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Intrauterine metabolic programming alteration increased susceptibility to non-alcoholic adult fatty liver disease in prenatal caffeine-exposed rat offspring

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HIGHLIGHTS

- Prenatal caffeine exposure increased susceptibility to NAFLD in rat offspring.
- It is mediated by intrauterine glucose and lipid metabolic programming alteration.
- Gender differences were observed in the manifestation of disease.

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ABSTRACT

An increase in susceptibility to metabolic syndromes (MetS) in rat offspring that experienced prenatal caffeine exposure (PCE) has been previously demonstrated. The present study aimed to clarify this increased susceptibility and elucidate the mechanism of foetal origin that causes or contributes to adult non-alcoholic fatty liver disease (NAFLD) as a result of PCE. Based on the results from both foetal and adult studies of rats that experienced PCE (120 mg/kg d), the foetal weight and serum triglyceride levels decreased significantly and hepatocellular ultrastructure was altered. Foetal livers exhibited inhibited insulin-like growth factor-1 (IGF-1), enhanced lipogenesis and reduced lipid output. In adult female offspring of PCE + lab chow, lipid synthesis, oxidation and output were enhanced, whereas lipogenesis was inhibited in their male conterparters. Furthermore, in adult offspring of PCE+ high-fat diet, catch-up growth appeared obvious with enhanced hepatic IGF-1, especially in females. Both males and females showed increased lipid synthesis and reduced output, which were accompanied by elevated serum triglyceride. Severe NAFLD appeared with higher Kleiner scores. Gluconeogenesis was continuously enhanced in females. Therefore, increased susceptibility to diet-induced NAFLD in PCE offspring was confirmed, and it appears to be mediated by intrauterine glucose and alterations in lipid metabolic programming. This altered programming enhanced foetal hepatic lipogenesis and reduced lipid output in utero, which continued into the postnatal phase and reappeared in adulthood with the introduction of a high-fat diet, thereby aggravating hepatic lipid accumulation and causing NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterised as liver steatosis and excessive fat (mainly triglyceride) storage, which may progress to steatohepatitis, fibrosis and cirrhosis (Neuschwander-Tetri, 2005). Intrauterine growth retardation (IUGR) is the failure of a foetus to achieve a predicted growth potential based on the genetic constitution and environmental influences (Scifres and Nelson, 2009), and it primary manifests as low birth weight. A population-based study showed that the risk of NAFLD in children born with IUGR was 4 times higher than that of children born with a normal weight (Nobili et al., 2008), indicating that NAFLD may have a foetal origin.

Caffeine is a xanthine alkaloid that is found primarily in coffee, tea, energy drinks and some soft drinks, and is consumed on a daily basis all over the world. Both clinical data and animal

Abbreviations: NAFLD, non-alcoholic fatty liver disease; PCE, prenatal caffeine exposure; IUGR, intrauterine growth retardation; GC, glucocorticoid.

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experiments indicate that caffeine has the potential to induce reproductive and embryonic toxicity (Fortier et al., 1993; Huang et al., 2012). Metabolic syndrome (MetS) is characterised by several cardiometabolic risk factors, including central obesity, hypertension, hyperglycaemia, and dyslipidaemia (Bruce and Byrne, 2009). Our previous studies (Liu et al., 2012; Xu et al., 2012a) showed that prenatal caffeine exposure (PCE) results in IUGR and an increase in susceptibility to MetS later in life, and that the mechanism associated with such effects may be related to alterations in intrauterine metabolic programming. However, the potential association between increased susceptibility to NAFLD and PCE, and whether such an association is derived from intrauterine metabolic programming, has not been clearly demonstrated.

Previous research has shown that de novo lipogenesis plays a key role in the development of NAFLD (Postic and Girard, 2008), and may even mediate the generation of original NAFLD (Yamada et al., 2011). The present study was designed to investigate the effects of PCE on the susceptibility to NAFLD in offspring with IUGR and to clarify the underlying mechanisms of this phenomenon.

2. Materials and methods

2.1. Materials

Caffeine (CAS No. C0750) was obtained from Sigma–Aldrich Co., Ltd. (St Louis, MO, USA). Isoflurane was purchased from Baxter Healthcare Co. (Deerfield, IL, USA). A glucose oxidase assay kit was provided by Mind Bioengineering Co., Ltd. (Shanghai, China). The triglyceride assay kit was from Sangon Biotech Co., Ltd. (Shanghai, China). Reverse transcription and real-time reverse-transcription PCR (RT-PCR) kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). GeXP multiplex gene expression analysis kits were purchased from Beckman-Coulter Inc. (Fullerton, CA, USA). The oligonucleotide primers for rat Q-PCR genes (PAGE purification) and GeXP multiplex gene expression analysis (HPLC purification) were synthesised by Sangon Biotech Co., Ltd. (Shanghai, China). All other chemicals and reagents used were of analytical grade.

2.2. Animals and treatment

Animal experiments were performed in the Center for Animal Experiments of Wuhan University (Wuhan, China), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All animal experimental procedures were approved by and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee.

Wistar rats (with weights of 200–240 g for females and 260–300 g for males) were obtained from the Experimental Center of Hubei Medical Scientific Academy (No. 2009-0004, Hubei, China). Animals were housed under standard conditions (room temperature: 18–22 °C; humidity: 40–60%) and allowed free access to rat chow and distilled water. After one week of acclimation, 2 females were mated with 1 male for one night. Upon confirmation of mating by the appearance of sperm in a vaginal smear, the day was taken as gestational day (GD) 0. Pregnant females were then transferred to individual cages.

Three independent experiments comprised this study: foetal rats, adult rats fed with normal diet and adult rats fed with high-fat diet. For the portion of foetal rats, pregnant rats were randomly divided into 2 groups: a control group and the caffeine group. From GD11 until term delivery (GD20), the rats in the caffeine group were each administered caffeine 120 mg/kg day by oral gavage, once a day (Xu et al., 2012b). Rats in control group were given the same volume of distilled water. On GD20, 8 randomly selected pregnant rats from the caffeine group and 8 from the control group with 10–14 live foetuses were anesthetised with isoflurane and sacrificed. The foetuses were quickly removed to weigh, and IUGR was diagnosed when the body weight of a foetus was two standard deviations less than the mean body weight of foetuses in the control group (Engelbregt et al., 2001). Serum was prepared from blood by centrifugation at 12,000 × g, 4 °C for 15 min. Foetal livers were separated under a dissecting microscope and collected. Samples collected from littermates were pooled together and immediately frozen in liquid nitrogen, followed by storage at −80 °C for subsequent analyses.

For the experiment of adult rats fed with normal diet, the pre-treatment of the animals was the same as the first portion, but pregnant rats ($n = 8$ for each group) were kept alive until normal delivery. On postnatal day 1, the numbers of pups were normalised to 8 pups per litter to ensure adequate and standardised nutrition. On postnatal week 1 (PW1), all the offspring were weighed and assessed to IUGR. After weaning (PW4), one male and one female pups randomly selected from each dam were fed with lab chow (providing 22% of its energy content as protein, 63% as carbohydrate, and only 5% as fat). Therefore, the offspring could be divided into 4 groups: male of control with lab chow, males of caffeine with lab chow, and so were the female. Each group contained 8 pups. Body weights of the rat offspring were measured weekly. The corresponding body weight growth rate was calculated as reported by Xu et al. (2012a) as follow.

$$\text{Growth rate}\,(\%) = \frac{\text{weight}\,(\text{PW}\chi) - \text{weight}\,(\text{PW}_1)}{\text{weight}\ (\text{PW}_1)} \times 100$$

At PW24, the rats were anesthetised with isoflurane and sacrificed in a room separate from the other animals. Serum was prepared from blood and stored at −80 °C until further analysis. The livers were resected, with a section of each of 5 randomly selected livers from each group cut off and fixed in 4% paraformaldehyde solution for histological examination. Livers from all 8 rats in each group were immediately frozen in liquid nitrogen followed by storage at −80 °C for subsequent analyses.

For the experiment of adult rats fed with high-fat diet, the animal treatment was the same as the above adult rats fed with lab chow except for the diet type. The high-fat diet was previously reported by our laboratory (Zhang et al., 2013b) (providing 18.9% of its energy content as protein, 61.7% as carbohydrate, and 19.4% as fat).

2.3. Analysis of blood samples

The levels of serum glucose and triglycerides were detected using biochemical assay kits following the manufacturer's protocol. An Agilent 7890A GC coupled to an Agilent 5975C quadrupole mass selective detector (Santa Clara, CA, USA) was used to measure the concentrations of serum caffeine, and the details were shown in Gas chromatograph for caffeine detection part in Supplementary data.

2.4. Histological measurement

For light microscopy analysis, tissue staining with haematoxylin and eosin (HE) was achieved using paraffin embedded tissue sections and standard procedures in graded alcohols and xylene. Sections were observed and photographed using an Olympus AH-2 light microscope (Olympus, Tokyo, Japan). The grading and staging of NAFLD in adult rats were scored according to the system reported by Kleiner et al. (2005). The scoring system comprises 3 histological features that are evaluated semi-quantitatively: steatosis (0–3), lobular inflammation (0–3) and hepatocellular ballooning (0–2). Five HE sections of each group were selected randomly, and 5 random fields from each section were scored under the microscope. A Kleiner score is calculated by the sum of the steatosis, inflammation and ballooning scores. Scores of ≥5 correlate with a diagnosis of Nonalcoholic steatohepatitis, and biopsies with scores of less than 3 were diagnosed as "NAFLD." Steatosis was graded on a 4-point scale: 0 = minimal, 1 = mild, 2 = moderate and 3 = severe.

For transmission electron microscopy (TEM) analysis, 1-mm^3 tissue blocks of liver samples were collected and placed in 3% glutaraldehyde/1.5% paraformaldehyde solution with 0.1 M PBS. Samples were postfixed for 1.5 h in 1% osmium tetroxide/1.5% potassium ferrocyanide solution and washed in 0.1 M PBS, dehydrated in graded concentrations of ethanol, and embedded in Epon 618. Epoxy blocks were sliced on an ultratome (LKB-V, LKB, Stockholm, Sweden, 70 nm), stained with uranyl acetate and lead citrate, and examined using a Hitachi H600 transmission electron microscope (Hitachi, Co., Tokyo, Japan). Digital images were computationally acquired.

2.5. RNA extraction, reverse transcription, and quantitative real-time PCR

The mRNA expression levels of insulin-like growth factor 1 (IGF-1), insulin-like growth factor 1 receptor (IGF-1R), insulin receptor substrate 2 (IRS-2), glucose transporter 2 (GLUT-2), SREBP1c, FASN, AMP-activated protein kinase (AMPKa), carnitine palmitoyltransferase 1a (CPT1a), microsomal triglyceride transfer protein (MTTP), forkhead box O1 (FoxOl) and glucose-6-phosphatase (G6Pase) were analysed by quantitative RT-PCR. Detailed protocols for RNA extraction and reverse transcription have been published elsewhere (Tan et al., 2012). Primers and procedure of quantitative real-time PCR were shown in Real-time PCR part in Supplementary data.

2.6. Multiplicative gene expression analysis

Multiplicative analysis containing 3 housekeeping and 24 target genes was completed using a multiplex primers designed using the GenomeLab™ Express Profiler software (Beckman-Coulter, Fullerton, CA). Multiplex optimisation (e.g., primer validation and attenuation) was completed according to the manufacturer's instructions. Details were shown in Multiplex gene expression analysis part in Supplementary data.

2.7. Statistical analysis

SPSS 13 (SPSS Science Inc., Chicago, IL, USA) and Prism (GraphPad Software, La Jolla, CA, USA) were used for data analysis. All measurement data, except the IUGR rates, are expressed as the mean ± S.E.M. For enumeration data, such as IUGR rates, the proportion of rats affected per litter was calculated first (IUGR rate (%) = $\frac{n\,(\text{number of fetuses with weight below IUGR standard})}{N\,(\text{total number of fetuses in one litter})} \times 100$), and then an IUGR rate was arcsine