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Research article

Excessive folic acid supplementation in pregnant mice impairs insulin secretion and induces the expression of genes associated with fatty liver in their offspring

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ABSTRACT

Objective: Previous human and animal studies have shown that excessive maternal intake of folic acid (FA) predisposes to impaired glucose tolerance in the offspring. However, the underlying mechanism is unknown. Therefore, we aimed to determine whether excessive supplementation with FA during pregnancy affects the glucose tolerance of mouse offspring.

Research methods & procedures: Pregnant C57BL/6J mice were fed AIN93G diet containing either 2 mg [control group (CN)] or 40 mg [high FA group (HFA)] FA/kg diet throughout their pregnancies. On postnatal days (PD)22 and 50, fasting blood glucose was measured in the offspring of both groups, and an oral glucose tolerance test (OGTT) was performed on PD50. On PD53, tissues were collected, and the tissue masses, area of insulin expression in the pancreas, liver triglyceride content, and gene expression were determined.

Results: The blood glucose concentrations at 60 and 120 min of the OGTT were higher in female HFA than CN offspring. The serum fasting and non-fasting insulin concentrations and the area of insulin expression in the pancreas were lower in HFA than CN offspring. The liver triglyceride content was higher in female, and tended to be higher in male ($P < 0.05$), HFA offspring than CN offspring ($P < 0.05$). The liver mRNA expression of fat synthesis genes, such as *Pparγ2* (male and female) and *Cidec* (male), was higher in HFA than CN offspring ($P < 0.05$).

Conclusion: Excessive maternal supplementation of FA in mice leads to lower insulin synthesis and an impairment in hepatic fat metabolism in the offspring.

1. Introduction

Folate is a member of the B-group vitamin family and is important for 1-carbon metabolism, which is involved in DNA synthesis and cell division. Folate deficiency in humans can cause pernicious anemia, which is characterized by a low red blood cell count [1]. Several large-scale intervention studies, such as that by Milunsky *et al.*, of 23,491 women undergoing maternal serum α-fetoprotein screening or amniocentesis at around 16 weeks of gestation, have demonstrated that maternal supplementation with folic acid (FA) reduces the incidence of neural tube defects (NTDs) in newborns [2, 3]. The findings of these intervention studies strongly imply that women require FA supplementation during pregnancy, and indeed, since 1998, many countries, including the United States and Canada, have implemented mandatory periconceptional FA supplementation by fortification of flour and grain products. In many countries, the FA fortification has been associated with substantial increases in the circulating concentration of folate and significant reductions in the incidence of NTDs in infants [4].

Furthermore, several animal studies have suggested that FA deficiency increases the risk of metabolic diseases, including type 2 diabetes

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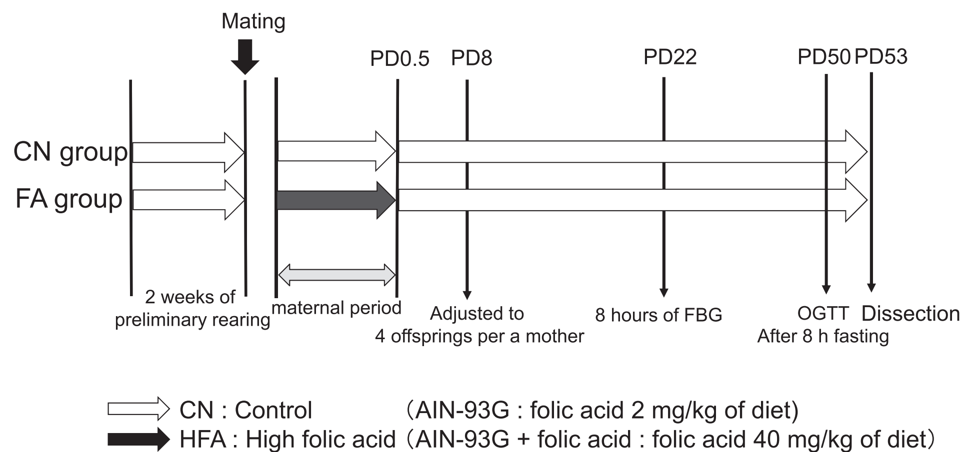

Figure 1. Experimental design. Diagram of the dietary intervention in the mice. Female C57BL/6J mice were fed a control or high-folic acid diet, and 8 h fasting blood glucose was measured in their offspring at PD22 and 50. The offspring were sacrificed at PD53. CN, control group (AIN93G diet: folic acid 2 mg/kg of diet); HFA, high-folic acid group (AIN93G + folic acid: folic acid 40 mg/kg of diet); PD, postnatal day; FBG, fasting blood glucose; OGTT, oral glucose tolerance test.

and dyslipidemia. FA deficiency of dams during pregnancy has been shown to lead to hyper-homocysteinemia and impaired glucose tolerance (IGT) in their neonatal offspring [5, 6]. In addition, it has been reported that offspring born to dams fed a low-FA diet exhibit IGT and post-prandial hyperinsulinemia when adult [7]. These animal studies indicate that FA deficiency is capable of inducing IGT, apparent insulin resistance, and other features of metabolic disease, as well as NTDs.

However, deleterious effects of excessive folate intake have also been reported [8, 9, 10, 11, 12, 13, 14, 15], and it is possible that excessive FA intake by pregnant women due to supplement abuse may have adverse effects. A cross-sectional study of 1,458 elderly participants with normal serum creatinine concentrations and no history of stroke, alcoholism, recent therapy of anemia, or diseases of the liver, thyroid, or coronary arteries, conducted as part of the National Health and Nutrition Examination Survey during 1999–2002, demonstrated that high plasma folate concentration is positively associated with subsequent exacerbation of both anemia and cognitive impairment, as well as higher plasma concentrations of homocysteine and methylmalonic acid, markers of vitamin B_{12} deficiency [8, 9]. However, it remains to be determined whether excessive FA intake during pregnancy induces adverse effects in newborns.

One case-control study showed that positional plagiocephaly can occur in children born to mothers who consume excessive amounts of FA during pregnancy [10]. Moreover, the Pune Maternal Nutrition Study of 700 consecutive eligible pregnant women in six villages in India showed that lower maternal vitamin B_{12} and higher folate concentrations during pregnancy are positively associated with subsequent insulin resistance in 6-year-old offspring [11]. In addition, a cohort study conducted at 30 ± 2 weeks' gestation in 654 women in India showed that maternal homocysteine concentration is positively associated with smaller newborns and a higher glucose concentration at the 30-min time point of an oral glucose tolerance test (OGTT) in the 5 and 9.5-year-old children [12]. The same study demonstrated that a higher folate concentration in pregnant women is positively associated with insulin resistance (assessed using the homeostasis model of assessment-insulin resistance [HOMA-IR]) in the 9.5 and 13.5-year-old children [12]. Taken together, these findings suggest that excessive intake of folate and deficiency of vitamin B_{12} could predispose to IGT and type 2 diabetes mellitus.

An animal study by Anderson *et al.* showed that body weight in 25-week-old male rats is higher in the offspring of dams fed a high-FA diet than in those born to a dam fed a regular AIN93G diet [13]. In another study, female offspring of dams fed a high-FA diet during pregnancy had a 6% lower body weight at 17 weeks of age than those born to dams fed a control diet [14]. In addition, it has been reported that high maternal FA intake induces increases in body weight, blood glucose, and insulin resistance in male offspring, but not in female offspring, under high-fat diet-fed conditions [15]. However, the mechanism underlying the development of IGT in the offspring of mothers that consume excessive FA during pregnancy remains to be established.

In this study, we aimed to determine the mechanism underlying excessive maternal FA supplementation-induced metabolic disorders in C57BL/6J mice, before the development of IGT, by assessing serum insulin concentration and insulin expression in pancreatic β-cells. We also aimed to evaluate the relationships between the reduction in insulin secretion in the offspring induced by excessive maternal FA supplementation and the hepatic expression of genes involved in the development of fatty liver.

2. Material and methods

2.1. Animals and diets

We prepared two diets based on the AIN93G [16] diet that contained either 2 mg (control; CN) or 40 mg (high FA: HFA) FA/kg diet (Oriental Yeast Co., Ltd., Saitama, Japan). Thus, the FA concentration was 20 times higher in the HFA diet than in the CN diet. The FA dose in the HFA diet was determined on the basis of a previous study that investigated the effect of HFA diet in mice [17]. In addition, equivalent doses of FA have been used in a study of glucose metabolism [15].

The study was performed in accordance with the guidelines of Ministry of the Environment and approved by the Committee of Animal Experiments of Jumonji University (No. 1505, 2015. 9. 7.).

Six-week-old male and female C57BL/6J mice were purchased from Japan SLC Inc. (Hamamatsu, Japan), and housed under a 12 h light/dark cycle (lights on 08:00–20:00), at an ambient temperature of 20–22 °C and relative humidity of 30–60%. The mice had free access to food and water throughout.

2.2. Experimental procedures

The mice were acclimated for 2 weeks after purchase, then mating was performed in cages containing one male and three females for 12 h. The following day, the female mice were checked for copulatory plugs, and pregnant individuals were moved to separate cages. The females were allocated to two groups and fed either the CN or HFA diet throughout their pregnancy. After the birth of their offspring, at postnatal day (PD)0.5, the diets were replaced with the regular AIN93G diet. On PD8, the number of offspring was adjusted to four per mother to standardize the conditions like access to milk. The offspring number in each

Table 1. Sequences of the RT-PCR primers used in the study.

Gene		Primer 1	bp		Primer 2	bp
Tbp	F	CTTCACCAATGACTCCTATGACC	23	R	ACAGCCAAGATTCACGGTAGA	21
Pparg1	F	ATCTTCTGTGCCACCTGCTAA	21	R	GTGCGTACAGTTCTGATTGC	20
Pparg2	F	AGAACACACGCTTCCTTCCA	20	R	CCGACATTCCATGTTGAGG	19
Acaca	F	GAACACCCAGAGCATTGTCC	20	R	AGTGGCCGTTCTGAAACTGT	20
Acacb	F	TAAGAAGCTCGTGGGACAGC	20	R	TGGTAGATGGGAAGCAGCA	19
Cidec	F	GAAGGTTCGCAAAGGCATC	19	R	CAGGGAGAAGGGCTTGTCTT	20
Acox1	F	CGCCAGTCTGAAATCAAGAGA	21	R	TCCGCATGTAGGTCTCCTTT	20
FaSn	F	TTCCTTGTGCACCCCATT	18	R	AGGTTCGGAATGCTATCCAG	20
Gpd1	F	TCCCCTTTACTACCCTTTCTG	21	R	TGTGGGCTCCGAGTATTTAAC	21
Pck1	F	ATGTGTGGGCGATGACATT	19	R	AACCCGTTTTCTGGGTTGAT	20
Pck2	F	CAGGGTCTTATCCGCAAACT	20	R	CACATCCTTGGGGTCTGTG	19
Ggat1	F	TGTGGCTGCATTTCAGATTG	20	R	GCTGGGAAGCAAATGATTGT	20
Ggat2	F	CCCCGTTGTGAGGTTATAAAG	21	R	CCACCCTAGATGAGCAGAAA	20
Plin1	F	AGCATCGAGAAGGTGGTAGAGT	22	R	CTCCTCACAAGGCTTGGTTT	20

Sequences of the oligonucleotide primers used for PCR amplification. F, forward; R, reverse; bp, base pairs; *Tbp*, TATA-box binding protein; *Pparγ1*, peroxisome proliferator-activated receptor γ-1; *Pparγ2*, peroxisome proliferator-activated receptor γ-2; *Acaca*, acetyl-coenzyme A (CoA) carboxylase alpha; *Acacb*, acetyl-coenzyme A (CoA) carboxylase beta; *Cidec*, cell death-inducing DFF45-like effector c; *Acox1*, acyl-coenzyme A oxidase 1, palmitoyl; *Fasn*, fatty acid synthase; *Gpd1*, glycerol-3-phosphate dehydrogenase 1; *Pck1*, phosphoenolpyruvate carboxykinase 1; *Pck2*, phosphoenolpyruvate carboxykinase 2; *Dgat1*, diacylglycerol O-acyltransferase 1; *Dgat2*, diacylglycerol O-acyltransferase 2; *Plin1*, perilipin 1.

group and the total number used in this study were 8–12 and 42, respectively.

On PD22, 8 h-fasted (07:00–15:00) blood was obtained from a tail vein and the glucose concentration (FBG) was measured using a Rabo Glucometer (Foracare Japan, Tokyo, Japan). On PD50, an OGTT (10 μL/g body mass of 20% glucose solution) was performed after 8 h of fasting. Blood samples were collected and their glucose concentrations were measured using a Rabo Glucometer, just before (0), 30, 60, and 120 min after the glucose administration.

The offspring were sacrificed on PD53, and their pancreas were rapidly removed and fixed in 4% paraformaldehyde. Samples of the left liver lobe were pulverized in liquid nitrogen and stored at −80 °C until use. The experimental design for this study is shown in Figure 1.

2.3. Measurement of serum insulin concentration

On PD22, 50, and 53, serum samples were obtained for the measurement of insulin concentration, using a Mouse Insulin ELISA Kit (Mercodia, Uppsala, Sweden). Ten microliters of serum were added to each well of a mouse monoclonal anti-insulin coated plate, then 1 × enzyme conjugate (peroxidase-conjugated mouse monoclonal anti-insulin antibody) was added, and the plate was agitated using a shaker (SR-1, Asone Corp., Osaka Japan) at 260 rpm at room temperature (RT, 15–25 °C). Each well was then washed with 300 μL wash buffer, 200 μL (3,3′,5,5′-tetramethylbenzidine) was added, and the plate was incubated for 15 min at RT. The reaction was terminated by adding 50 μL of 0.5 M H_2SO_4, the absorbance at 450 nm was measured using a spectrophotometer (DTX880 Multimode Detector, Beckman Coulter Inc., Brea CA), and the insulin concentration (pmol/L) was calculated using a calibration curve.

2.4. Immunohistochemistry for insulin

Stored pancreatic tissue was dehydrated using increasing concentrations of ethanol, embedded in paraffin, then cut into 5 μm sections and mounted on poly-L-lysine-coated slides. The sections were then deparaffinized using xylene twice for 15 min, and subsequently rehydrated in 100%, 95%, 90%, 80%, and 70% ethanol for 5 min each, and then drained. The prepared sections were immersed in 10 mM citrate buffer (pH 6.0) for antigen retrieval in a microwave for 15 min, and then returned to RT for 30 min. The sections were washed for 5 × 3 min in phosphate-buffered saline (PBS) and then blocked using methanol containing 3% hydrogen peroxide in PBS for 10–15 min at RT.

One hundred microliters of anti-insulin antibody (Nichirei Biosciences Inc., Tokyo, Japan) were added dropwise to each slide, with the exception of a negative control slide. The sections were then incubated for 1 h at RT, and washed three times with PBS. One hundred microliters of the secondary antibody were then added and the slides were incubated for a further 30 min at RT, before being rinsed well with PBS. The slides were then incubated for 3 × 5 min with PBS, a solution of polymer-antibody-peroxidase complex applied, and they were then incubated again at RT for 30 min. Two drops (100 μL) of 3,3′-diaminobenzidine tetra hydrochloride solution were then added dropwise and left for 5–20 min at RT, before thorough rinsing with purified water. The sections were counterstained with hematoxylin, then dehydrated in 70%, 80%, 90%, 95%, and 100% ethanol for 3 min each, incubated with xylene, and sealed using a water-insoluble encapsulating agent. Up to 10 images were obtained using a light microscope (Keyence Corp., Osaka, Japan) at ×40 magnification. For each section, the diameters of all insulin-positive cells were quantified using ImageJ ver. 1.46 software [18, 19].

2.5. Liver triglyceride content

Liver samples collected on PD53 were homogenized at RT using five volumes of radioimmunoprecipitation assay buffer (1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 150 mM NaCl), and the triglyceride concentrations were measured using a commercial kit (Triglyceride E-Test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.6. RNA extraction and quantitative RT-PCR

Total RNA was extracted using the acidified guanidinium thiocyanate method [20] and converted to cDNA by reverse transcription. To quantify the mRNA expression of selected genes, PCR amplification was performed in a Light Cycler Instrument (Roche Applied Science, Upper Bavaria, Germany). Real-time RT-PCR amplifications were carried out in a total volume of 10 μL, containing 400 nM of each gene-specific primer (Rikaken, Aichi, Japan), cDNA, and Light Cycler 480 Probes Master (Roche Applied Science). The primer sequences are given in Table 1. The cycle threshold (CT) values for each target gene and a reference gene (TATA box binding protein [*Tbp*] determined by real-time RT-PCR were

Table 2. Basic characteristics of mothers fed a control diet or a folic acid-oversupplemented diet and their offspring.

A) Mothers					
	GD (days)	CN		HFA	
Body mass (g)	0	18.8 ± 0.9		18.5 ± 0.5	
	18	34.8 ± 1.4		33.4 ± 0.6	
Food intake /day (g)	0	2.0 ± 1.2		2.2 ± 1.1	
	18	3.6 ± 0.4		3.4 ± 0.2	
	18	3.6 ± 0.4		3.4 ± 0.2	
Number of offspring (n)		6.7 ± 0.4		5.5 ± 0.3*	
B) Offspring					
	PD (days)	Male	Female	Male	Female
Body mass (g)	8	4.3 ± 0.3	3.8 ± 0.1	3.8 ± 0.1	3.6 ± 0.1
	22	9.0 ± 0.2	9.1 ± 0.3	9.1 ± 0.2	8.9 ± 0.3
	53	22.2 ± 0.6	18.2 ± 0.4	22.3 ± 1.2	17.5 ± 0.3
Food intake (g) (PD22-53days)		86.3 ± 6.3		90.3 ± 4.7	

A) Body mass and food intake of C57BL/6J mice fed a CN or an HFA diet at GD 0 and 18. The numbers of offspring born were counted. The number of offspring were less than CN mice by using Student's t-test (*$P < 0.05$). B) The mean offspring body mass for each group at PD 8, 22, and 53 and their food intake (PD 22–53) were measured. Values are expressed as mean ± SEM for 10 mice. CN, control; HFA, high-folic acid; GD, gestational day; PD, postnatal day.

Table 3. The organ masses of offspring whose mothers were fed a control diet or a folic acid-oversupplemented diet.

		♂		♀	
Offspring	PD (days)	CN	HFA	CN	HFA
Mass of pancreas (g)	53	0.13 ± 0.01	0.17 ± 0.02 *	0.13 ± 0.02	0.14 ± 0.01
Mass of liver (g)	53	0.97 ± 0.04	0.93 ± 0.08	0.56 ± 0.15	0.74 ± 0.04

Offspring organ masses were recorded. Values are mean ± SEM for 10 mice. The mass of pancreas of HFA mice were heavier than CN mice by using Student's *t*-test (*$P < 0.05$). CN, control; HFA, high-folic acid; GD, gestational day; PD, postnatal day.

converted into signal intensities using the delta-delta CT method: $2^{(\text{CT of } \textit{Tbp} - \text{CT of } \textit{target gene})}$ [21]. Sequences of the oligonucleotide primers used for PCR amplification. F, forward; R, reverse; bp, base pairs; *Tbp*, TATA-box binding protein; *Pparγ1*, peroxisome proliferator-activated receptor γ-1; *Pparγ2*, peroxisome proliferator-activated receptor γ-2; *Acaca*, acetyl-coenzyme A (CoA) carboxylase alpha; *Acacb*, acetyl-coenzyme A (CoA) carboxylase beta; *Cidec*, cell death-inducing DFF45-like effector c; *Acox1*, acyl-coenzyme A oxidase 1, palmitoyl; *Fasn*, fatty acid synthase; *Gpd1*, glycerol-3-phosphate dehydrogenase 1; *Pck1*, phosphoenolpyruvate carboxykinase 1; *Pck2*, phosphoenolpyruvate carboxykinase 2; *Dgat1*, diacylglycerol O-acyltransferase 1; *Dgat2*, diacylglycerol O-acyltransferase 2; *Plin1*, perilipin 1.

2.7. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and normal distribution was tested, thereafter they were analyzed using two-way ANOVA and Tukey's HSD test (SPSS Statistics ver.21; IBM, Armonk, NY).Comparison between CN and HFA groups, or male and female groups were analyzed using Student's *t*-test. Statistical significance was accepted when $P < 0.05$.

3. Results

3.1. Basic characteristics of the mothers and offspring in the CN and HFA groups

The body mass and food intake of the mothers are shown in Table 2A. There was no significant difference in food intake between the two groups. There was also no difference in the gestation period between the groups (20 days).

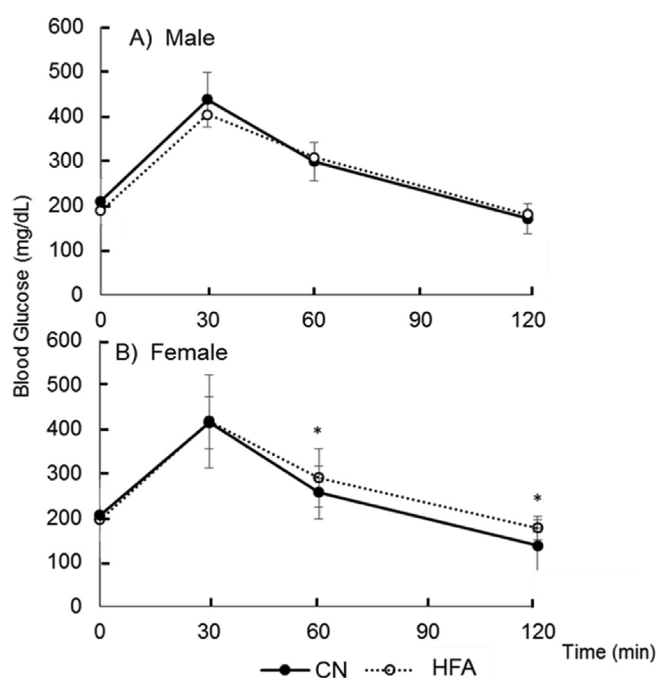

Figure 2. Profiles of oral glucose tolerance test on PD50. Plasma glucose concentrations in HFA (open circles) and CN (closed circles) offspring during an OGTT. A) Male offspring and B) female offspring. Significant differences between CN and HFA were identified in females using Student's *t*-test (*, $P < 0.05$). CN, control group; HFA, high-folic acid group; OGTT, oral glucose tolerance test.

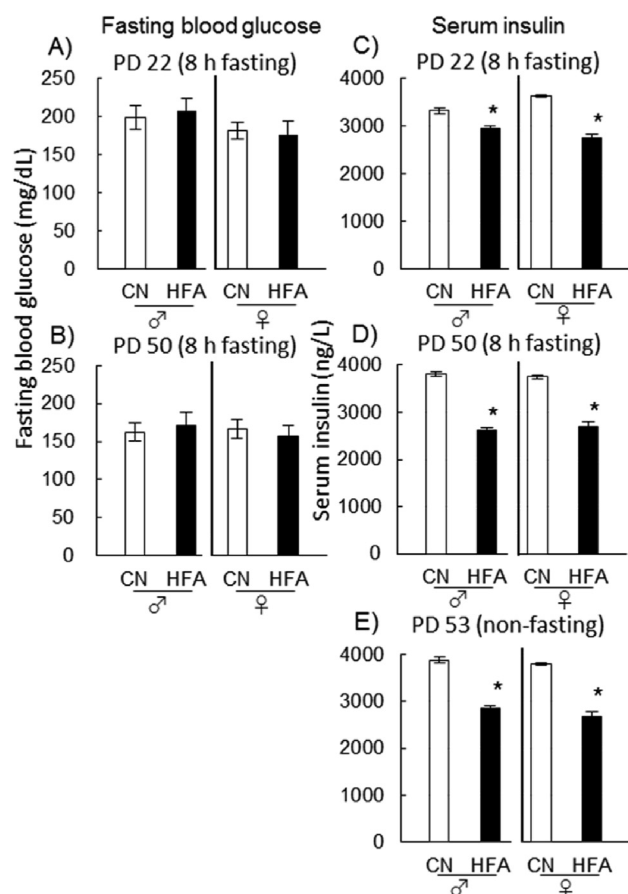

Figure 3. Fasting blood glucose and serum insulin concentration. Fasting blood glucose and serum insulin concentration in HFA (black) and CN (white) offspring. Fasting blood glucose on A) PD22 (after 8 h of fasting) and B) PD50 (after 8 h fasting). Insulin concentration on C) PD22 (after 8 h of fasting), D) PD50 (after 8 h of fasting), and E) PD53 (no fasting) were significantly lower in HFA than CN male and female mice, according to Student's *t*-test (*, $P < 0.05$). CN, control group; HFA, high-folic acid group.

The offspring parameters are shown in Table 2B. Offspring born to HFA dams were compared with those born to CN dams. There was no significant differences in body mass or food intake between the two groups. The organ masses of the offspring are shown in Table 3. Offspring born to HFA dams were compared with those born to CN dams. The mass of the pancreas was significantly higher in the male offspring of the HFA group than in those of the CN group ($P < 0.05$, Table 3).

3.2. Oral glucose tolerance testing, fasting blood glucose, and serum insulin concentration

The glucose concentrations at the 60- and 120-min time points of the OGTT on PD50 were significantly higher in the HFA group than the CN group in female ($P < 0.05$, Figure 2B), but not male ($P < 0.05$, Figure 2A) mice.

There was no significant difference in fasting glucose concentration between the two groups on PD22 ($P < 0.05$, Figure 3A) and PD50 ($P < 0.05$, Figure 3B), respectively. The fasting serum insulin concentrations on PD22 (Figure 3C) and PD50 (Figure 3D) were significantly lower in the HFA group than the CN group ($P < 0.05$, Figure 3C, D).

The Figure 3E shows the non-fasting concentration on PD53. It was also significantly lower in the HFA group than in the CN group ($P < 0.05$).

3.3. Immunohistochemistry for insulin

Typical images of the immunohistochemical staining of pancreatic sections for insulin are shown in Figure 4A. The mean diameter of the insulin-positive cells in an individual pancreas section were significantly lower in both male and female mice of the HFA group than in those of the CN group ($P < 0.05$, Figure 4C), but the difference was greater in the females.

3.4. mRNA expression of genes involved in hepatic carbohydrate and fat metabolism

The liver triglyceride content at PD53 was higher in females and tended to be significantly higher in males of the HFA group than of the CN group ($P < 0.05$, Figure 5).

Figure 6 shows the expression of *Pparγ1*, *Fasn,* and *Acacb* was higher in female than male offspring. The expression of Cidec was was higher in both male and female HFA offspring than CN offspring, and the expression of *Pparγ2* was higher in the HFA males only ($P < 0.05$, Figure 6).

4. Discussion

In the present study, we have shown that excessive FA supplementation during pregnancy reduces fasting (PD22 and 50) and non-fasting (PD53) serum insulin concentrations, and reduces β-cell mass in both male and female offspring. These results suggest that excessive FA supplementation during pregnancy impairs insulin secretion in the offspring from a young age. In general, animals or humans with a low insulin secretory capacity tend to exhibit IGT. Our data suggest that one of the major causes of IGT in the offspring of mothers who undergo excessive FA supplementation during pregnancy is a reduction in islet β-cell mass.

Previous studies have demonstrated that consumption of a high-FA diet during pregnancy induces weight gain, insulin resistance, and IGT in adult rat offspring [13, 14, 15]. However, the mechanism whereby these metabolic abnormalities are induced in the offspring remains to be established. It has also been shown that protein malnutrition during pregnancy reduces the synthesis and secretion of insulin by pancreatic β-cells and peripheral insulin sensitivity in rat offspring [22]. Furthermore, the proportion of β-cells in the islets is lower in offspring born to dams fed a low-protein diet than in those born to control dams at PD7-21, which explains the observed reduction in insulin secretion in the offspring. It has also been reported that the exposure of fetuses to high concentrations of FA, combined with subsequent consumption of a high-fat diet between 7 and 15 weeks of age, induces insulin resistance in male, but not female offspring [15]. Moreover, consumption of a high-folate diet during pregnancy in C57BL/6 mice is associated with lower insulin secretion at 23 weeks of age. Therefore, we hypothesized that fetal overexposure to FA may accelerate the development of the impairment in insulin secretion that occurs secondary to high-fat diet-induced insulin resistance. Therefore, we assessed insulin expression and the circulating insulin concentration in mice exposed to excessive FA as fetuses.

However, the present study has not shown how excessive FA exposure of a fetus leads to lower β-cell mass in the offspring. It has been reported that feeding a low-protein diet during gestation alters the development of the endocrine pancreas and favors the development of diabetes in later life. It has also been reported that consumption of a low-protein diet during gestation increases microRNA375 expression in β-cells, which negatively regulates their proliferation and insulin secretion [23], and induces mitochondrial dysfunction [24]. In addition, the adult offspring of mice that consume a low-energy diet during pregnancy demonstrate lower expression of insulin and the transcription factor insulin promoter factor (*Pdx*)-1, which induces insulin expression in the pancreas [25]. Thus, it is possible that excessive fetal FA exposure may have its effect *via* miR375 and *Pdx-1* in β-cells, and this possible mechanism should be investigated in a future study. However, it is unclear whether excessive

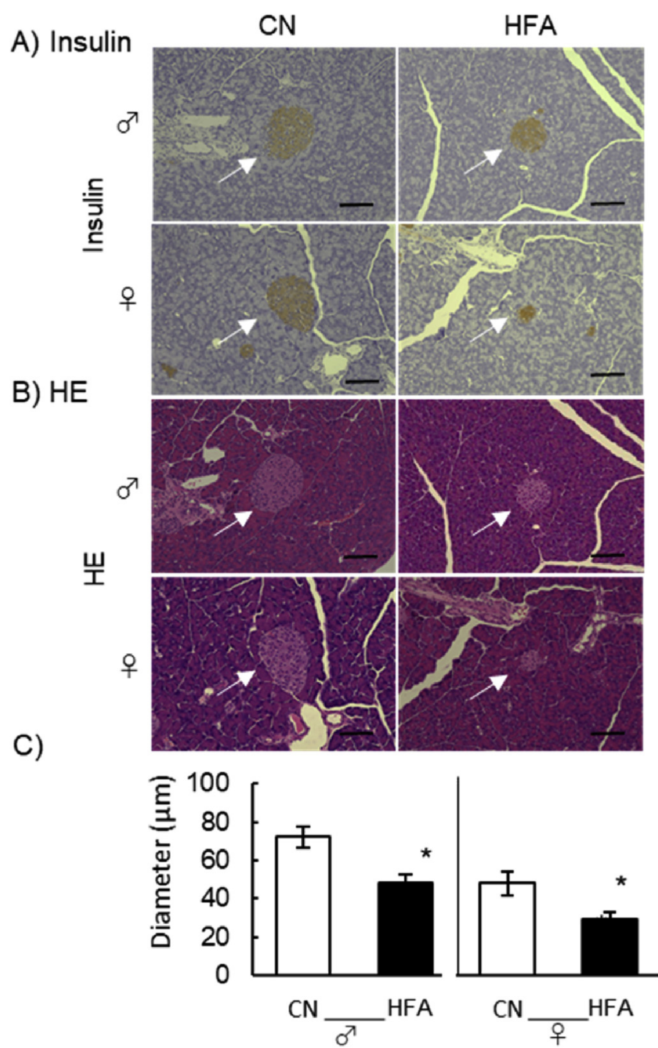

Figure 4. Immunohistochemistry for insulin in pancreatic sections on PD53. Immunohistochemical and hematoxylin and eosin (HE) staining of sections prepared from pancreas removed from the offspring of each group after sacrifice. Sections were processed for A) insulin and B) HE staining. Magnification 20× (scale bars: 50 μm). C) Mean ± SE diameter (μm) of insulin-positive cells in the pancreas. Significant differences between the CN and HFA of male or female mice were identified using Student's *t*-test (*, $P < 0.05$). CN, control group; HFA, high-folic acid group.

fetal exposure to FA reduces the expression of genes involved in insulin secretion and β-cell maturity, such as *Pdx*-1 and *Ins*. In addition, it is still unclear whether the consumption of a high-fat and/or high-sucrose diet by mice that were exposed to excess FA *in utero* exacerbates their insulin resistance and IGT. These questions should be addressed in further studies.

A limitation of the present study was that we did not assess the areas or numbers of pancreatic islets. It is possible that excessive fetal FA exposure increased the total β-cell mass and number. Indeed, it has previously been reported that β-cell mass increases when mice develop insulin resistance [26, 27]. Furthermore, in the present study, the pancreatic mass of the male offspring on PD53 was increased by excessive fetal FA treatment. Therefore, it may be that insulin resistance is induced in these mice, which may affect the β-cell mass and islet number. The effect of excessive FA supplementation on these parameters should therefore also be determined in a future study.

In the present study, we found that liver triglyceride content at PD53 was higher in females and tended to be higher in males in the HFA group than in those in the CN group. To determine whether the increases in

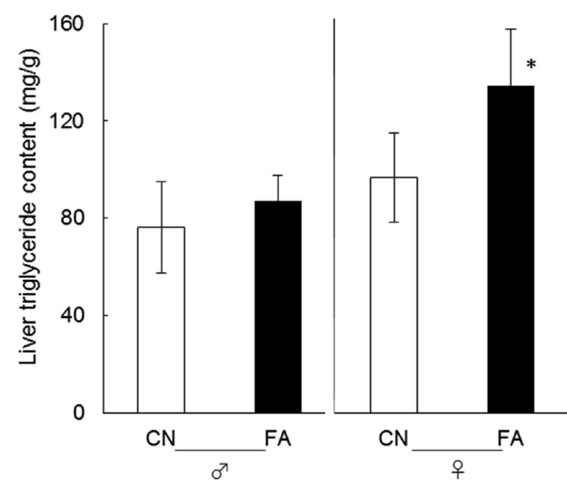

Figure 5. Liver triglyceride content on PD53. Data were expressed as Mean ± SE triglyceride content in the liver. Significant differences between the CN and HFA females were identified using Student's *t*-test (*, $P < 0.05$). CN, control group; HFA, high-folic acid group.

liver triglyceride were the consequence of the excessive fetal exposure to FA, we measured the mRNA expression of genes encoding proteins involved in hepatic carbohydrate and fat metabolism in the offspring, including those involved in gluconeogenesis and fatty acid synthesis (*Fasn*, *Acaca*, and *Acacb*), and in triglyceride synthesis (glycerol-3-phosphate dehydrogenase 1 [*Gpd1*] and diacylglycerol O acyltransferase 1 [*Dgat1*]). In general, lower insulin secretion is associated with higher expression of hepatic gluconeogenic genes. However, we did not find any differences in the expression of these genes. In contrast, we did identify higher expression of genes encoding proteins involved in lipid metabolism (*Pparγ2* and *Cidec*), in the liver of HFA offspring. *Pparγ* is a transcription factor that promotes hepatic lipid uptake and lipid droplet formation [28], and *Cidec* is a target gene of *Pparγ* that encodes a protein involved in lipid droplet formation, which is dramatically induced in fatty liver [29]. Consistent with this, we have demonstrated higher liver triglyceride content in offspring exposed to excess FA during fetal development. It should be noted that the increase in liver triglyceride content was greater in females than in males, despite the reduction in insulin secretion in both sexes. However, the expression of genes involved in lipid accumulation, such as *Cidec*, in the liver, was higher in male, but not female, offspring born to mothers fed the high FA-diet than in those born to mothers fed a normal diet. The reason for this remains to be determined. In general, the major causes of fat accumulation in the liver are an increase in *de novo* fat synthesis in the liver or an increase in delivery to the liver, because of dysfunction in other tissues, such as adipose and skeletal muscle. Therefore, the increase in triglyceride content in female mice that were exposed to excess FA *in utero* may have been caused by defects in adipose or skeletal muscle. However, we did not analyze these tissues. Therefore, the phenotypes of adipose and skeletal muscle, with respect to metabolism and insulin sensitivity, should be characterized in adult male and female offspring in future studies.

Our findings suggest that the excessive intake of FA during pregnancy may reduce glucose utilization in the liver and increase the risk of fatty liver in the offspring. However, it should be noted that IGT was not induced in the offspring. This may be because the mice were only studied at quite a young age. Nevertheless, consumption of a high-FA diet during pregnancy reduces serum insulin concentration and insulin resistance, assessed using HOMA-IR, in 15-week-old C57BL/6 mice [15]. In the present study we evaluated the offspring phenotype on PD22, 50, and 53 in mice consuming a standard low-fat diet. Therefore, it should also be determined whether excessive exposure to FA *in utero* induces IGT at

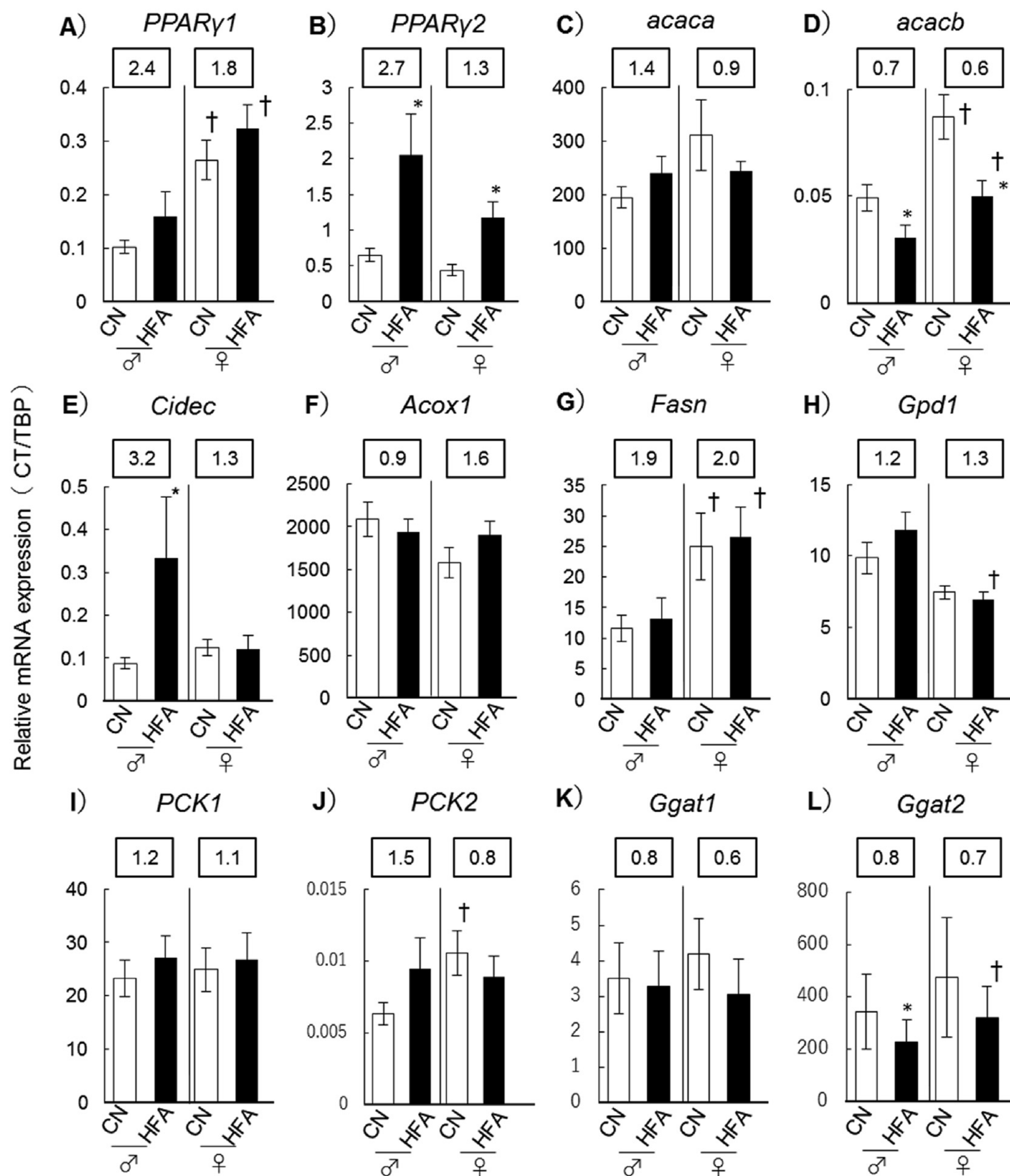

Figure 6. mRNA expression of genes involved in carbohydrate metabolism. Analysis of the mRNA expression of genes involved in carbohydrate metabolism was performed in offspring liver using real-time RT-PCR. A) *Pparγ1*, B) *Pparγ2*, C) *Acaca*, D) *Acacb*, E) *Cidec*, F) *Acox1*, G) *Fasn*, H) *Gpd1*, I) *Pck1*, J) *Pck2*, K) *Ggat1*, and L) *Ggat2*. Control group (CN; white bars); high-folic acid group (HFA; black bars), The fold difference between the HFA and CN groups are shown at the top of the graphs (*, $P < 0.05$), CN *versus* HFA, (**†**, $P < 0.05$), male *versus* female using two-way ANOVA and Tukey's HSD Test (*, $P < 0.05$). *Pparγ1*, peroxisome proliferator-activated receptor γ-1; *Pparγ2*, peroxisome proliferator-activated receptor γ-2; *Acaca*, acetyl-coenzyme A (CoA) carboxylase alpha; *Acacb*, acetyl-coenzyme A (CoA) carboxylase beta; *Cidec*, cell death-inducing DFF45-like effector c; *Acox1*, acyl-coenzyme A oxidase 1, palmitoyl; *Fasn*, fatty acid synthase; *Gpd1*, glycerol-3-phosphate dehydrogenase 1; *Pck1*, phosphoenolpyruvate carboxykinase 1; *Pck2*, phosphoenolpyruvate carboxykinase 2; *Dgat1*, diacylglycerol O-acyltransferase 1; *Dgat2*, diacylglycerol O-acyltransferase 2.

other life stages, in mice consuming either standard chow or a high-fat diet.

In the present study, we found that offspring born to mothers in the HFA group ate more than those born to mothers in the CN group. Lower insulin secretion and excessive food consumption in young mice may induce IGT and fatty liver in later life, and this possibility will be investigated in further studies, in which it will be determined whether the protein levels of PPARG2 and CIDEC in the liver and liver fat accumulation in HFA offspring are different from controls.

Recent studies have demonstrated that energy deficiency during pregnancy induces features of non-alcoholic fatty liver disease (NAFLD) in offspring [30], as do a number of other animal models of diseases with a developmental origin, including excessive FA consumption during pregnancy. Consistent with this, Nobili *et al.* [31] identified NAFLD in 35 (39%) of 90 children born small for gestational age, when they were 11–12 years old. Deteriorating β-cell function, in combination with increasing hepatic insulin resistance and declining suppression of hepatic glucose output, leads to hyperglycemia [32]. Therefore, one of the factors linking NAFLD with intrauterine growth retardation, fetal malnutrition, or excessive FA exposure may be impaired insulin secretion.

5. Conclusions

We have shown that excessive FA ingestion by pregnant mice is associated with lower β-cell mass and insulin concentration, and higher expression of genes associated with the development of fatty liver in the offspring.

Declarations

Author contribution statement

Yuri Kintaka, Kazuki Mochizuki: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nobuhiro Wada, Sadako Nakamura, Yuko Yamazaki: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Seiji Shioda: Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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