in offspring induced by diet type

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ABSTRACT

Accumulating evidence implicates that offspring are susceptible to paternal alterations in numerous fetal disorders, such as growth and metabolic defects. However, less study has been conducted to define the relationship between paternal zinc deficiency (ZnD) and energy metabolism of offspring. In the present study, we used a paternal ZnD exposure (Zn at 0.3 μg/g) model to test energy metabolism of male and female offspring with the intervention of diet type (high-fat diet and low-fat diet). Our results demonstrated that paternal ZnD decreased body weight (BW) gain per week ($P < 0.01$) and ME intake per week ($P < 0.05$) at 11 weeks in male offspring with high-fat diet intervention but not in female offspring. Further, anabolism and catabolism of hepatic energy products also exhibited alterations. ZnD attenuated liver glucose but increased lipids content accompanied with elevated adiponectin and reduction in leptin level in serum, which exhibited lipid metabolic disturbance and smaller ratio of liver weight to BW in male but not female offspring. The qRT-PCR and liver energy metabolites analysis revealed that paternal ZnD mainly induced reduction in glucose tolerance and lowered glucose uptaking ability in male offspring and thereby alleviated glycolysis and the tricarboxylic acid cycle (TCA) cycle, which displayed a male gender-dependency. Therefore, we propose that paternal ZnD abolishes metabolic effects in male offspring induced by diet type intervention. Our findings reveal a novel link between paternal Zn-D and offspring energy metabolism.

1. Introduction

Zinc (Zn) as an essential micronutrient that is well known for its role in energy generation, fatty acids ratio of liver and subcellular membranes, and has been studied over many years since the early 1930s (Burke and Fenton, 1985; Barnett et al., 2013; Li et al., 2018a). A well-maintained Zn homoeostasis in animals is mediated mainly by Zn absorption and metabolism in the small intestine (Karayalcin et al., 1988; Riggio et al., 1992), regulation in the liver (Himoto and Masaki, 2018) and dietary factor/content of oral Zn intake, etc. (Chen et al., 2017). Meanwhile, Zn uptaking and efflux transporter proteins and factors, for example, solute-linked carrier family 30, solute-linked carrier family 39, metal response element-binding transcription factor-1 and metallothioneins also play a crucial role in Zn homoeostasis (Dufner-Beattie et al., 2004; Liuzzi and Cousins, 2004; He et al., 2006; Huang and Tepaamorndech, 2013; Chen et al., 2017). However, zinc deficiency (ZnD) is well documented in liver diseases, such as chronic liver damage, liver cirrhosis, and impaired Zn absorption of small intestine with low Zn uptake and transport (Himoto and Masaki, 2018).

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Increasing evidence suggests that ZnD could exaggerate organ systems dysfunction. A previous study has shown that sequestration of Zn is essential to brain development and function, and is neurotoxic when released Zn concentration during neurotransmission in the synapse is lower than 40 mmol/L (Smith et al., 2006). ZnD could also evoke mitochondrial oxidative stress and consequently initiate a variety of metabolic abnormalities, including insulin resistance, hepatic steatosis, and iron overload (Himoto and Masaki, 2018; Huang et al., 2018). Moreover, ZnD could modify the ratios of phospholipid to protein and cholesterol to phospholipid in erythrocyte membranes (Driscoll and Bettger, 1991; Verstraeten et al., 2004) and alter the mutual transformation of unsaturated fatty acids (Burke and Fenton, 1985; Burke et al., 1987). In pregnant rats, ZnD modified liver fatty acid synthetase and affected the metabolism of essential fatty acids (Dib and Carreau, 1986).

It is reported widely that paternal behaviors could affect multiple behavioral and physiological phenotypes in subsequent generations (Beeler et al., 2019). Paternally inherited alterations are emerging as relevant factors in numerous disease states, including the growth and metabolic defects observed in fetal disorders (Chang et al., 2019). For example, paternal high-fat (HF) diet could impair glucose tolerance in female offspring (Li et al., 2018b); besides, smoking, and alcohol consumption, etc. involved in fetal programming of maternal origin may also alter the epigenome and phenotype of offspring (Hawkey et al., 2019; Wu et al., 2019). However, there is less recognition of the effect of paternal ZnD on the energy metabolism of offspring. Therefore, we hypothesized that the influence of paternal ZnD could alter energy metabolism of offspring.

2. Materials and methods

2.1. Animal care and experimental design

This study was approved by the animal welfare committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. Twenty male (20.28 ± 0.03 g, 6 weeks old) and 30 female (16.89 ± 0.03 g, 6 weeks old) C57BL/6J mice were purchased from the SLAC Laboratory Animal Central (Changsha, China). All mice were housed in pathogen-free colonies conditioned at 22 ± 2 °C, a relative humidity of (50 ± 5)% and a 12-h light and 12-h dark cycle and had free access to feed and water (Zhou et al., 2018). After 3 d of adaption, 10 male mice (20.56 ± 0.04 g, 6 weeks old) were assigned to receive the ZnD diet (zinc at 0.3 μg/g), another 10 male mice (20.01 ± 0.06 g, 6 weeks old) and 30 female mice (16.89 ± 0.03 g, 6 weeks old) were fed the control diet (control diet with zinc at 30 μg/g), respectively, for 4 weeks. Table 1 provides details of basal diets composition for parental generation (F0) mice. One month later, the male mice fed ZnD diet were mated with the female mice fed the control diet to produce the first filial generation (F1) offspring. Table 2 shows details of the low-fat diet and high-fat diet composition for F1 mice. Then weaned F1 offspring (3 weeks old) were allocated into 8 groups: 1) control-LF male offspring (BW = 9.11 ± 0.09 g), 2) control-LF female offspring (BW = 7.99 ± 0.24 g), 3) ZnD-LF male offspring (BW = 9.08 ± 0.13 g), 4) ZnD-LF female offspring (BW = 8.29 ± 0.15 g), 5) control-HF male offspring (BW = 9.21 ± 0.13 g), 6) control-HF female offspring (BW = 8.14 ± 0.14 g), 7) ZnD-HF male offspring (BW = 7.47 ± 0.08 g), 8) ZnD-HF female offspring (BW = 8.08 ± 0.08 g) (Fig. 1). The feed intake and BW of these mice were weighed weekly till mice were 11 weeks old. At the end of the experimental period, the blood was collected, and the liver, epididymal fat, and subcutaneous fat were separated and weighed. The liver samples were collected immediately after delivery and frozen in liquid nitrogen before analysis.

Table 1
Composition of the experimental diets of F0 mice (g/kg, DM basis, by formulation).[a]

Item	Control diet	ZnD diet
Ingredients		
Egg white (spray dried)	203	203
Corn starch	397.486	397.486
Maltodextrin 10	132	132
Sucrose	100	100
Cellulose, BW200	50	50
Soybean Oil	70	70
t-Butylhydroquinone	0.014	0.014
Mineral Mix S19409 (Ca, P, K or Zn not added)	7	7
Potassium phosphate (monobasic)	4.6	4.6
Calcium carbonate	6.45	6.45
Calcium phosphate (dibasic)	8.25	8.25
Zinc carbonate (52.1% Zn)	0.058	0
Vitamin Mix V10037	10	10
Biotin (1%)	0.4	0.4
Choline bitartrate	2.5	2.5
Pure FD&C Yellow #5 Dye	0.005	0
Pure FD&C Red #40 Dye	0	0.005
Total	991.76	991.71
Nutrients levels		
Ca, %	5.01	5.01
P, %	3.16	3.16
K, %	3.60	3.60
Zn, mg/kg	30.5	0.3
Protein	200	200
Carbohydrate	640	640
Fat	70	70
ME, MJ/kg	16.736	16.736

[a] Diets are bought from Research Diets, Inc., USA. The complete compositions of the diets are available through the supplier's website, http://www.researchdiets.com (D10012G new diet 1 for control diet, D10012G new diet 2 for zinc-deficient diet). ZnD = zinc deficiency.

Table 2
Composition of the experimental diets of F1 mice (g/kg, DM basis, by formulation).[a]

Item	Low-fat diet	High-fat diet
Ingredients		
Casein (30 mesh)	200	200
L-Cystine	3	3
Corn starch	315	0
Maltodextrin 10	35	125
Sucrose	350	68.8
Cellulose (BW200)	50	50
Soybean oil	25	25
Lard	20	245
Mineral mix S10026	10	10
Dicalcium phosphate	13	13
Calcium carbonate	5.5	5.5
Potassium citrate	16.5	16.5
Choline bitartrate	2	2
FD&C Yellow Dye #5	0.05	0
FD&C Blue Dye #1	0	0.05
Vitamin mix V10001	10	10
Total	1055.05	773.85
Nutrients levels		
Protein	192	260
Carbohydrate	673	260
Fat	43	350
ME, MJ/kg	16.10	21.92

[a] Bought from Research Diets, Inc., USA. The complete compositions of the diets are available through the supplier's website, http://www.researchdiets.com (Catalog#s D12450B for the low-fat diet, D12492 for the high-fat diet).

2.2. Relative organ weights

The liver, epididymal fat, and subcutaneous fat were separated and weighed. The ratios of organ weight to BW were calculated according to the previous method (Yin et al., 2015).

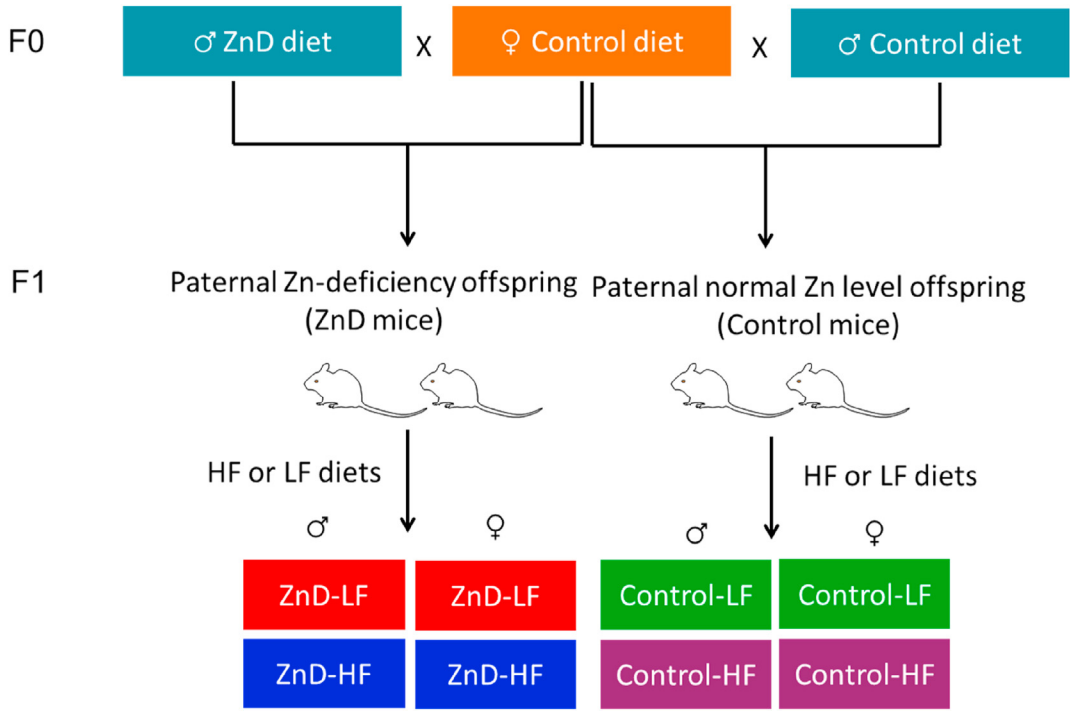

Fig. 1. Experimental design for the F1 offspring. F1 mice were randomly divided into 8 groups according to the diet type, Zn status and sex of mice. Control-LF, mice were fed a low-fat diet. ZnD-LF, mice with Zn deficiency (ZnD) were fed a low-fat diet. Control-HF, mice were fed a high-fat diet. ZnD-HF, mice with ZnD were fed a high-fat diet.

2.3. Biochemical assays

Plasma was separated by centrifugation at 3,000 × *g* for 10 min at 4 °C and then the indexes of glucose (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), triglyceride, cholesterol (Applygen Technologies Inc. Beijing, China), insulin, adiponectin, and leptin (Cusabio Biotech Co., Ltd. Wuhan, China) were detected according to the manufacturer's instruction.

2.4. Measure and analysis of Oil Red O staining and hepatic lipid content level

Oil Red O staining was performed as per a previous study (Leika DM3000, Germany). (Zhou et al., 2018). The liver specimens were frozen in a freezer (Sakura, Tokyo, Japan) at −80 ± 1 °C and 6-μm sections were stained with filtered Oil Red O. Subsequently, hepatic cholesterol (Applygen Technologies Inc. Beijing, China), glycerin trilaurate (Applygen Technologies Inc. Beijing, China) and protein content (Applygen Technologies Inc. Beijing, China) were assayed according to the manufacturer's instruction.

2.5. Glucose tolerance and insulin residence test

Six hour-fasted mice were administered intraperitoneal injection of glucose at 2 g/kg BW in PBS. Blood glucose levels were measured 0, 15, 30, 60, and 120 min after injection using Accu-Chek Active glucometer and glucose strips (Roche Diagnostics). Similarly, 4 h-fasted mice were i.p. injected with insulin at 1 IU/kg BW in PBS (100 IU/mL stock; Actrapid Novo Nordisk, Vienna, Austria). Blood glucose levels were measured 0, 15-, 30-, 45-, 60-, and 120-min post injection using Accu-Chek Active glucometer and glucose strips.

2.6. Real time PCR (RT-PCR)

Extraction of total RNA and its reverse transcription were performed according to the previous reports (Zhou et al., 2018). The mRNA expressions of energy metabolism related genes were detected (Lightcycler 480II, Roche, Swiss) as described previously. The primers are listed in Table 3. The relative expression of genes was expressed as a ratio of the target gene to the control gene using a threshold cycle (Ct) formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{target} - Ct_{\beta\text{-actin}})$ treatment - $(Ct_{target} - Ct_{\beta\text{-actin}})$ control. The β-actin was chosen as the housekeeping gene.

2.7. Determination of hepatic energy metabolite by LC-MS/MS

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using high performance liquid chromatography (Agilent 1290 Infinity LC, US) coupled to a QTRAP 5500 mass spectrometer (AB SCIEX, US). Energy metabolites were

Table 3
Primers used for real-time PCR.

Gene	Primers	Sequence (5′-3′)	Size, bp
β-actin	Forward	CATTGCTGACAGGATGCAGAAGG	138
	Reverse	TGCTGGAAGGTGGACAGTGAGG	
SREBP1	Forward	CGACTACATCCGCTTCTTGCAG	143
	Reverse	CCTCCATAGACACATCTGTGCC	
SREBP2	Forward	AAGCTGGGCGATGGATGAGA	278
	Reverse	ATGGGACCTGGCTGAATGAC	
Glut2	Forward	GTTGGAAGAGGAAGTCAGGGCA	129
	Reverse	ATCACGGAGACCTTCTGCTCAG	
FASD1	Forward	ACCTGTCAGTCTTTGGCACCTC	139
	Reverse	TCCTTGCGGAAGCAGTTAGGCT	
FASD2	Forward	TTCCTGGAGAGCCACTGGTTTG	132
	Reverse	GAAGAAGGACTGCTCCACATTGC	
ELOVL-2	Forward	CTACCCTGGACAGCGCATC	187
	Reverse	CCAGCCATATCGAGAGCAGG	

β-actin = beta-actin; *FASD1* = delta 5-desaturase; *FASD2* = delta 6-desaturase; *ELOVL2* = fatty acid elongase 2; *SREBP* = sterol regulatory element binding protein; *Glut2* = glucose transporter 2.

extracted from nearly 60 mg samples and finally resuspended in 100 μL acetonitrile-water solution (1:1, vol/vol). An Acquity UPLC BEH Amide column (2.1 mm × 150 mm, 1.7 μm) was utilized at 45 °C. Samples (5 μL) were injected at 4 °C. Positive and negative modes were used for the analysis of hydrophilic and hydrophobic energy metabolites. Ammonium formate (positive mode) or acetonitrile (negative mode) were used as mobile phases A and B, respectively. The flow rate was 0.3 mL/min. The gradient of mobile phase B was as follows: 0 to 18 min, 90% changed to 40% linearly; 18 to 18.1 min, 40% changed to 90% linearly as reverse; 18.1 to 23 min, 90%. The MS system was operated with a QTRAP ESI ion source in negative mode under the following conditions: source temperature 450 °C; ion source gas 1, 45 units; ion source gas 2, 45 units; curtain gas, 30 units; ion spray voltage floating (ISVF), 4.5 kV.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) followed by Duncan's multiple-range test was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL). Values were presented as the mean ± SEM. Probability values of <0.05 and < 0.01 were considered statistically significant and extremely significant, respectively, and $0.05 \leq P \leq 0.10$ were considered as a tendency.

3. Results

3.1. Paternal Zn deficiency decreased energy intake and growth development in male offspring

As shown in Fig. 2C, paternal ZnD decreased BW from 5 to 11 weeks (5 weeks, $P = 0.06$; 6 weeks, $P < 0.05$; 7 weeks, $P < 0.05$; 8 weeks, $P = 0.08$; 9 weeks, $P = 0.07$; 10 weeks, $P < 0.05$; 11 weeks, $P < 0.05$) in HF diet-induced male offspring when compared with control-HF offspring. Energy intake from 9 to 11 weeks was attenuated consequently in ZnD-HF (Fig. 2A) ($P < 0.01$). Paternal ZnD significantly decreased BW of LF-diet induced male mice at 5 weeks but failed to affect energy intake from 5 to 11 weeks when compared with control-LF mice ($P < 0.01$). Interestingly, no significance was found in female offspring no matter the energy intake and BW ($P > 0.05$).

3.2. Paternal Zn deficiency decreased relative organ weights in male offspring

As the results revealed, relative liver weights decreased significantly regardless of HF-diet treated or LF-diet treated in male offspring (Fig. 3A) when compared to the corresponding control

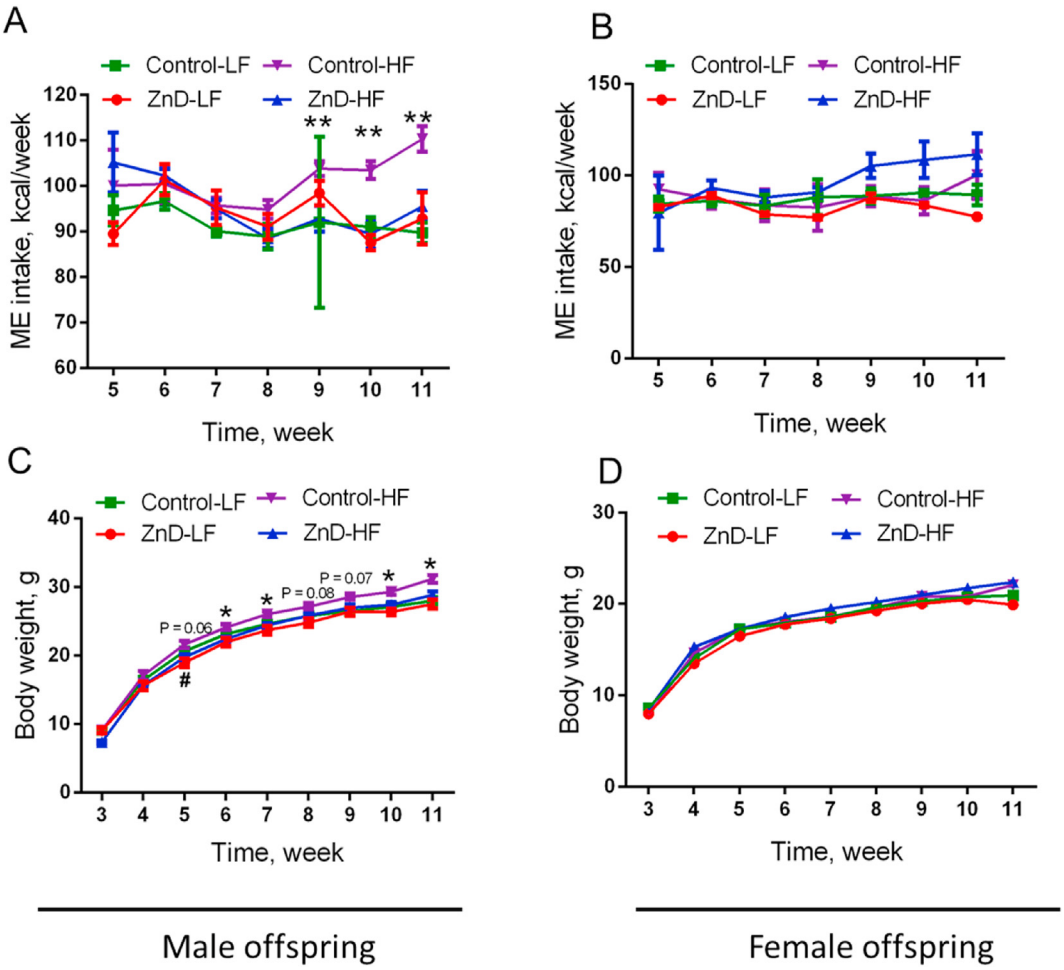

Fig. 2. Effect of Zn-deficiency (ZnD) on the energy intake and growth development in (A and C) male offspring and (B and D) female offspring. Control-LF, mice were fed a low-fat diet. ZnD-LF, mice with Zn deficiency were fed a low-fat diet. Control-HF, mice were fed a high-fat diet. ZnD-HF, mice with ZnD were fed a high-fat diet. Data were analyzed by student's *T* test (IBM SPSS 22 software) and expressed as the mean ± SEM. *, ** represent the difference between Control-HF and ZnD-HF group (*$P < 0.05$, **$P < 0.01$).

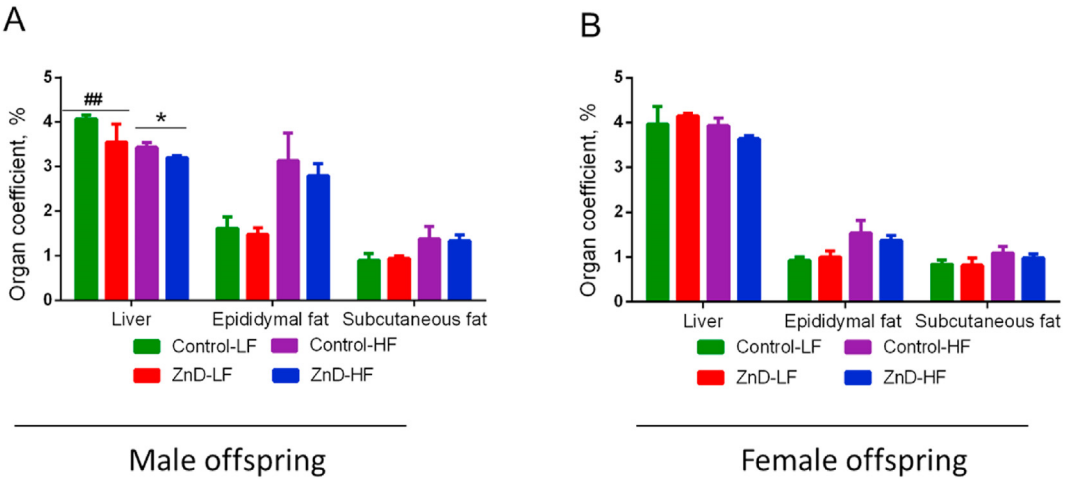

Fig. 3. Effect of paternal Zn-deficient (ZnD) on relative organ weights in (A) male offspring and (B) female offspring. Control-LF, mice were fed a low-fat diet. ZnD-LF, mice with paternal ZnD were fed a low-fat diet. Control-HF, mice were fed a high-fat diet. ZnD-HF, mice with paternal ZnD were fed a high-fat diet. Data were analyzed by student's *T* test (IBM SPSS 22 software) and expressed as the mean ± SEM. $^{\#\#}$ represents the difference between Control-LF and ZnD-LF group ($^{\#\#}P < 0.01$). * represents the difference between Control-HF and ZnD-HF group (*$P < 0.05$).

male offspring ($P < 0.05$). However, there was no significance in relative epididymal fat weight and relative subcutaneous fat weight in either LF-treated groups or HF-treated groups when compared to the corresponding control male offspring ($P > 0.05$). In contrast, no significant change was found among these data of organ coefficient in female offspring ($P > 0.05$).

3.3. Serum biochemical assays

Serum glucose (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), triglyceride and cholesterol (Applygen Technologies Inc. Beijing, China) in male offspring (Fig. 4A) and female offspring (Fig. 4B) were observed, respectively. Moreover, serum insulin (Fig. 4B; Fig. 4F) (male; female), adiponectin (Fig. 4C; Fig. 4G) (male; female), and leptin (Fig. 4D; Fig. 4H) (male; female) (Cusabio Biotech Co., Ltd. Wuhan, China) were also detected. Much like the sex-dependent association with effects of paternal Zn status or diets type on energy intake, growth development and relative organ weights, there was no significant difference in these serum biochemical indexes in female offspring ($P > 0.05$).

3.4. Paternal Zn deficiency increased hepatic lipid accumulation mainly in male offspring

To further identify the intracellular lipid content of hepatocytes, hepatic triglyceride, cholesterol, and Oil Red O staining were conducted. As expected, the quantitative assay of hepatic lipid content showed that the hepatic content level of hepatic cholesterol ($P < 0.01$) and triglyceride ($P < 0.05$) in male offspring were increased in LF and HF groups via the paternal ZnD intervention, respectively, when compared to the control group (Fig. 5A). Moreover, paternal ZnD also increased the hepatic triglyceride level with the LF treatment in female offspring ($P < 0.05$) relative to the control group (Fig. 5B). Accompanied by an increased content of cholesterol and triglyceride, lipid droplets accumulation via Oil Red O staining also demonstrated lipid accumulated in paternal ZnD offspring (Fig. 5C).

3.5. Paternal Zn-deficiency decreased glucose tolerance in male offspring

Results showed that ZnD intervention decreased glucose intolerance when male offspring were fed with HF diet (Fig. 6A) ($P < 0.01$), whereas no significance was found in insulin sensitivity or glucose intolerance in male mice with LF-diet after 2-h glucose or insulin injection (Fig. 6C) ($P > 0.05$). Glucose intolerance and insulin resistance were not affected in female offspring by HF or LF diet intervention at 2 h after glucose or insulin injection (Fig. 6B; Fig. 6D) ($P > 0.05$).

3.6. Paternal Zn-deficiency alleviated energy metabolism related gene expressions in offspring

According to our previous study, paternal ZnD most modified offspring energy metabolism. Thus, genes sterol regulatory element binding protein 1 (*SREBP-1*), sterol regulatory element binding protein 2 (*SREBP-2*), Delta 5-desaturase (*FADS1*), Delta 6-desaturase (*FADS2*), glucose transporter 2 (*Glut2*), and fatty acid elongase 2 (*ELOVL-2*) related to energy metabolism in male and female offspring liver were conducted. It was found that paternal ZnD decreased the relative expression of *Glut2* ($P < 0.01$) and increased the *ELOVL-2* expression in male offspring with LF diet ($P < 0.01$) (Fig. 7A). In female offspring, paternal ZnD increased the *SREBP-1* and *Glut2* significantly no matter whether treated with LF or HF diet ($P < 0.05$) (Fig. 7B).

3.7. Metabolites

As shown in Fig. 8, guanosine monophosphate content was decreased whereas lactate and pyruvate were increased in ZnD male offspring induced by LF diet when compared to the control ($P < 0.05$); with the HF diet treatment, paternal ZnD could decrease α-ketoglutaric acid content but increase the level of dihydroxyacetone phosphate ($P < 0.05$), succinate ($P < 0.01$) and thiamine pyrophosphate ($P < 0.05$) in male offspring. For female offspring, paternal ZnD exhibited high content of cyclic-adenosine monophosphate (cyclic-AMP) ($P < 0.01$), nicotinamide adenine dinucleotide phosphate (NAD^+) ($P < 0.05$), reduced nicotinamide adenine dinucleotide phosphate (NADH) ($P < 0.01$) and oxaloacetate

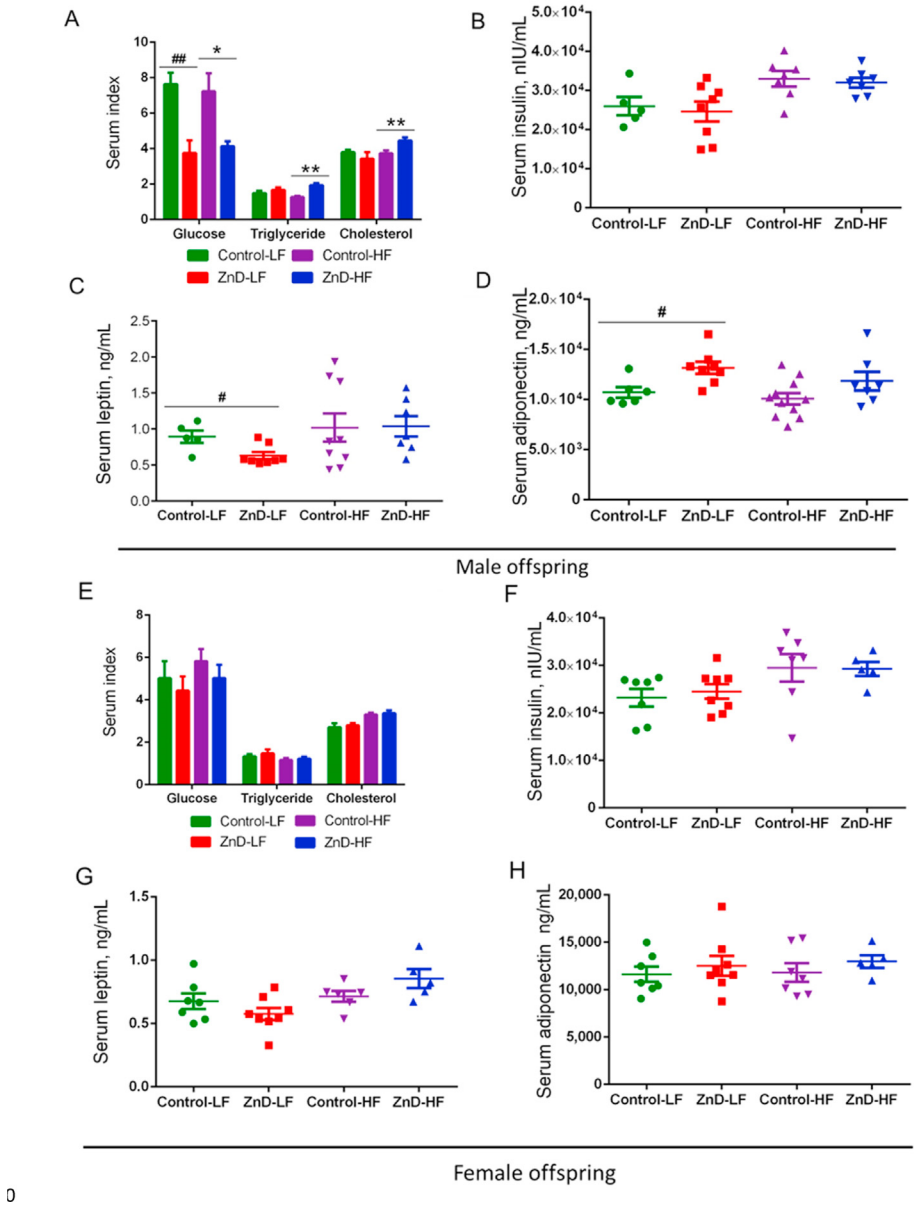

Fig. 4. Effect of paternal Zn-Deficiency (ZnD) on the serum indexes in (A to D) male offspring and (E to H) female offspring. Control-LF, mice were fed a low-fat diet. ZnD-LF, mice with paternal ZnD were fed a low-fat diet. Control-HF, mice were fed a high-fat diet. ZnD-HF, mice with paternal ZnD were fed a high-fat diet. Data were analyzed by student's *T* test (IBM SPSS 22 software) and expressed as the mean ± SEM. $^{\#\#}$ represents the difference between Control-LF and ZnD-LF group ($^{\#\#}P < 0.01$). * represents the difference between Control-HF and ZnD-HF group (*$P < 0.05$, ** $P < 0.01$).

($P < 0.05$) with LF diet treatment when compared to the control group. However, there was no significant difference between paternal ZnD group and the control group with HF diet treatment in female mice ($P > 0.05$).

4. Discussion

Zinc, as a component of over 200 enzymes, participates in many physiological functions in animals, especially playing a pivotal role in the balance of lipids metabolism (Janczyk et al., 2015). In recent years, more and more studies have focused on Zn deficiency. It is reported putatively that ZnD has a negative impact on body fat accumulation in rodents accompanied by diminished food intake and a subsequent reduction with BW gain (Huang et al., 2007). Previous studies have also demonstrated that ZnD could attenuate energy intakes and final BW in about 5-week-old rats, however, there was no significance in older mice e.g., 32-week-old mice (Salgueiro et al., 2002; Beattie et al., 2006; Weigand and Egenolf, 2017), which is confirmed that final BW after 4 to 6 weeks are indeed not significantly different in the ZnD rats (Cunnane et al., 1984). In this study, we found that average BW gain per week was decreased in paternal Zn deficient mice no matter with the intervention of HF diet or LF diet in male mice and manifested as lower ME intake. However, in these studies, no direct evidence was obtained to support that paternal Zn status could attenuate ME intake and weight gain in female mice, which may be caused by the age and gender variance in mice (Weigand and Egenolf, 2017). Moreover, it was reported that elevated leptin and abrogated adiponectin concentrations may reduce appetite and caloric intake (Kroll et al., 2019), thus it could be inferred that increasing

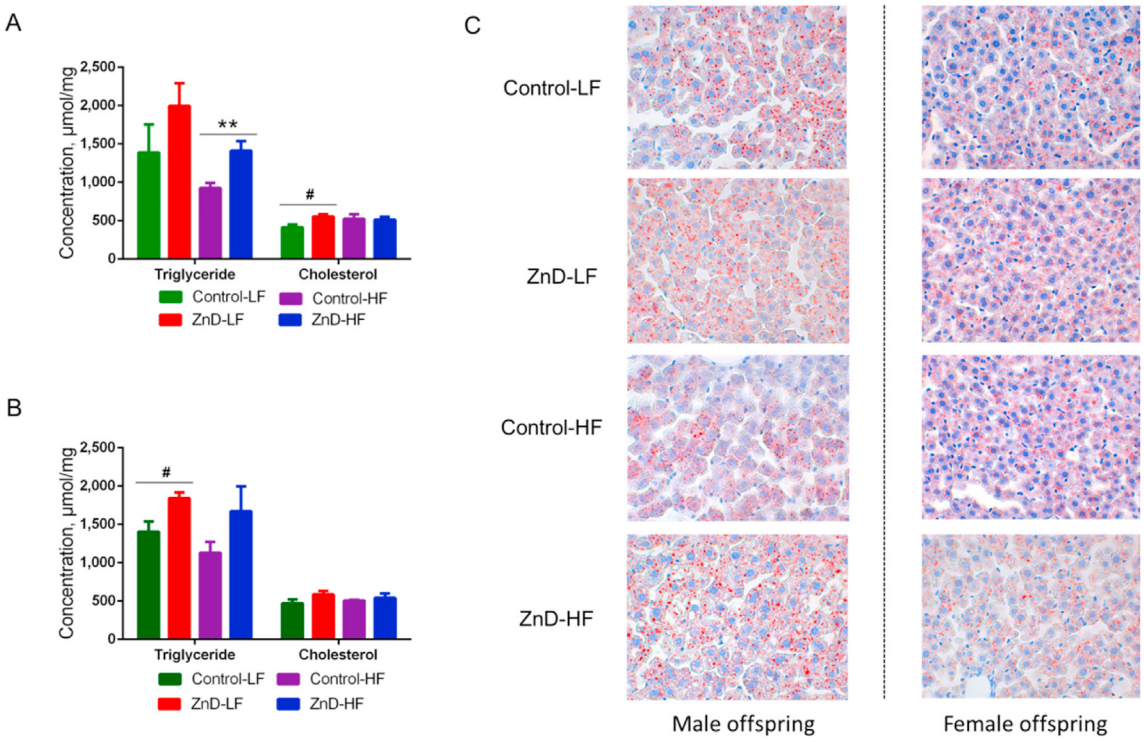

Fig. 5. Paternal ZnD increased hepatic lipid accumulation. The hepatic lipid content of cholesterol and triglyceride in (A) male and (B) female offspring. (C) The hepatic lipid droplet formation in male and female mice via Oil Red staining (magnification, 400×). Control-LF, mice were fed a low-fat diet. ZnD-LF, mice with paternal ZnD were fed a low-fat diet. Control-HF, mice were fed a high-fat diet. ZnD-HF, mice with paternal ZnD were fed a high-fat diet. Data were analyzed by student's *T* test (IBM SPSS 22 software) and expressed as the mean ± SEM. # represents the difference between Control-LF and ZnD-LF group ($^{\#}P < 0.05$). **represents the difference between Control-HF and ZnD-HF group ($^{**}P < 0.01$).

adiponectin and decreasing leptin concentrations may be considered to be a feedback regulation on the negative energy balance. A previous study also showed that paternal ZnD could induce a variety of metabolic abnormalities in liver (Himoto and Masaki, 2018). In this study, irrespective of LF diet or HF diet, paternal ZnD caused reduction in relative organ weight of liver in male offspring, but not female offspring, which might be caused via the interruption in lipids synthesis (Dib and Carreau, 1986).

Detection of energy metabolism genes expressions and liver lipids contents were processed to further confirm that paternal ZnD affects the lipids synthesis and metabolism in offspring. Accumulating evidence implicates that hepatic or serum triglyceride and cholesterol were not significantly different with the low-level paternal zinc intervention or not (Payahoo et al., 2013; Weigand and Egenolf, 2017). Some opposite evidence demonstrated that paternal ZnD could increase not only serum cholesterol concentrations but essential fatty acid concentrations in triglycerides and phospholipids (Cunnane 1988). Our study showed that serum lipids that accompanied liver lipid contents were alleviated in mice with the co-intervention of paternal zinc status and diet type. Serum lipid profiles were elevated in HF diet male mice under treatment of paternal ZnD, which manifested as gender-dependent. Hepatic lipids concentration was increased both in male and female mice, which was also confirmed via the staining of hepatic lipid droplets.

Significantly, glucose tolerance was attenuated in male offspring with the intervention of HF diet and paternal ZnD, which also displayed gender-dependent. According to a previous study, Zn supplementation improved glucose tolerance and reduced the risk of diabetes in obese subjects (Ishikawa et al., 2005) and low glucose uptake occurred in mice deficient for Zn transporter 7 protein (Tepaamorndech et al., 2016), which means that ZnD might result in glucose tolerance decreasing and low glucose uptake in the body.

To have a further finding of paternal ZnD effects on hepatic energy metabolism, principal lipogenic genes that regulate fatty acid biosynthesis, including *FASD1*, *FASD2*, *ELOVL2*, *SREBP1* and *SREBP2*, glucose transporter gene, and *Glut2* were measured by real-time PCR (McNamara et al., 2008; Zhou et al., 2017). Consistent with lower glucose content in serum and glucose tolerance, there was a significant reduction in *Glut2* gene expression, which exhibited that paternal ZnD might disrupt the normal transport of glucose. Further, gene expression of *ELOVL2* was up-regulated, which might explain why triglyceride content in male offspring was increased when treated with LF diet (Zhou et al., 2017). Interestingly, paternal ZnD increased *SREBP1* and *Glut2* expression in female offspring, treated either with the LF or HF diet. Although there was no change in glucose content in serum in female offspring, it was found that glucose tolerance increased in ZnD-HF female offspring at 30 min after insulin intervention. Moreover, paternal Zn deficiency also increased ZnD-HF glucose tolerance when these female offspring were fasted for 6 h, which was manifested as the up-regulated expression of Glut2 in female offspring (Low et al., 2021).

These results were also confirmed by pivotal metabolic pathways impairment, such as glycolysis and the tricarboxylic acid cycle (TCA) cycle. As known to all, the fundamental function of glycolysis is to provide energy, thus connect carbohydrate metabolism with growth and development. Glucose is the main substrate of glycolysis in the body and supplies the majority of the necessary carbon to support cell growth (Luo et al., 2019; Schcolnik-Cabrera et al., 2019). Glycolysis converts glucose into pyruvate and produces ATP for living cells (Yang et al., 2018). Our targeted metabolomics analysis revealed that pyruvate was decreased in male, but not female paternal ZnD mice with either LF or HF diet intervention, which might be caused via the low intake of glucose in ZnD mice

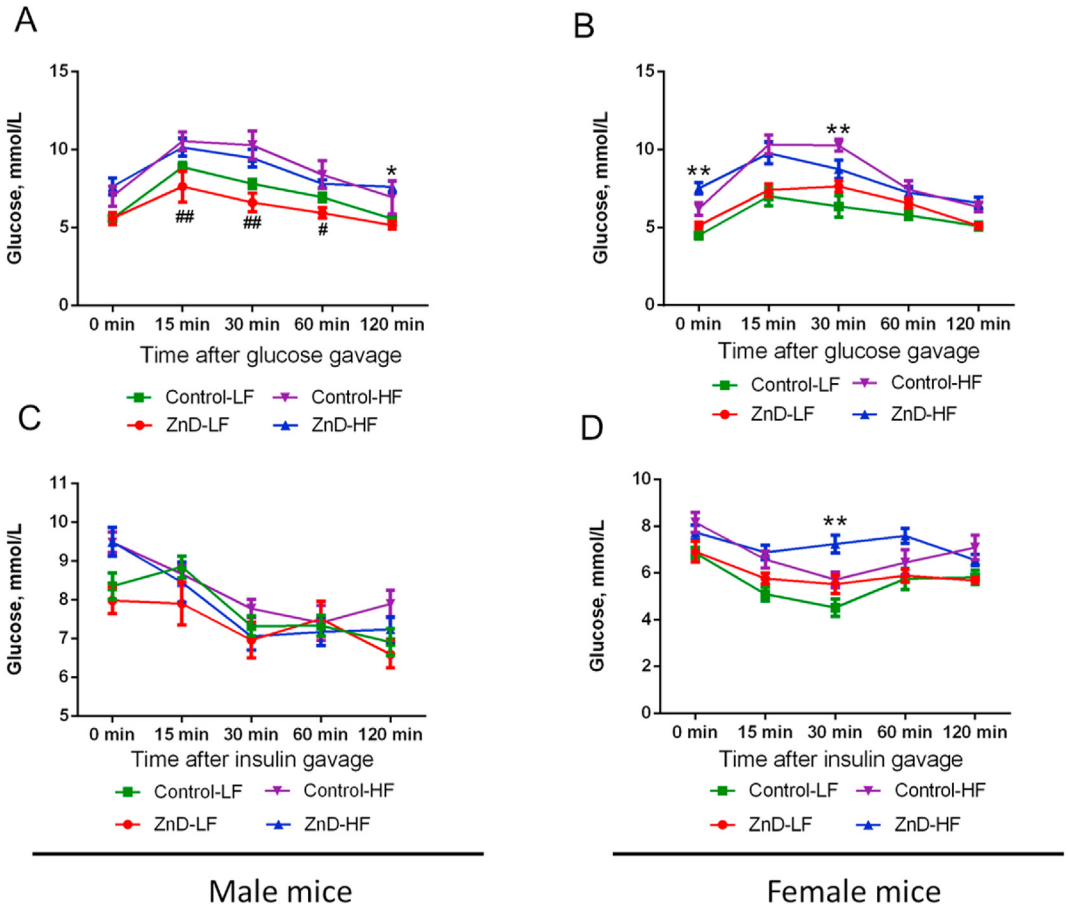

Fig. 6. Paternal Zn deficiency (ZnD) decreased glucose tolerance in male offspring. Glucose intolerance (A) and insulin sensitivity test (C) in male mice. Glucose intolerance (B) and insulin sensitivity test (D) in female mice. Control-LF, mice were fed a low-fat diet. ZnD-LF, mice with Zn deficiency were fed a low-fat diet. Control-HF, mice were fed a high-fat diet. ZnD-HF, mice with ZnD were fed a high-fat diet. Data were analyzed by student's *T* test (IBM SPSS 22 software) and expressed as the mean ± SEM. ** represents the difference between Control-HF and ZnD-HF group (**$P < 0.01$). GLU = serum glucose.

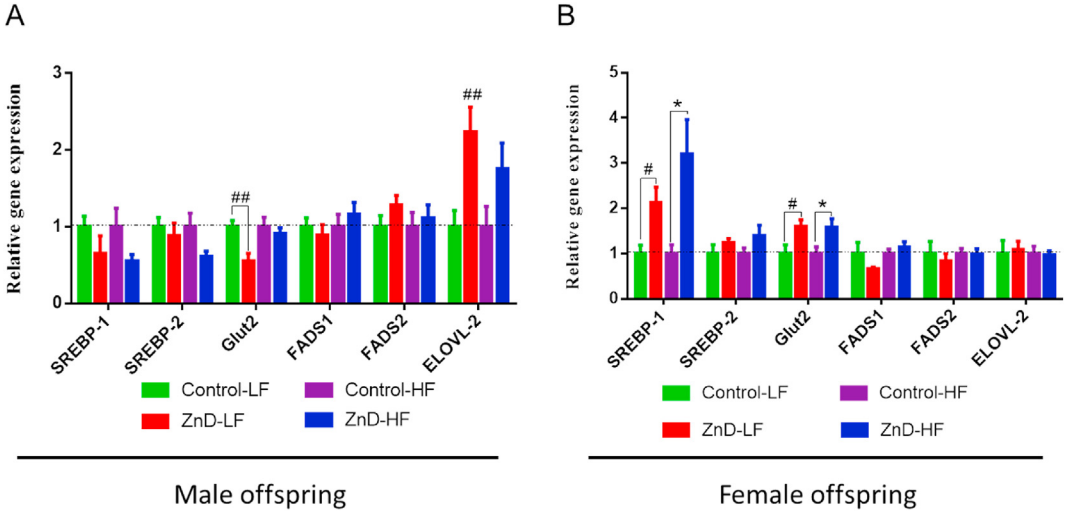

Fig. 7. Paternal Zn deficiency (ZnD) alleviated energy metabolism related genes expressions in (A) male and (B) female offspring. Control-LF, mice were fed a low-fat diet. ZnD-LF, mice with Zn deficiency were fed a low-fat diet. Control-HF, mice were fed a high-fat diet. ZnD-HF, mice with ZnD were fed a high-fat diet. Data were analyzed by student's *T* test (IBM SPSS 22 software) and expressed as the mean ± SEM. $^{\#}$, $^{\#\#}$ represent the difference between Control-LF and ZnD-LF group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). * represents the difference between Control-HF and ZnD-HF group (*$P < 0.05$). *SREBP* = sterol regulatory element binding protein; *Glut2* = glucose transporter 2; *FASD1* = delta 5-desaturase; *FASD2* = delta 6-desaturase; *ELOVL2* = fatty acid elongase 2.

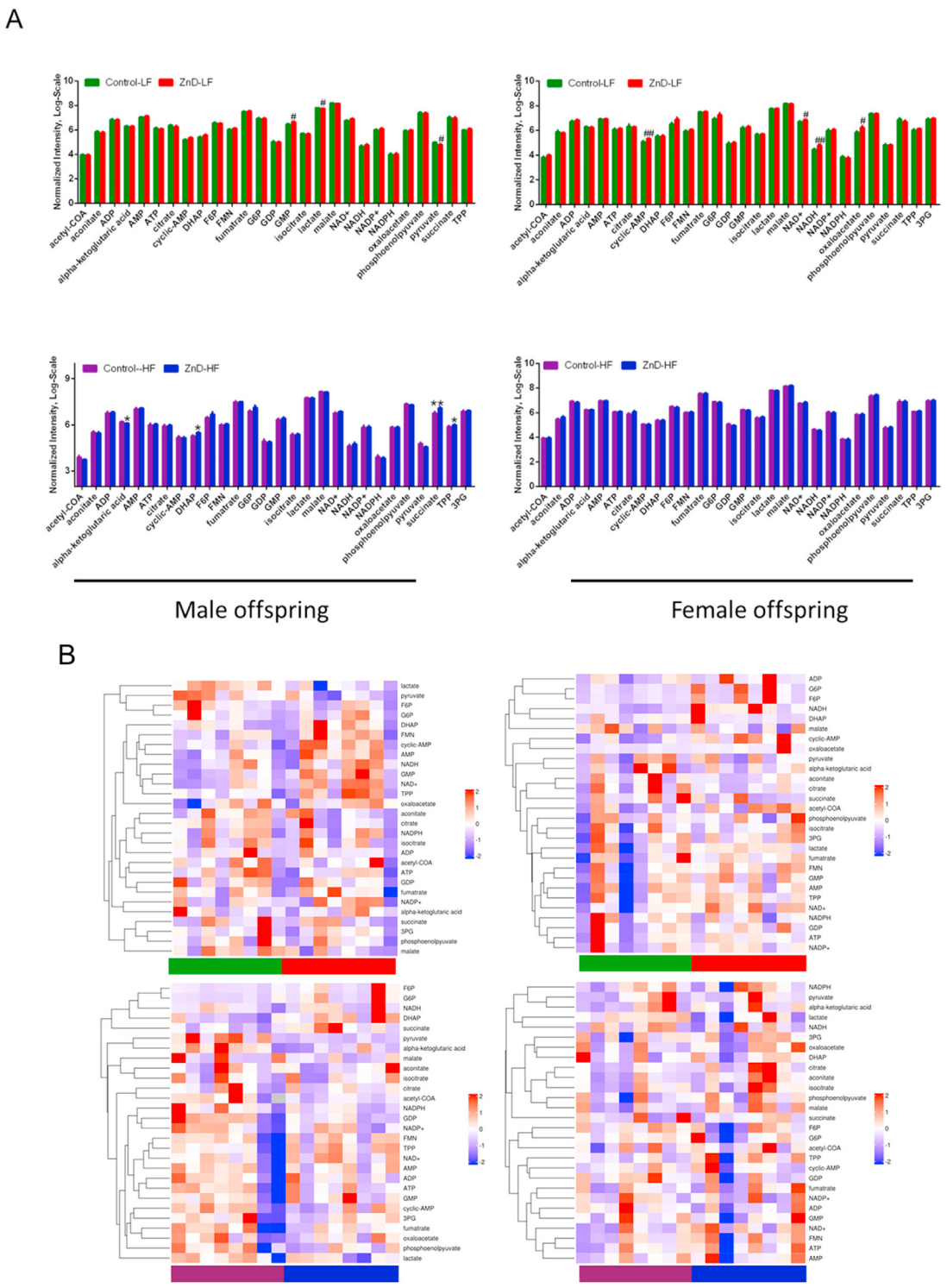

Fig. 8. Paternal Zn deficiency (ZnD) changed liver metabolites in mice. (A) Liver metabolites analysis by LC-MS/MS. (B) LC-MS/MS–derived ion counts were normalized to the control and plotted as a heat map relative to the control. Control-LF, mice were fed a low-fat diet. ZnD-LF, mice with Zn deficiency were fed a low-fat diet. Control-HF, mice were fed a high-fat diet. ZnD-HF, mice with ZnD were fed a high-fat diet. Data were analysed by student's *T* test (IBM SPSS 22 software) and expressed as the mean ± SEM. $^{\#, \#\#}$ represent the difference between Control-LF and ZnD-LF group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). $^{*, **}$ represent the difference between Control-HF and ZnD-HF group ($^{*}P < 0.05$, $^{**}P < 0.01$). GMP = guanosine monophosphate; DHAP = dihydroxyacetone phosphate; TPP = thiamine pyrophosphate; NAD^+ = nicotinamide adenine dinucleotide phosphate; NADH = reduced nicotinamide adenine dinucleotide phosphate.

(Jin et al., 2020; Yin et al., 2015). Further, reduction of liver α-ketoglutaric acid and lactate, two critical intermediates for glycolysis, also suggested paternal ZnD altered the balance of glycolysis and affected TCA cycle. It is reported that succinate could provide an energy supply and catecholamine-like influence on the hypothalamus and increase the organ's sensitivity to input signals from peripheral endocrine glands, therefore elevating succinate in the liver, which may in turn affect the TCA cycle (Radzinsky et al., 2019).

Nevertheless, a lesser effect of paternal ZnD on glycolysis was found. Interestingly, levels of cyclic-AMP, NAD+, NADH and oxaloacetate were increased in female mice with LF diet intervention. As previous research has revealed, NAD+ is a pivotal intermediate product of glycolysis and oxidative phosphorylation, which could be reduced to NADH during glycolysis and then oxidized to generate the proton gradient necessary for ATP production (Xu et al., 2019). The altered levels of NADH and NAD + suggest that the redox state might be imbalanced in female mice. Elevated oxaloacetate also showed that TCA cycle was substantially altered, which might be a feedback response in the TCA cycle (Yang et al. 2018, 2019).

5. Conclusion

Currently, the present study shows a link between paternal ZnD and offspring, especially male offspring, energy metabolism. Male offspring are susceptible to paternal ZnD and this manifests as disrupted glucose metabolism, lipids metabolism and is deeply revealed in lower BW. Therefore, paternal ZnD plays a pivotal role in offspring energy status. Meaningfully, monitoring parental Zn levels may change the metabolic state of the offspring, so as to prevent an energy metabolism disorder of the offspring.

Declaration of competing interest

The authors declare no competing financial interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Author contributions

Dan Wan and **Yulong Yin** designed research. **Guanya Li** and **Zhenglin Dong** collected data and samples. **Guanya Li, Shusheng Yue** and **Zhenglin Dong** conducted the laboratory analyses. **Guanya Li** analyzed data and wrote the original draft. **Dan Wan** reviewed and edited the paper.

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